Identification of NTRK3 as a potential prognostic biomarker associated with tumor mutation burden and immune infiltration in bladder cancer

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Abstract

Background

Bladder cancer (BLCA) is a common malignant tumor of urinary system with high morbidity and mortality. In recent years, immunotherapy plays a significant role in the treatment of BLCA. Tumor mutation burden (TMB) has been reported to be a powerful biomarker to predict tumor prognosis and efficacy of immunotherapy. Our study aimed to explore the relationship between TMB, prognosis and immune infiltration to excavate the key genes in BLCA.

Methods

Clinical information, somatic mutation and gene expression data of BLCA patients were downloaded from The Cancer Genome Atlas (TCGA) database. According to the calculated TMB scores, patients were divided into high and low TMB groups. Gene Set Enrichment Analysis (GSEA) was performed to screen significantly enriched pathways. Differentially expressed genes (DEGs) between the two groups were identified. Univariate cox analysis and Kaplan-Meier survival analysis were applied for screening key genes. Immune infiltration was performed for TMB groups and NTRK3.

Results

Higher TMB scores were related with poor survival in BLCA. After filtering, 36 DEGs were identified. NTRK3 had the highest hazard ratio and significant prognostic value. Co-expressed genes of NTRK3 were mainly involved in several pathways, including DNA replication, basal transcription factors, complement and coagulation cascades, and ribosome biogenesis in eukaryotes. There was a significant correlation among TMB scores, NTRK3 expression and immune infiltration.

Conclusions

Our results suggest that NTRK3 is a TMB-related prognostic biomarker, which lays the foundation for further research on the immunomodulatory effect of NTRK3 in BLCA.

1. Background

Bladder cancer (BLCA) is one of the most common urinary malignancies and the incidence is gradually increasing, accounting for nearly 550,000 new cases and 200,000 deaths worldwide annually [1]. 75% of newly diagnosed patients are nonmuscle-invasive bladder cancers (NMIBC) and, although their 5-year overall survival (OS) rate is as high as 90% after treatment, suffer from a high rate of recurrence which carries a large social and economic burden [2, 3]. Muscle-invasive bladder cancer (MIBC) represents about 20% of newly diagnosed cases and approximately 15–20% of NMIBCs can progress to MIBCs [4].

Despite radical cystectomy and neoadjuvant chemotherapy are available, the MIBC patients have a poor prognosis with a 5-year OS rate of less than 50%. Besides, about half of the patients will eventually progress to distant metastases [5, 6].

In the past 30 years, the systemic treatment for BLCA has remained unchanged and the curative effect has not made a breakthrough, especially for advanced disease [7]. Nowadays, with the rapid evolution of immunotherapy, the use of immune checkpoint inhibitors (ICIs) is becoming an important emerging treatment strategy for advanced BLCA [8]. Treatment with ICIs such as anti-programmed cell death protein 1 (PD-1), anti-programmed death-ligand 1 (PD-L1), and anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) can significantly increase the OS and result in durable remission for many advanced cancers, including melanoma, non-small cell lung cancer, renal cancer and urothelial cancer [9–12]. However, these effects only exist in a small subset of patients who can respond to ICIs. Many studies have shown that the tumor mutation burden (TMB) is a powerful biomarker for predicting the efficacy of ICIs and can be used to identify patients who will benefit from immunotherapy [13]. Tumors can be caused by the accumulation of somatic mutations in the cells affected by carcinogens. Some mutant cells returned to normal after DNA self-modification, some died, and a minority of them expressed neoantigens on surface. Tumor cells can prevent these mutant cells from being recognized by the immune system through abnormal expressions of antigens or regulating the tumor microenvironment (TME), thereby achieving immune escape [14]. The higher TMB accounts for more neoantigens and increases the probability of activating the body's anti-tumor immune response, which can improve the patients' response to ICIs [15, 16]. Thus, exploring the relationship between TMB and immunoregulation and discovering the related genes or biological mechanisms will help to better understand the role of immunotherapy in cancer treatment.

In our study, mutation information and expression profiles were acquired from The Cancer Genome Atlas (TCGA) database. Bioinformatic analysis was applied for calculating TMB scores and evaluating their relationships with prognosis and immune infiltration. Finally, we identified one potential key biomarker with prognostic value, which might help to filter patients suitable for immunotherapy in BLCA.

2. Methods

2.1 Data source and data processing

Transcriptome data and corresponding clinical information of BLCA patients were downloaded from the TCGA data portal (<http://cancergenome.nih.gov/>). Then we downloaded the Simple Nucleotide Variation data from “Masked Somatic Mutation” category processed with VarScan software in TCGA. The “maftools” R package was used to analyze the Mutation Annotation Format (MAF) file and visualize the somatic mutation data [17].

2.2 Calculation of TMB scores and prognostic evaluation

TMB is also defined as mutation frequency. We calculated TMB scores with the number of all nonsynonymous mutations, divided by exon length (38 Mb). We used the X-tile software to calculate the best cut-off value of TMB scores and divided the patients into high and low TMB groups [18]. Survival analysis was conducted while clinicopathological characteristics were compared between high and low TMB groups. The Wilcoxon rank-sum test was for comparing two groups and the Kruskal–Wallis test was for more groups.

2.3 Gene Set Enrichment Analysis (GSEA) between high and low TMB groups

GSEA version was 4.0.0 based on JAVA platform and the reference gene set (c2.cp.kegg.v7.0.symbols.gmt) was downloaded from the Molecular Signatures Database (MSigDB) (<http://software.broadinstitute.org/gsea/msigdb/>) [19]. The pathways were considered to be statistically enriched with $P < 0.05$ and FDR (false discovery rate) < 0.25 .

2.4 Screening of key genes associated with TMB and survival

We identified the differentially expressed genes (DEGs) between high and low TMB groups. The “edgeR,” “limma,” and “DEseq2” packages in R software were used for this analysis. Univariate cox and KM methods were performed for survival analysis on every DEG to obtain the target genes. $|\log_2FC| > 1$ and P value < 0.05 were set as the thresholds.

2.5 Co-expression analysis of NTRK3

LinkedOmics (<http://www.linkedomics.org/login.php>) is a publicly available portal that includes multi-omics data from all 32 TCGA cancer types and provides a web-based platform for analyzing [20]. Co-expression of NTRK3 was analyzed statistically on the website using the Pearson correlation coefficient and the results were visualized by volcano plot and heat map. The LinkInterpreter module performed GSEA to get the related Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. The rank criterion was $P < 0.05$ and 500 simulations were tested.

2.6 Immune infiltration analysis

The “estimate” package in R software was used to calculate scores for tumor purity, the level of stromal cells presents, and the infiltration level of immune cells in tumor tissues based on expression data. CIBERSORT script in R software was used to analyze 22 types of immune cell fractions in each patient [21]. Samples with p value ≥ 0.05 were excluded for the next step. Wilcoxon rank-sum tests were conducted to analyze the different abundances of 22 immune cell types between high and low TMB groups. The TIMER database (<https://cistrome.shinyapps.io/timer>) was used to analyze the correlation between NTRK3 expression and immune cell infiltration level [22]. The TISIDB web portal (<http://cis.hku.hk/TISIDB/index.php>) was applied to explore the association between NTRK3 and multiple immune regulatory factors [23].

3. Results

3.1 Overview of mutation information in BLCA patients

We analyzed and visualized the somatic mutation information of BLCA patients using the “maftools” package. Among all the mutation types, missense mutation is the most common, whose frequency is far higher than other types (Fig. 1A). Single nucleotide polymorphism (SNP) represented the largest fraction in the variant type than insertion or deletion (Fig. 1B). The most common single nucleotide variant (SNV) was C > T, followed by C > G and C > A (Fig. 1C). The waterfall plot showed the top 15 most frequently mutated genes (Fig. 1D). Figure 1E showed the co-occurrence and exclusive associations between mutated genes, in which TP53 was tightly associated with FGFR3 and RB1.

3.2 Clinical relevance and pathways analysis for TMB

The TMB score of each BLCA patient was calculated and the best cut-off value of TMB was 6.4 computed by X-tile software (Fig. 2A). Then we divided the patients into a high TMB group with 164 cases and a low TMB group with 244 cases based on this cut-off. The result of KM survival analysis showed that the overall survival of the high TMB group was significantly better than the low TMB group (Fig. 2B, log-rank $P < 0.0001$). Clinical correlation analysis revealed high-grade advanced tumors had higher TMB scores than low-grade tumors (Fig. 2E, $P < 0.01$). However, no significant associations were found between TMB scores and clinical stage, T stage, smoking level (Fig. 2C, D, F).

In addition, GSEA analysis was performed between high and low TMB groups to explore the biological pathways that may be affected by TMB. The results showed that BLCA samples in the low TMB group were significantly enriched for 41 biological processes, and the following four biological processes with high normalized enrichment scores and big sizes of gene counts were selected, including ECM (extracellular matrix) receptor interaction, focal adhesion, leukocyte transendothelial migration and MAPK signaling pathway (Fig. 2G). But for the high TMB group, no significant result was enriched.

3.3 Screening and identification of key DEGs between high and low TMB groups

Three methods were applied to screen for DEGs between high and low TMB groups. The intersection among the results of the three methods is presented in Venn diagram and Upset plot (Fig. 3A). 36 genes were selected and the expression level of each gene in high and low TMB groups was shown in the form of heatmap (Fig. 3B). Univariate cox analysis for each of the 36 genes showed that 10 genes were significantly related to overall survival ($P < 0.05$). Then we performed KM survival analysis for these 10 genes and only four genes were with log-rank $P < 0.05$, including NCAM1, SPON1, NTRK3 and PRND. The Forest plot showed the result of univariate cox analysis for these genes (Fig. 3C). Figure 3D-G showed the KM survival curves for them. Among them, NTRK3 had the largest Hazard Ratio (HR) value, which meant that the high expression of NTRK3 may be a risk factor. In addition, we validated the prognostic value of

NTRK3 in another independent cohort (GSE48276, Fig. 3H). Therefore, we selected NTRK3 and included it in further analysis.

3.4 NTRK3 co-expression in BLCA

To explore the biological significance of NTRK3 in BLCA, the co-expression profiles of NTRK3 in the BLCA cohort were examined by LinkedOmics. As shown in Fig. 4C, 1,640 genes were significantly negatively correlated with NTRK3, and 5,027 genes were significantly positively correlated with NTRK3 (FDR < 0.01). The top 50 significant genes positively and negatively correlated with NTRK3 were shown in heatmaps (Fig. 4A, B). GSEA enrichment results based on NTRK3 were dealt with “Weighted set cover” in LinkedOmics. The significant KEGG pathways included DNA replication, basal transcription factors, complement and coagulation cascades, ribosome biogenesis in eukaryotes (Fig. 4D).

3.5 Relationship between TMB scores, NTRK3 expression level and ESTIMATE results

In the low TMB group, though the $P > 0.05$, the expression of NTRK3 was obviously higher than that of the high TMB group (Fig. 5A). Then we calculated immune and stromal scores for patients by ESTIMATE algorithm. In Fig. 5B, patients were divided into two groups according to the median value of NTRK3 expression. We found that the high expression group had a higher immune and stromal score than the low group significantly. Next, we measured the immune/stromal scores between high and low TMB groups. Compared with the high TMB group, the low TMB group had higher immune and stromal scores (Fig. 5C, D). All these results indicated that there was an evident correlation between TMB, NTRK3 and the infiltration level of immune cells and stromal cells. There may be a potential mechanism of interaction and mutual influence among them.

3.6 Comparison of immune cell infiltration between high and low TMB groups and analysis of NTRK3 immune correlation

Immune cell fractions for each sample were estimated by CIBERSORT algorithm based on transcriptome data. After excluding samples with P values ≥ 0.05 , 134 low-TMB samples and 102 high-TMB samples were selected for comparing fractions of 22 leukocyte subtypes between high and low TMB groups. The results showed that high-TMB samples had higher fractions of CD 8⁺ T cells, memory activated CD4⁺ T cells and T follicular helper cells (Tfh) while Low-TMB samples had higher fractions of memory resting CD4⁺ T cells and resting mast cells (Fig. 6A). TIMER was used to assess the correlation between NTRK3 expression and common immune infiltrating cells. The expression level of NTRK3 was positively correlated with infiltrating levels of immune cells (B cell, CD8⁺ T cell, CD4⁺ T cell, macrophage, neutrophil, and dendritic cell) (Fig. 6B). Besides, we also evaluated the relationship between NTRK3 and immunomodulators through the TISIDB portal (Fig. 7A-D). The results showed that NTRK3 in BLCA has a significant positive correlation with chemokine CCL14, immunoinhibitor ADORA2A, and immunostimulator CXCL12 (Fig. 6C, D, E). Figure 7E-G showed the relationship between the three immunomodulators and overall survival in BLCA. Thus it can be seen, NTRK3 interacts with immune

regulation in TME and may become a potential biomarker which has an important impact on the development of tumor and prognosis of patients.

3.7 Validation for NTRK3 by immunohistochemistry

The immunohistochemistry data of NTRK3 was acquired from the Human Protein Atlas database (<http://www.proteinatlas.org>). As shown in Fig. 8, NTRK3 staining was higher in tumor samples than that in normal tissue, which was consistent with the result of survival analysis, indicating that high expression of NTRK3 was a risk factor in bladder tumor.

4. Discussion

Prior to the advent of ICIs, surgery and platinum-based systemic chemotherapy were the standards of care. However, only 20% of IMBC patients are fit to receive chemotherapy and almost half of them may have sequelae with poor prognosis [24]. About 30% – 50% of metastatic IMBCs cannot receive cisplatin due to comorbidities and until recent years platinum-based regimes were still the only treatment option, with no inspiring effect in clinical outcomes[25]. Nowadays, immunotherapy represented by ICIs has gradually revolutionized the treatment paradigm of BLCA. Indeed, the intravesical use of bacillus Calmette–Guérin (BCG) for treating NMIBCs has proven the immune response characteristic of BLCA since the 1970s [26]. Tumorigenesis is a process of mutation accumulation. BLCA has the third-highest level of somatic mutations and is highly antigenic which may facilitate the immunological recognition[27]. Consequently, it is crucial to fully understand and explore the role of TMB in the immunotherapy of BLCA.

In the present study, we found that BLCA patients with higher TMB had more survival benefits. This is consistent with the results of some previous studies, such as cutaneous melanoma and ovarian carcinoma [28]. For patients treated with ICIs, high TMB has been confirmed to be associated with good response and better OS [29]. However, Cao et al. made a pooled analysis of 103,078 patients to evaluate the predictive efficiency of TMB and found the prognostic effect of TMB varied in patients with or without ICIs treatment, which might be related to tumor types [30]. Another research also indicated that the patients without ICIs treatment in some tumors may suffer a worse prognosis despite having high TMB [31]. Therefore, for the real application of TMB, it is still necessary to determine and assess its exact role in different tumors by more clinical trials. Moreover, meaningful GSEA results were all enriched in the low TMB group and most were immune-related, indicating that low TMB might enhance the tumor heterogeneity in BLCA. Of the GSEA results, ECM receptor interaction plays a crucial role in regulating invasion and progression of tumors. Cancer cells in the ECM of TME can release signals to mislead immune cells to avoid being attacked [32]. Besides ECM, the overactivation of focal adhesion pathway can result in dysfunction of cell migration and influence immune cells chemotaxis, which lead to tumor metastasis [33].

In terms of immune cell infiltration, the high proportion of CD4⁺ T cell, CD8⁺ T cell and Tfh may be an important factor leading to better OS in the high TMB group. Tumor-infiltrating immune cells are a

hallmark of immune surveillance and an integral part of complex microenvironment regulating tumor progression [34]. CD8⁺ T cells have strong cytotoxic activity for killing cancer cells, being considered as main drivers of anti-tumor immunity. The depletion in numbers and dysfunction of CD8⁺ T cells in TME create a favorable condition for cancer cell proliferation and metastasis [35]. CD4⁺ T cells and Tfh are also required and play a prominent role in anti-tumor immunity. CD4⁺ T cells can directly eliminate tumor cells through cytotoxicity or indirectly regulate TME to target tumor cells [36]. And Tfh may conduce to the formation of tertiary lymphoid structures in primary site and thereby promote intertumoral immune response of CD8⁺ T cells and B cells [35]. ICIs can help T lymphocytes restore activity and break through the physical barrier of TME to promote T cell homing, thus activating anti-tumor immunity and improving the effect of immunotherapy [37].

After strict screening, NTRK3 was finally selected as a potential biomarker due to its excellent prognostic value and immune infiltration correlation. It encodes TRKC protein, a member of neurotrophic tropomyosin receptor kinases (TRK) family which regulates many aspects of neuronal development and function. After binding to the ligand neurotrophin-3 (NT-3), TRKC autophosphorylates and motivates various signaling pathways such as MAPK and PI3K/AKT pathways which can regulate cellular growth and differentiation [38]. In recent years, many studies have reported that TRK pathway aberrations such as single nucleotide variation, gene fusion and gene overexpression are involved in the pathogenesis of many cancers, among which NTRK3 gene fusion is the most fully verified carcinogenic event [39]. Except for the functional relevance of TRKC in the nervous system, the overexpression of TRKC is observed in many types of tumors, including neuroblastoma, breast cancer, hepatocellular carcinoma and metastatic melanoma. TRKC plays an important role in regulating angiogenesis, inducing tumor growth, preventing apoptosis and promoting metastasis. Abnormal activation of NTRK3 and its fusion proteins may regulate the epithelial-mesenchymal transition (EMT) process, tumor growth rate and tumorigenicity by activation of several signaling pathways [40].

In our results, the expression level of NTRK3 was significantly positively correlated with the patients' immune/stromal scores, suggesting that NTRK3 may be inherently regulated by the TME. Moreover, NTRK3 also showed good correlation with a variety of immune lymphocytes. Among the related immunomodulators, we are particularly interested in CCL14 and CXCL12, both of which were strongly positively co-expressed with NTRK3 and had prognostic value. CCL14 represents a C-C type chemokine with high concentrations in human plasma, mainly involved in the transport of lymphocytes and inflammatory cells [41]. The role of CCL14 in tumor progression is unclear and has been poorly reported, especially in bladder cancer. Yu et al. found that high expression of CCL14 played a protective role which can promotes apoptosis of cancer cells and improve survival time in hepatic carcinoma[42]. However, Li et al. found that inhibiting the expression of CCL14 could effectively suppress the metastatic potential and angiogenesis of breast cancer [43]. In addition, there is a very interesting theory. Tumors can actively release chemokines to regulate the microenvironment so as to transfer the host immune response from immunogenic to tolerogenic, thereby achieving immune escape and promoting tumorigenesis and metastasis. Shields et al. expounded this view in their research on CCL21 [44]. Thus, the underlying

mechanism of the interaction between CCL14 and NTRK3 and how they jointly affect the progression of BLCA still needs more studies to explore. As for CXCL12, it has been extensively studied in the field of cancers. CXCL12, also known as stromal cell derived factor-1 (SDF-1), was first characterized as a pre-B cell growth factor and combines its receptor CXCR4 as a signaling axis which mainly participated in a lot of physiological processes such as hematopoiesis, immune responses and vascular formation [45]. Studies have shown that cancer cells in TME can induce overexpression of CXCL12 by autocrine or paracrine, and then activate downstream pathways to affect immune status and promote cancer cell proliferation and distant metastasis. Blocking CXCL12/CXCR4 signaling axis may inhibit tumor growth and provide new ideas for immunotherapy [46].

The current study exists several limitations. First, our study is retrospective based on public databases and problems such as insufficient and limited data are inevitable. Second, the relationship between TMB and the response to ICIs cannot be evaluated in BLCA due to a lack of immunotherapy information of patients. Third, our results need to be verified by other independent patient cohorts with mutation information and by laboratory or clinical experiments.

5. Conclusion

In conclusion, the present study systematically explored the relationship between TMB and prognosis as well as immune infiltration in BLCA. With further exploration, our data revealed that the expression of NTRK3 was closely correlated with survival and immune response. Accordingly, NTRK3 may prove to be a novel TMB-related biomarker and contribute to the development of immunotherapy in bladder cancer.

Abbreviations

BLCA
Bladder cancer
TMB
Tumor mutation burden
TCGA
The Cancer Genome Atlas
GSEA
Gene Set Enrichment Analysis
DEGs
Differentially expressed genes
NMIBC
Nonmuscle-invasive bladder cancer
MIBC
Muscle-invasive bladder cancer
ICI
Immune checkpoint inhibitor

TME
Tumor microenvironment
FDR
False discovery rate
KEGG
Kyoto Encyclopedia of Genes and Genomes
SNP
Single nucleotide polymorphism
SNV
Single nucleotide variant
OS
Overall survival
ECM
Extracellular matrix
NTRK3
NT-3 growth factor receptor

Declarations

Ethics approval and consent to participate

Any repository data used in this study are open access and do not require any permissions. Ethics approval and consent to participate are not applicable for them.

Consent for publication

Not applicable.

Availability of data and materials

All the data in our study can be accessed from the online public database and all the hyperlinks to publicly archived datasets were listed in the "Methods" part.

Competing interests

The authors declare that they have no conflict of interest.

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Authors' contributions

ZZ and HTN contributed to conception and design. YBY and PFZ contributed to collection and assembly of data. ZZ, GFM and MXZ contributed to data analysis and interpretation. YL and WJ contributed to manuscript writing. HTN contributed to the manuscript revision and finalization.

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Figures

Figure 1

Overview of mutation information in BLCA patients. (A-C) Variant classifications, variant types and SNV classes in BLCA samples. SNP: single nucleotide polymorphism, INS: insertion, DEL: deletion, SNV: single nucleotide variants. (D) Waterfall plot showing the mutation information for top 15 genes. (E) The coincident and exclusive associations across the top 30 mutated genes.

Figure 2

Clinical relevance and pathways analysis for TMB. (A) The best cut-off value of TMB was 6.4 computed by X-tile. (B) KM curves of overall survival of high and low TMB groups. (C-F) The correlation between TMB scores and clinical stage, T stage, pathology grade and smoking level. (G) The top 4 results of GSEA between high and low TMB patients.

Figure 3

Screening of DEGs between high and low TMB groups associated with overall survival. (A) Upset plot and Venn plot of the results of three DEG-analysis methods. (B) The heatmap showed expression levels of 36 DEGs between high and low TMB groups. (C) The forest plot showed the results of univariate cox analysis for 4 genes selected. (D-G) KM survival curves of 4 genes selected. (H) Validation of prognostic value for NTRK3 in GSE48276.

Figure 4

NTRK3 co-expression analysis in HCC (LinkedOmics). (A, B) Heat maps showing top 50 genes positively and negatively correlated with NTRK3 in BLCA. Red and blue represent positive and negative correlations, respectively. (C) The total NTRK3 highly correlated genes identified by Pearson test in BLCA cohort. (D) The significant KEGG pathways of NTRK3 in BLCA cohort.

Figure 5

Relationship between TMB scores, NTRK3 expression level and ESTIMATE results. (A) Comparison of NTRK3 expression level between high and low TMB groups. (B) Comparison of immune/stromal scores between high and low groups based on NTRK3 expression. (C, D) Comparison of immune/stromal scores between high and low TMB groups.

Figure 6

Immune infiltration analysis in BLCA. (A) Comparison of the infiltration of 22 leukocyte types between high and low TMB groups. (B) The correlation between NTRK3 and immune infiltrating cells. (C-E) The correlation between the expression of NTRK3 and the expression of CCL14, ADORA2A, and CXCL12 respectively.

Figure 7

Relationship between NTRK3 expression and immunoregulators. (A-D) Spearman correlations between expression of NTRK3 and immunoregulators (Y axis) across human cancers (X axis). (E-G) The KM survival curves of CCL14, ADORA2A and CXCL12 in BLCA.

A

Normal

B

Tumor

Figure 8

Immunohistochemistry of NTRK3 based on the Human Protein Atlas. (A) IHC staining of NTRK3 in bladder normal tissue. (B) IHC staining of NTRK3 in bladder tumor tissue.