Identification and validation of RB1 as an immune-related prognostic signature based on tumor mutation burdens in bladder cancer

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Bladder cancer (BCa) is one of the most common malignant tumors in the urinary system. Developing effective prognostic gene and exploring the immune cells that affect the prognosis of tumor are required. Full transcriptome data ($n = 433$), clinical information ($n = 581$) and mutation sequencing ($n = 412$) were obtained from The Cancer Genome Atlas and independent mutation sequencing data of 101 samples were acquired from International Cancer Genome Consortium. Statistical processing was conducted using R packages. Gene biologically functional research was performed with gene set enrichment analysis based on Kyoto Encyclopedia of Genes and Genomes database. Twenty-two types of immune cell infiltration were assessed and calculated in 398 samples of BCa. Furthermore, the expression of immune-related prognostic signature was verified. The relationship between prognostic gene and immune cells was explored preliminarily. Tumor mutation burdens of mutant-type groups were higher than wild-type groups of 19 genes, except for FGFR3 and CREBBP. Kaplan–Meier analysis showed that high frequency of retinoblastomal 1 (RB1) mutation led to poor prognosis of BCa patients and was an independent prognostic factor ($P = 0.004$; HR = 1.776). Proportions and correlation of 22 types of immune cells in 433 samples were determined. We found that RB1 expression decreased in BCa validated through quantitative PCR and immunohistochemistry. In addition, regulatory T cells (Tregs) were detected as a negatively correlated type of immune cell to mutation of RB1, whereas fluorescence costaining showed that Foxp3 expression of Tregs infiltration was negatively related to the expression of RB1. Mutation of RB1 can be identified as an independent prognostic predictor of BCa, and it may suppress the infiltration of Tregs in BCa tissues, increasing the incidence of tumor immune escape. *Anti-Cancer Drugs* 34: 269–280 Copyright © 2022 The Author(s). Published by Wolters Kluwer Health, Inc.

Anti-Cancer Drugs 2023, **34**:269–280

Keywords: International Cancer Genome Consortium, prognostic signature, RB1, The Cancer Genome Atlas, tumor mutation burden, Tregs

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Received 12 August 2022 **Revised form accepted** 12 August 2022

Introduction

According to the latest research, bladder cancer (BCa) is known as the 11th most common malignant tumor all over the world [1]. In Europe and America, the mortality caused by BCa is the fourth among males, ranking after prostate cancer, lung cancer and colon cancer till 2020 [2]. BCa can be classified into nonmuscular invasive BCa and muscular invasive BCa (MIBCa), and almost 25% of patients were identified as MIBCa at first diagnosis, suffering from relatively poorer 5-year survival of 50% [3,4]. Given that the existing therapy strategies were unable to provide satisfying improvements of long-term outcomes, new regimens targeting promising biomarkers are urgently needed.

Currently, a novel therapy aimed at the discovery of immune checkpoint inhibitors has been demonstrated as a prospective and practical means to resist cancers [5]. For instance, inhibition of programmed cell death-1 (PD-1) can temporize the programmed death of immune cells, thus increasing immunotherapy targets against various tumors appear. However, the theory in BCa remains to be proved [5]. As a consequence, exploring feasible immunosuppressive targets would provide prescient guidance when selecting treatment for patients with BCa.

Notably, the associations among tumor mutation burden (TMB), cancer immunotherapy and tumor-related prognosis have been persuasively demonstrated [6,7]. Rizvi *et al.* [8] argued that either in various oncogenes or tumor suppressor genes, the higher nonsynonymous mutation burden accumulates, the more potential neoantigens could emerge, which may increase the immune response against advanced and metastatic tumor cells. In terms of

one certain carcinoma, the higher TMB is measured, the stronger response to immunotherapy could happen [9,10]. In this updated study, The Cancer Genome Atlas (TCGA) and International Cancer Genome Consortium (ICGC) were employed in order to probe into the relations among degrees of TMB, prognosis and immune response.

Materials and methods

Data retrieval and tumor mutation burden-statistical processing

TCGA (https://cancergenome.nih.gov/) was applied to retrieve full transcriptome data of 433 BCa samples, clinical information of 581 patients and somatic mutation data of 412 individuals. Somatic mutation data of extra 101 samples were outrightly acquired from ICGC (https://dcc.icgc.org/) databases. The landscape genome mutations of different genes from two databases were demonstrated as waterfall plots separately. Statistics were processed and analyzed with the 'oncoprint' R package. Only nonsynonymous missense mutation was counted for the calculation. According to the corresponding gene mutation, samples were divided into wild and mutation groups. The difference of TMB calculation between two groups was performed in box plots, using two independent samples *t*-test, of which $P < 0.05$ was supposed to be statistically significant. Statistics analyses were processed with 'maftools' R package.

Prognostic factors analysis

Clinical data of 581 samples qualified with available prognostic information and baseline characteristics were downloaded from TCGA database. For different genes, Kaplan–Meier curves (K–M curves) with log-rank test were applied to characterize the difference of overall survival rates between wild cohort and mutation cohort. Hazard ratios (HRs) were used as effective value, of which $P < 0.05$ were supposed to be statistically significant. We performed univariate Cox regression as well as multivariate Cox regression analysis to distinguish specific genes as independent prognostic predictors from other features of BCa patients. The clinical baseline variables included age, sex, tumor grade, tumor/node/metastasis (TNM) stage and TMB occurrence.

Gene functional exploration

Gene set enrichment analysis (GSEA) was applied to predict the pathways relevant genes participating in between the wild and the mutation groups of TCGA-BCa samples, based on Kyoto Encyclopedia of Genes and Genomes (KEGGs) database [11,12]. Enrichment scores of most related genes were assessed for functional prediction. Statistics were processed and visualized with 'clusterProfiler' R package.

Immune cell infiltration assessment

The proportions of major 22 types of immune cells of 433 Bca samples were analyzed and visualized in the bar plot [13,14], including B cells, T cells, plasma cells, monocytes, macrophages, natural killer cells, eosinophils, neutrophils, etc. The correlations degree between each type of immune cell was evaluated with pair-wise correlation analyses and by Spearman's rank correlation analyses, of which $P < 0.05$ was considered to be statistically significant. The vioplot demonstrated the infiltrations of 22 types of immune cells in wild and mutation groups by using 'vioplot' R package, of which Wilcoxon rank-sum test's $P < 0.05$ was statistically significant criterion.

Immunohistochemistry and immunofluorescence

For immunohistochemistry, antibody against retinoblastomal 1 (RB1) was obtained from Cell Signaling Technology (Boston, Massachusetts, USA). Bladder tissue was fixed with 4% paraformaldehyde, embedded in paraffin, and then sectioned. Tissue sections were placed into a box and then heated in a microwave oven for antigen retrieval. Then, we blocked endogenous peroxidase by treating the sections with 3% hydrogen peroxide. Sections were then incubated with a primary antibody followed by an appropriate secondary antibody. Antibody binding was then visualized by diaminobezidin 3 (DAB) treatment. The nuclei were then stained, and the sections were dehydrated. Finally, the sections were mounted on glass slides for analysis. For immunofluorescent analysis, BCa tissue was frozen and then embedded in paraffin. The specimen was sealed in the sealed buffer for 60 min. Drain the sealing buffer and add diluted primary antibody. The fluorescently labeled secondary antibody was diluted with antibody dilution buffer, and the specimens were incubated at room temperature without light for 1–2 h. Similarly, antibody binding was then visualized by DAB treatment. Image-pro Plus software (version 6.0 , Media Cybernetics, Bethesda, Maryland, USA) was then used to evaluate the expression of RB1 and FOXp3.

Quantitative real-time PCR analysis

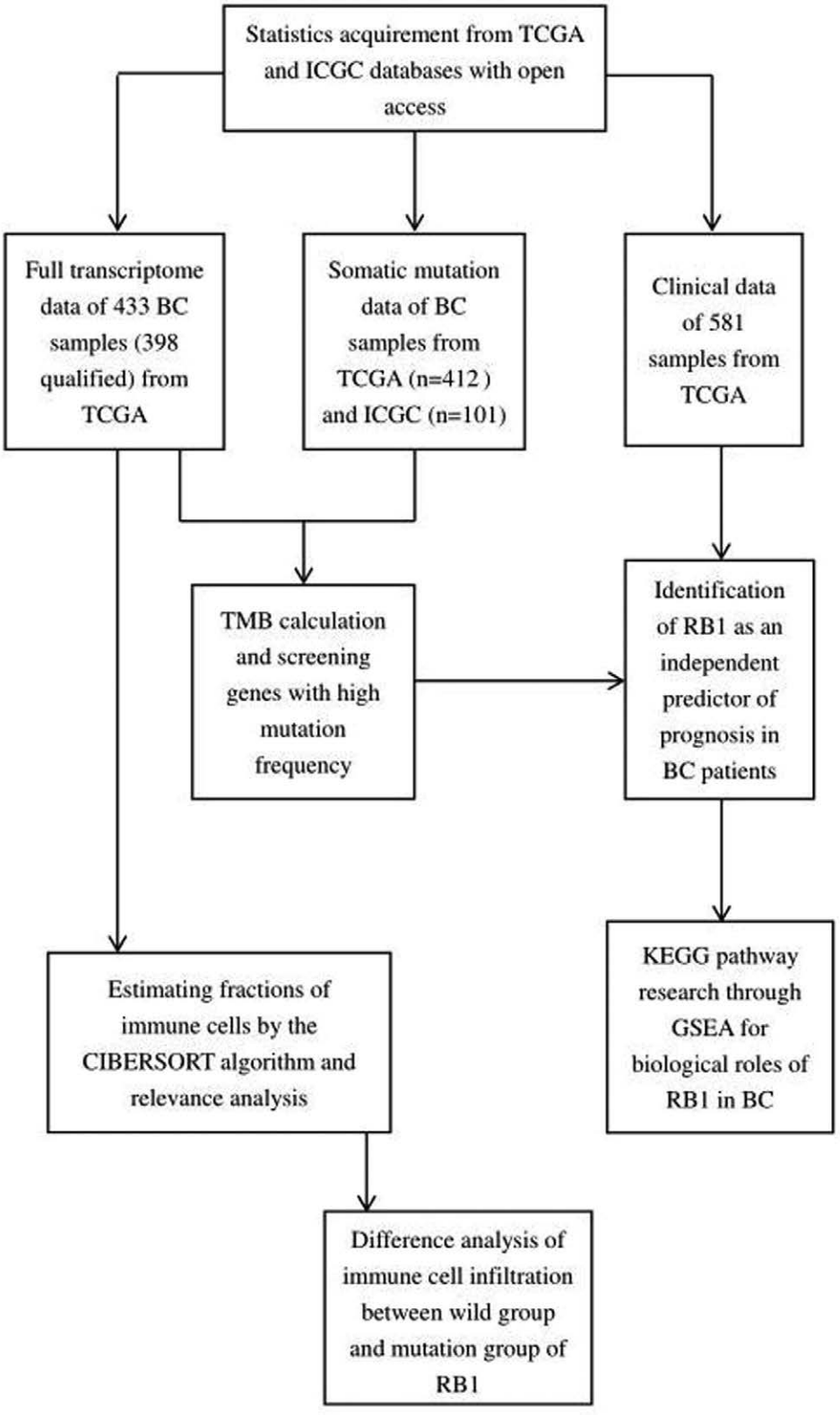
RNA extraction kits (TIANGEN, Beijing, China) were used to extract RNA of bladder tissue. The specific primers used were as follows: 5′GCGTGTTTGTGCCTGTCCTG (forward) and 5′TGGTTTCTTCTTTGGCTGGG (reverse). mRNA expression was normalized to β-actin expression. Every experiment was repeated at least three times.

Result

Description of tumor mutation burden in bladder cancer

The research process was showed in Fig. 1. Full transcriptome data belonging to 398 individual BCa samples were retrieved from TCGA database and 101 samples were retrieved from ICGC database, both with outrightly open acquisition access. Then we analyzed the whole-exome sequencing data using the 'maftools' package to visualize an exhaustive presentation of mutational information. The waterfall plots of mutational landscapes of TCGA and ICGC samples were separately established in Fig. 2a and b.

Fig. 1



The flow chart of the whole research process.

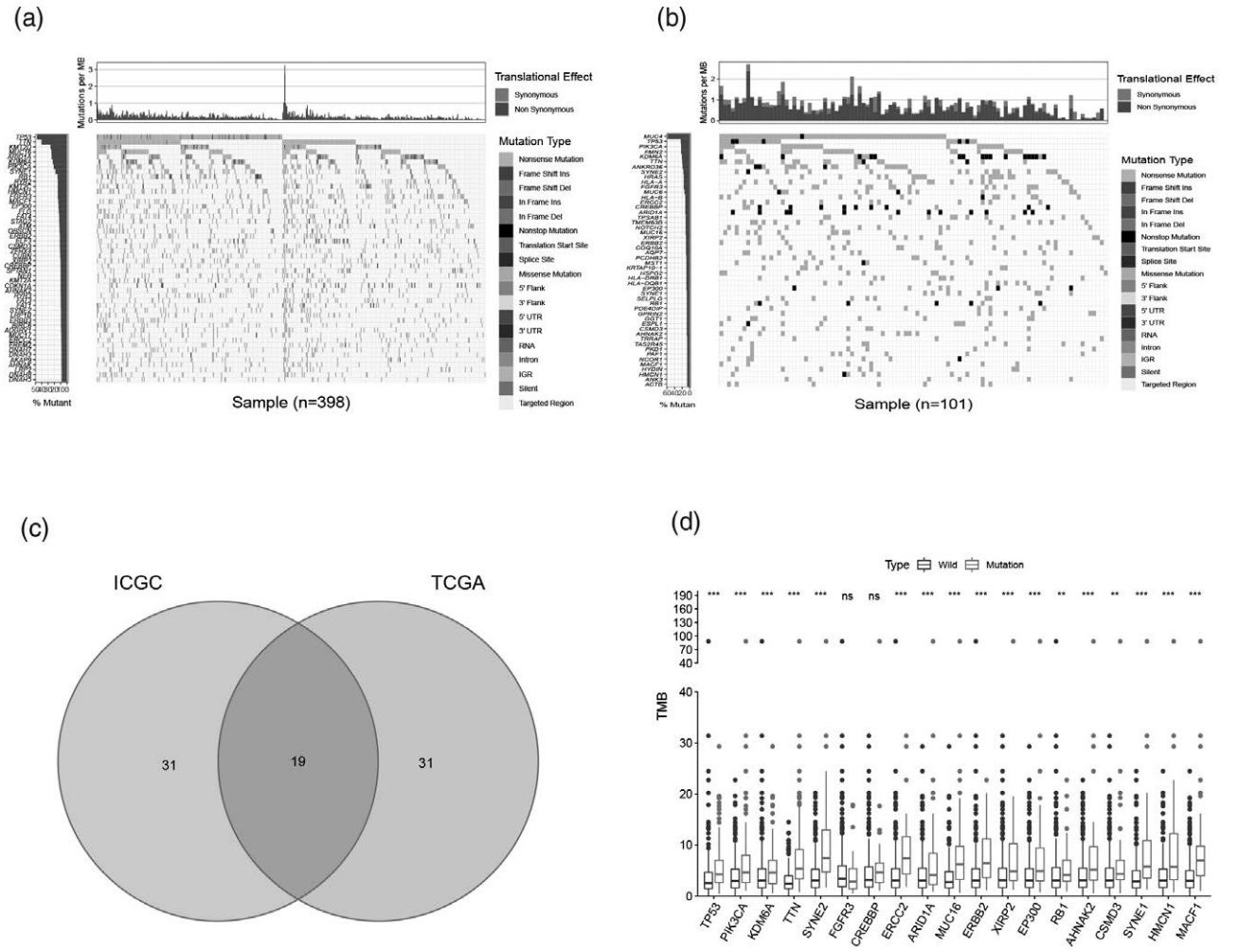
An intersection of 19 highly mutated genes was identified for further analyses after screening from the top 31 mutated genes of each database, including TP53, PIK3CA, KDM6A, RB1, etc. (Fig. 2c). Then the difference between wild-type groups and mutant-type groups of 19 included genes was analyzed. The results showed that TMB of mutation type BCa samples were higher than wild type in sets of TP53, PIK3CA, KDM6A, TTN, SYNE2, ERCC2, ARID1A, MUC16, ERBB2, XIRP2, EP300, RB1, AHNAK2, CSMD3, SYEN1, HMCN1 and MACF1, except for FGFR3 and CREBBP (Fig. 2d).

Overall survival analysis and prognostic factors identification

In order to figure out the influence of mutated genes on prognosis, we performed survival analyses of the 19 genes mentioned above. Nineteen K–M curves of overall survival were drafted to compare survival rates and times shown in Fig. 3a–s. We found that, among all of the 19 genes, the wild group presented significant survival advantage to the mutation group only in RB1 set (log-rank $P = 0.039$), which meant the high mutation frequency on RB1 had a negative effect on the prognosis of BCa patients.

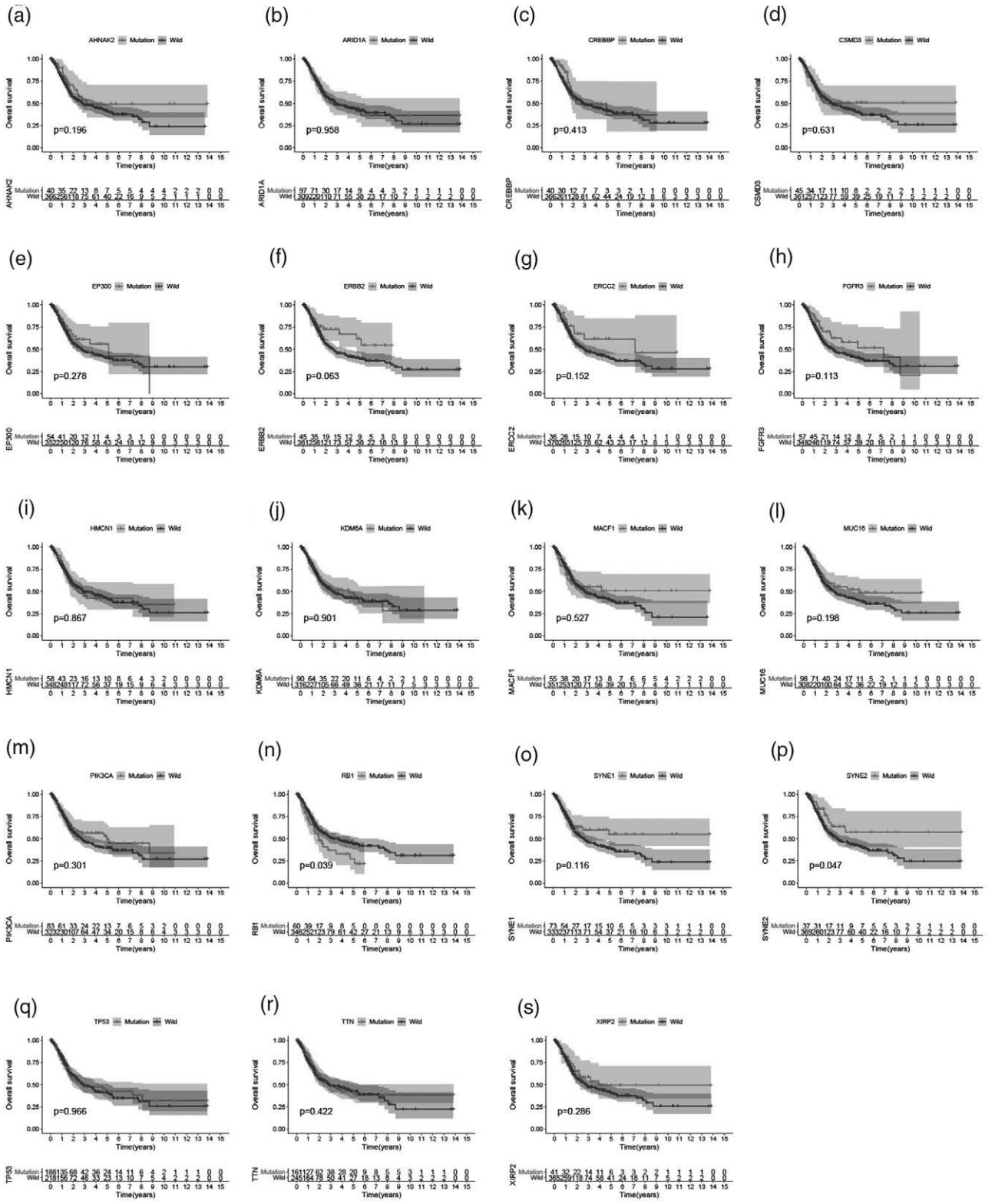
For further investigation of whether RB1 could be identified as an independent survival factor from other clinical characteristics, univariate Cox regression analysis and multivariate Cox regression analysis were separately performed to comprehensively analyze the HRs of multiple potential prognostic factors. Forest plots were shown in Fig. 4a and b. It was implied that age ($P = 0.001$; HR = 1.704), TNM stage ($P < 0.001$; HR = 2.285), TMB ($P < 0.001$; HR = 0.911) and

Fig. 2



Description of TMB features in BCa. (a) The waterfall plots of mutational information from TCGA ($n = 412$); (b) The waterfall plots of mutational information from ICGC ($n = 101$); (c) Vene plot of high-mutated genes of interest were selected after intersecting TCGA and ICGC. (d) Boxplot of TMB between wild and mutation groups. BCa, bladder cancer; ICGC, International Cancer Genome Consortium; TCGA, The Cancer Genome Atlas; TMB, tumor mutation burden.

Fig. 3



Kaplan–Meier curves (K–M curve) of 19 mutated genes. (a) AHNAK2; (b) ARID1A; (c) CREBBP; (d) CSMD3; (e) EP300; (f) ERBB2; (g) ERCC2; (h) FGFR3; (i) HMCN1; (j) KDM6A; (k) MACF1; (l) MUC16; (m) PIK3CA; (n) RB1; (o) SYNE1; (p) SYNE2; (q) TP53; (r) TTN; (s) XIRP2. RB1, retinoblastomal 1.

mutation of RB1 (P = 0.004; HR = 1.776) could be identified as independent prognostic factors affecting overall survival (Table 1). More evidently, according to the regression analyses, either TMB or mutation of RB1 is associated negatively with prognosis of BCa patients. This discovery was consistent with the conclusion that the RB1 mutation and TMB served together in the development of BCa.

Biological roles of RB1 in Kyoto Encyclopedia of Genes and Genomes pathways

To further predict the potential biological roles of RB1 in BCa, the GSEA analysis was employed for KEGG pathways research. In accordance with the GSEA score, KEGG pathways such as DNA replication, mismatch repair, cell cycle, oocyte meiosis and regulation of autophagy (Fig. 5), which mostly relate to cell proliferation and cell differentiation, enrich on the mutation region of RB1 band. This finding signifies that mutation types of RB1 might be more inclined to take part in cell proliferation and development of BCa.

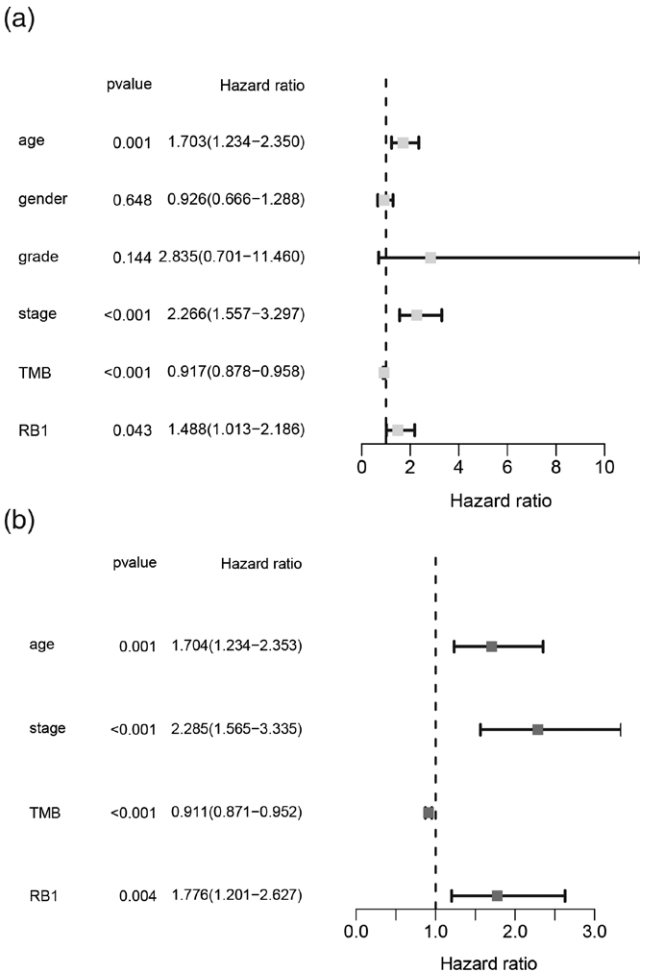
Quantitative PCR of RB1 expression in bladder cancer

To further validate the results *in vivo*, human bladder tissues were used to confirm the expression level of RB1. As the figure showed, compared with tumor-adjacent tissues, the expression of RB1 was lower in BCa (Fig. 6a and b). What is more, mRNA of RB1 was also lower in BCa (Fig. 6c and d).

Patterns of immune cell infiltration in bladder cancer tumor

The population proportions of 22 types of immune cells in 398 samples were visualized as a bar plot in BCa, and these types include B cells, T cells, plasma cells, monocytes, macrophages, natural killer cells, eosinophils, neutrophils, etc. As was presented in the bar plot, 398

Fig. 4



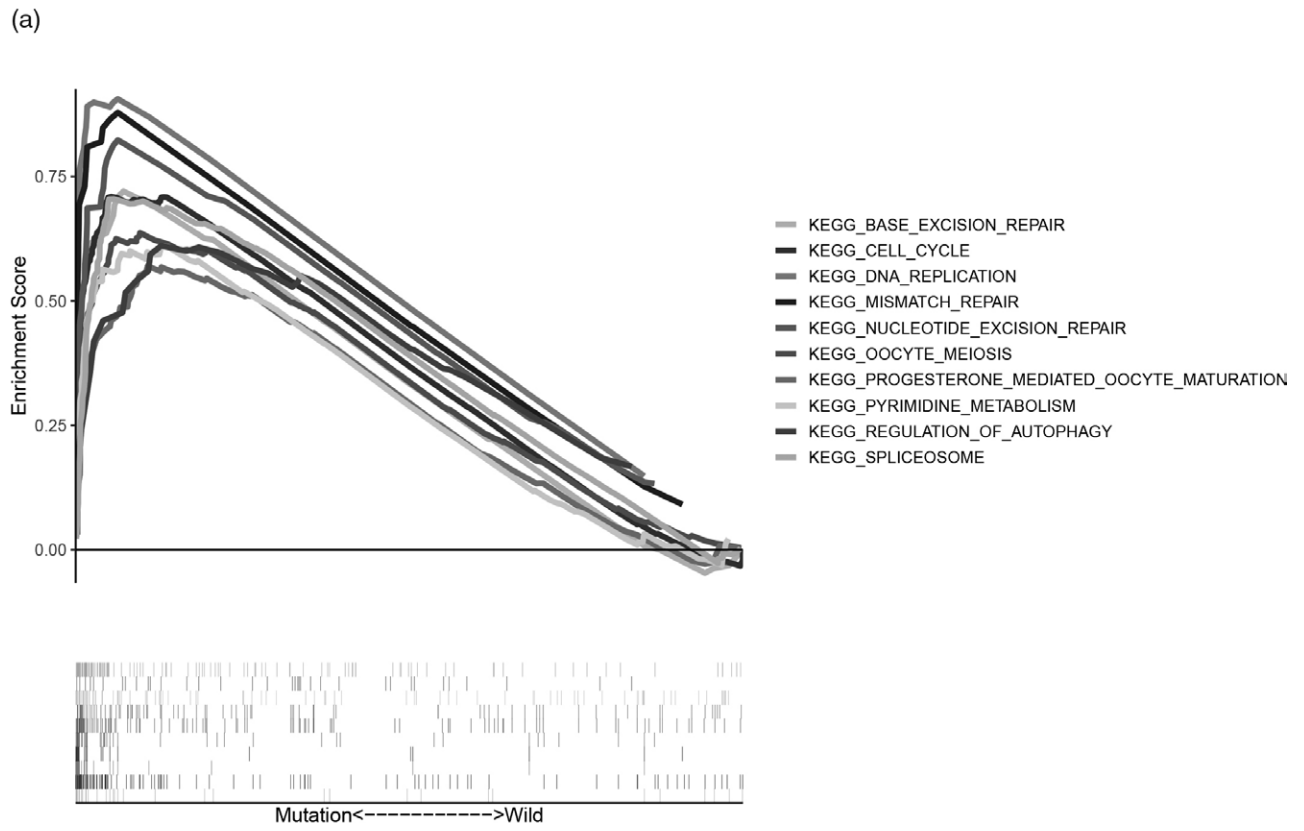
Identification of RB1 as an independent predictor of clinical outcomes in BCa patients. (a) Univariate Cox regression analysis; (b) Multivariate Cox regression analysis. BCa, bladder cancer; RB1, retinoblastomal 1.

Table 1 **Univariate and multivariate Cox regression analysis of bladder cancer patients**

Variables		Univariable model		Multivariable model	
		HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Age	>65/≤65	1.70 (1.23–2.35)	0.001	1.70 (1.23–2.35)	0.001
Sex	Female/male	0.93 (0.67–1.29)	0.648	-	-
Grade	High/low	2.83 (0.70–11.46)	0.143	-	-
Stage	(3–4)/(1–2)	2.27 (1.56–3.30)	<0.001	2.28 (1.57–3.33)	<0.001
TMB	High/low	0.92 (0.88–0.96)	<0.001	0.91 (0.87–0.95)	<0.001
RB1	High/low	1.49 (1.01–2.19)	0.042	1.78 (1.20–2.63)	0.004

CI, confidence interval; HR, hazard ratio; RB1, retinoblastomal 1; TMB, tumor mutation burden.

Fig. 5



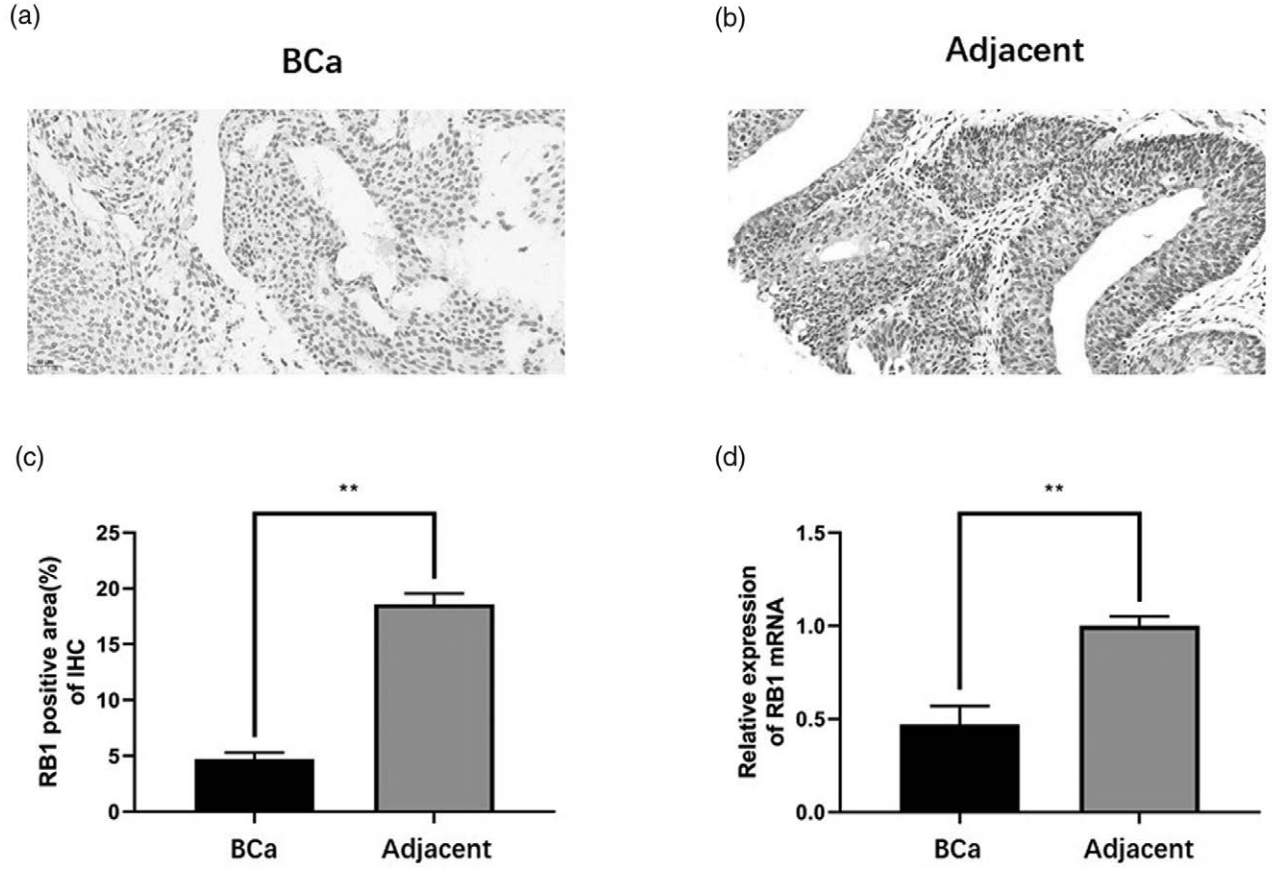
The potential biological roles of RB1 in BCa. GSEA of KEGG pathways RB1 takes part in after mutation. BCa, bladder cancer; GSEA, gene set enrichment analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes; RB1, retinoblastomal 1.

samples from TCGA database displayed the respective distributions of 22 immune cell infiltration (Fig. 7a).

As for the correlation patterns, relevance between each of the 22 immune cells was multifarious. CD4 memory-activated T cell showed significantly positive correlations with CD8 T cell, natural killer (NK) resting cell, macrophage 1 (M1) and follicular helper T cell. Whereas, both of CD8 T cell and CD4 memory activated T cell associated, respectively, negatively with CD4 memory resting T cell and macrophage 0 (M0) (Fig. 7b). This finding indicated that immune cells cooperating together are inclined to infiltrate in tumors. In contrast, those with conversed function tended to exist mutually opposite to each other in the tumor microenvironment of BCa.

In terms of RB1mutation, fractions of 22 immune cell types were established in the vioplot. The result revealed that the amount of Tregs) infiltrating in the wild-type group was significantly higher than in mutant-type group (Wilcoxon rank-sum test, $P = 0.031$; Fig. 7c).

Fig. 6



Validation of RB1 as a prognostic factor. (a) Immunohistochemistry of RB1 in tumor-adjacent tissues and BCa; (b) quantitative analysis of immunohistochemistry; (c) mRNA expression of RB1 in tumor-adjacent tissues and BCa. BCa, bladder cancer; RB1, retinoblastomal .

In-vivo validate the coexpression of FOXp3 and RB1

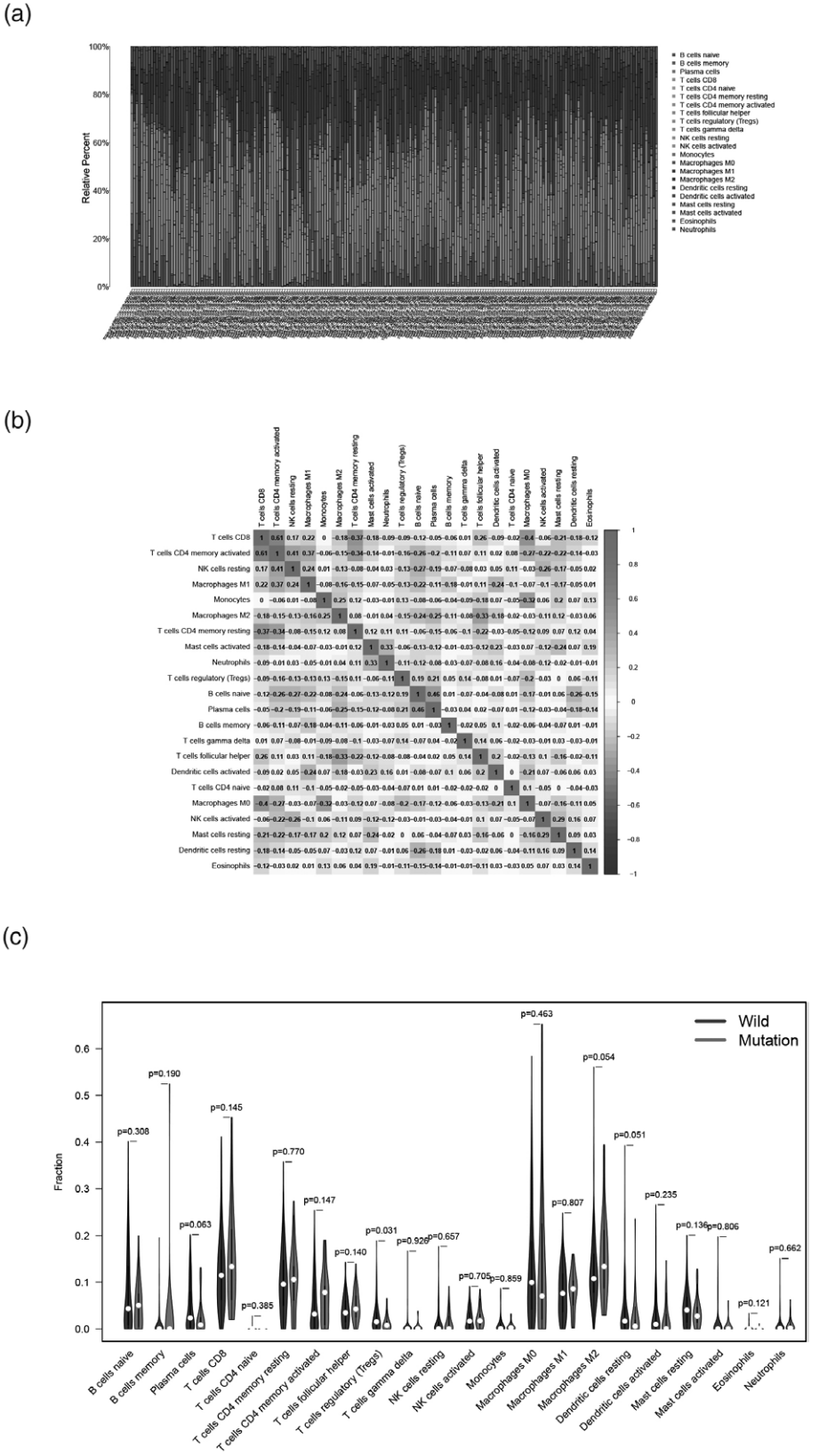
To explore the relationship between RB1 expression and Tregs infiltration in BCa, immunofluorescence multiple staining was employed. According to the TNM stage system, BCa were divided into high degree of malignancy and low degree of malignancy. Two kinds of BCa were stained separately. Results showed that RB1 expression in high degree of malignancy decreased when compared with low degree of malignancy. At the same time, Tregs infiltration increased reflected by expression of the marker Foxp3 (Fig. 8).

Discussion

BCa was the most prevalent malignant tumor of urinary system, and an increasing attention has been paid to cancer immunotherapy for its effectiveness and accuracy in treating advanced and metastatic carcinoma [14–16]. In 2017, Goodman *et al.* [10] pointed out that tumor mutational burden can function as an independent predictor of response to immunotherapy in various cancers. Subsequently, Fan *et al.* [17] identified three specific long-noncoding RNAs (lncRNAs) as correlated factors with TMB and clinical outcomes of triple-negative breast cancer in 2019, revealing that lncRNAs can be predictors of TMB and prognosis. Till 2020, Wang *et al.* [18] complementary of microRNA as a marker of TMB and immune cell infiltration in lung adenocarcinoma, which led to further refinement of the association among noncoding genes, TMB and immune cell infiltration. Moreover, the communication among coding RNAs expression, TMB level, prognosis and immune cell infiltration was currently described by Wang *et al.* [19] in breast cancer. However, researches on the gene mutation as a TMB-related factor are rarely reported. Therefore, given this imperfection of prognostic or immunotherapeutic model construction, we carried out this comprehensive analysis to explore the relationship among mutation, TMB and immune cell infiltration in BCa.

We found 19 genes with high levels of missense mutation in BCa samples by intersecting sequencing data from TCGA (n = 412) and ICGC (n = 101), of which 17 gene sets showed significant difference of TMB calculation

Fig. 7



Patterns of immune cell infiltration in BCa. (a) Barplot of immune cell population proportions in BCa; (b) Corrplot of immune cell populations in BCa; (c) Vioplot of immune cells between wild and mutation type groups. BCa, bladder cancer.

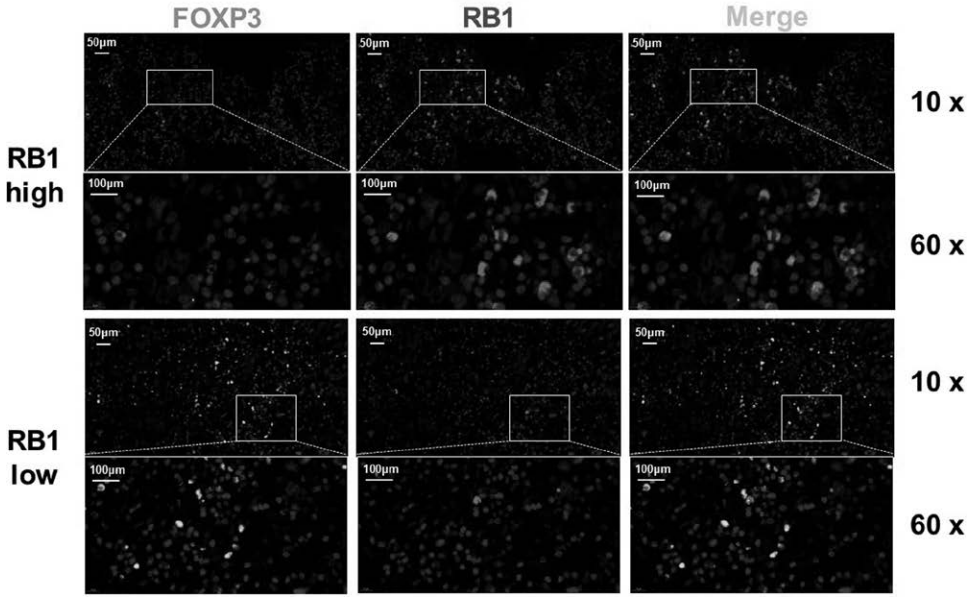
between wild type and mutation type. With the present or absent mutations of the 17 genes, levels of TMB, respectively, appear high and low, verifying that the mutation frequencies of TP53, PIK3CA, KDM6A, TTN, SYNE2, ERCC2, ARID1A, MUC16, ERBB2, XIRP2, EP300, RB1, AHNAK2, CSMD3, SYEN1, HMCN1 and MACF1 were all associated with the high TMB calculation of BCa samples. Notably, TP53 and RB1 were widely known as important antioncogenes in diverse range of cancers, whereas KDM2A was acknowledged as a core gene that promotes tumor development by participating in histone methylation [20–23]. Besides, according to the case report of Cao *et al.* [24] in Jun 2021, both high TMB and mutation of RB1 were detected in MIBCa patients, who showed less sensitive response to chemotherapy plus immunotherapy and poorer prognosis. The former promising discovery promoted us to further explore the potential effects of mutation of RB1 on immunotherapy to BCa. Thus, we analyzed the relationship between the mutation of RB1 and immune cell infiltration in future steps.

In the further study of the influence of mutation on prognosis, mutation occurring to RB1 significantly contributed to poorer clinical outcome. As is referred to before, RB1 represses the proliferation and malignant progression through negatively regulating the cell cycle in BCa. Consequently, it can be inferred that the high mutation occurring in RB1 sequence likely depresses its original function of cell cycle delaying. In other words, the high mutation in RB1 might promise the high TMB of BCa patients and coaccelerates malignant development of the tumor and lead to the poor prognosis, which is consistent with the argument of Cao *et al.* [24] and Luo *et al.* [25]. Moreover, performed univariate Cox regression as well as multivariate Cox regression analysis and further validated the mutation of RB1 was an independent prognostic predictor of BCa patients.

The subsequent analysis of the KEGG pathways reinforces the findings above. RB1 gene was more likely to participate in signal pathways related to cell proliferation and cell differentiation like DNA replication, oocyte meiosis and regulation of autophagy. Thus, mutation might alter its original roles in pathways. In a word, the biofunctions of the TMB-related genes could participate in complex biological signal pathways associated with the occurrence and development of BCa, including RB1.

In the tumor microenvironment, infiltration of inflammatory cells was one of typical features, which affected tumor proliferation and development [26,27]. Mutual effects between inflammatory cytokines and immune cells were of major concern. Previous clinical studies revealed that the immune cell infiltration gives rise to different denouements of carcinomas [28,29]. Thus, the relationship between TMB and immune cell infiltration was taken into consideration in this study. A landscape of immune infiltrating clusters on the basis of transcriptome from clinical samples has been shown without discernible patterns. Among the 22 types of immune cells, those with similar biological functions (CD4 memory

Fig. 8



Coexpression of Foxp3 and RB1. High degree of malignancy and low degree of malignancy were stained by immunofluorescence multiple staining method. RB1, retinoblastomal 1.

activated T cell, CD8 T cell, NK resting cell, M1 and follicular helper T cell) manifest positive correlations with each other, whereas functionally opposite cell types were mutually incompatible. This finding supported the current discovery of Narayanan *et al.* [30] in colorectal cancer. Interestingly, Tregs interact apparently negatively with proinflammatory cells, such as CD4 memory activated T cell, CD8 T cell, NK resting cell and (M1).

Furthermore, Tregs have been reported as a particular regulatory target of RB1 and able to suppress the infiltration of leukocytes and maintain immune homeostasis [31]. The specific trait of Tregs allows it to depress the immune responses against viruses, tumors and self-antigens [32]. According to our preliminary bioinformatic analyses, mutation of RB1 suggested lower infiltration of Tregs in BCa. However, the contrary results were discovered when we conducted immunofluorescence assay on BCa tissues for further in-vivo validation. We found that relatively lower expression of RB1 and higher expression of FOXp3 was detected in BCa tissue of higher pathological grade, whereas the opposite result was found in that of lower pathological grade. This discovery indicated that the high mutation of RB1 could result in the significant reduction of the immune infiltration of Tregs in BCa samples and might stimulate the malignant development of BCa, which agreed with the reported immunological function of Tregs in Bca [33]. From the perspective of the mechanism, chances were RB1 could inhibit the Tregs-associated inflammatory suppression, which was reversed by its mutation occurred in BCa. This way to undermine immune responses against tumor might also be a plausible answer to tumor immune escape of BCa.

In the present research, according to bioinformatics analyses, we put forward that the high mutation of RB1 in BCa could promise the high TMB and the extensive infiltration of Tregs. Moreover, mutations in RB1 detected by genetic testing in BCa patients were suggested to be a tool for prognostic prediction, according to our results. However, there were some inevitable deficiencies in our study. For example, because of the external data extracted from databases, we performed experimental methods for validation. Although prospective validations based on experimental results would make our findings more persuasive, feasible experiments were restricted due to the limitation of equipment and the specimens not readily available. Biases would also generate during the progress of genes selection. For example, significance was observed on SYNE 2 in TMB calculation and survival analysis, but its association with cancers is still inconclusive.

To conclude, the high mutation in RB1 implied high TMB in BCa, which indicated that RB1 mutated BCa patients might be potential candidates for PD-1 therapy. It could serve as an independent predictor of poor prognosis in BCa patients, as well. Meanwhile, the mutations in RB1 were inclined to protect BCa from inflammatory cell infiltration against cancer, through enhancing the infiltration of Tregs in tumor microenvironment, in turn leading to further malignant development.

Acknowledgements

This work was supported by the Key Program Project of Jiangsu Province [grant number BE2019751]. ; Natural Science Foundation of China [grant number 82100732]; Natural Science Foundation of Jiangsu Province [granted number BK20200360]; Excellent Youth Development Fund of Zhongda Hospital, SEU [grant number 2021ZDYYQPY04].

H.C., W.L. and M.C. designed the study; N.L. and T.W. conducted the study and maintained the data; Y.M. analyzed the data and made the figures; all authors drafted the paper and approved this version of the manuscript.

Ethics approval: the research procedure was in accordance with the ethical standards of the Declaration of Helsinki and Istanbul. The study protocol involving human bladder tissues was approved by the local ethics committee of Zhongda Hospital Southeast University. Written informed consent was obtained from all patients.

Data availability: the datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

Conflicts of interest

There are no conflicts of interest.

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