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Original Article

Tumor Microenvironment in Clear Cell Renal Cell Carcinoma: A Comprehensive Analysis

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Abstract

Background: M6A RNA methylation and the tumor microenvironment (TME) have been reported to play important roles in the progression and prognosis of clear cell renal cell carcinoma (ccRCC). However, whether m6A RNA methylation regulators affect the TME in ccRCC remains unknown. Thus, we aimed to evaluate comprehensively the effect of m6A RNA methylation regulators on the TME in ccRCC.

Methods: Transcriptome data of ccRCC were obtained from TCGA database. Consensus clustering analysis was conducted based on the expression of m6A RNA methylation regulators. Survival differences were evaluated by Kaplan–Meier analysis between the clusters. The DESeq2 package was used to analyze the differentially expressed genes (DEGs) between the clusters. GO and KEGG pathway analyses were performed by the ClusterProfiler R package. The CIBERSORT algorithm was used to evaluate immune infiltration.

Results: The expression of 15 m6A regulators significantly differed between ccRCC and normal kidney tissues. Based on the expression of these 15 m6A regulators, two clusters were identified by consensus clustering, in which cluster 1 had better overall survival (OS). Overall, 4,429 DEGs were identified between the two clusters and were enriched in immune-related biological processes. Cluster 1 had lower immune and ESTIMATE scores, higher expression of HLA and lower expression of immune checkpoint molecules. Moreover, immune infiltration and expressions of Th1/IFNγ gene signature were also significantly different between the two clusters.

Conclusion: Our study revealed m6A regulators were important participants in the development of ccRCC, with a close relationship with the TME.

Keywords: M6A RNA methylation regulator; Tumor microenvironment; Clear cell renal cell carcinoma

Introduction

Renal cell carcinoma (RCC) ranks sixth in males and tenth in females among the most common cancers (1). Clear cell renal cell carcinoma (ccRCC), which accounts for 70%–80% of all RCCs, is the most frequent primary RCC (2). In recent years, the treatment of RCC has made great progress with the development of surgical resection, chemotherapy and radiotherapy. However, overall survival (OS) is still dismal (3). One-third of patients relapse after an average of 1.9 yr (4), and patients who have local or distant metastasis further experience high mortality (5). Unfor-

tunately, effective RCC prognostic markers are still lacking due to the high heterogeneity and complex disease process. Therefore, it is of great significance to analyze the pathogenesis at the molecular level and to predict accurately the prognosis of ccRCC patients.

N6-methyladenosine (m6A) has gained renewed interest as a new layer of gene expression regulation, participates in more than 60% of RNA methylation and has become the most common posttranscriptional modification of mRNA (6-8). The regulation of m6A exists in a reversible dynamic equilibrium (9). M6A can be produced by m6A methyltransferases ("writers") and removed by m6A demethylases ("erasers"). Additionally, m6A-binding proteins ("readers"), including the YT521-B homology (YTH) domain family, insulin-like growth Factor 2 mRNA-binding proteins (IGF2BPs), and heterogeneous nuclear ribonucleoproteins (HNRNPs), are responsible for the identification of m6A-modified targeted RNAs (10). M6A RNA methylation regulators are involved in tumorigenesis and have a great impact on the survival of patients with various cancers, including ccRCC (11-14).

Cancer progression is inevitably related to an abnormal tumor microenvironment (TME). Deterioration of the TME promotes cancer cell escape from immune surveillance and accelerates the occurrence, development and metastasis of RCC (15). In addition, the interactions of chemokines with their receptors can recruit immune cell subsets into the TME to further modulate tumor progression and metastasis (16,17).

Abnormal m6A regulators are associated with the TME and immunotherapy responses in melanoma (18), colorectal cancer (19), gastric cancer (20) and head and neck squamous cell carcinoma (21), however, the interaction of m6A regulators with the microenvironment in ccRCC remains unclear. Thus, the purpose of this study was to evaluate comprehensively the correlation of m6A RNA methylation regulators with prognosis, immune checkpoint molecules, and the TME in ccRCC to better understand the etiology and provide new insight for immunotherapy of ccRCC.

Methods

Identification of m6A RNA methylation regulators

Twenty-one m6A regulators were identified in previous study (22). By using R package TCGAbiolinks, RNA-sequencing data and fragments per kilobase of exon model per million mapped fragments (FPKM) were downloaded from the TCGA database (https://portal.gdc.cancer.gov/), including mRNA expression data from 539 ccRCC and 72 normal samples. Next, the ensemble ID was converted to gene symbol by downloading the annotation file from GENCODE (https://www.gencodegenes.org/human/), extracting the mapping information of Gene Symbol and ENSG_ID and finally mapping ENSG_ID to Gene Symbol. The expression levels of m6A regulators were then obtained using R package Limma.

Consensus clustering analysis

We used unsupervised consensus clustering analysis to classify ccRCC patients into different groups with distinct m6A modification patterns based on the overall expressions of m6A regulators that had significantly different expressions between ccRCC and normal kidney samples. Consensus clustering performed by "ConsensusClusterPlus" R package is a reliable unsupervised machine learning method, in which we repeated 1000 repetitions by subsampling 80% of sample size and divided each subsample using k-means into multiple groups. The optimal number of clusters were determined by consensus matrix plot and cumulative distribution function (CDF) plot. The difference in OS between different clusters was assessed by the Kaplan–Meier method and log-rank test.

GO and KEGG pathway enrichment analysis of DEGs

The DEGs between different clusters were identified using the DEseq2 package of R. A $P<0.05$ and $|\log_2(\text{Fold change})| > 1$ were used to judge the statistical significance of gene expression dif-

ferences. The "ClusterProfiler R package" was used to analyze the GO and KEGG pathway enrichment. The GO terms included biological process, cellular component and molecular function. An adjusted $P<0.05$ was considered statistically significant.

Evaluation of tumor microenvironment

The immune score and ESTIMATE score were calculated by the ESTIMATE algorithm using the "ESTIMATE" R package. CIBERSORT was used to predict the proportion of 22 types of tumor infiltrating immune cells (memory B cells, naive B cells, activated dendritic cells, resting dendritic cells, eosinophils, M0 macrophages, M1 macrophages, M2 macrophages, activated mast cells, resting mast cells, monocytes, neutrophils, activated NK cells, resting NK cells, plasma cells, activated $CD4^+$ memory T cells, resting $CD4^+$ memory T cells, $CD4^+$ naive T cells, $CD8^+$ T cells, follicular helper T cells, gamma delta T cells, regulatory T cells) in each sample.

Estimation of immunotherapeutic response

The TIDE algorithm and subclass mapping were used to analyze the difference in response to PD-1 and CTLA-4 blockade between different clusters.

Statistical analysis

All data were analyzed by R (version 4.0.0), the Mann-Whitney-Wilcoxon test was used to compare the data from different clusters, and a $P<0.05$ was considered statistically significant.

Results

The expression of m6A regulators is related to the development of ccRCC

We selected 21 m6A regulators for further investigation. As shown in Fig. 1A-B, the expression of 15 m6A regulators was significantly different between ccRCC and normal kidney tissues, among which the expression levels of *METTL3*, *WTAP*, *RBM15*, *FTO*, *ALKBH5*, *YTHDC2* and *IGF2BP3* were higher in ccRCC samples than in normal samples. Compared with ccRCC samples, the expression of *METTL14*, *ZC3H13*, *RBM15B*, *YTHDF2*, *YTHDF3*, *IGF2BP2*, *HNRNPC* and *HNRNPA2B1* was significantly increased in normal kidney samples. The results of Kaplan–Meier analysis showed that 9 m6A regulators, including METTL3, WTAP, YTHDC2, IGF2BP3, METTL14, ZC3H13, YTHDF3, IGF2BP2, and HNRNPA2B1, were associated with the prognosis of ccRCC patients. ccRCC patients with higher expression of *METTL3*, *IGF2BP3*, *WTAP*, *IGF2BP2* and *HNRNPA2B1* had a more favorable prognosis, while patients with higher expression of *METTL14*, *ZC3H13*, *YTHDF3* and *YTHDC2* had a shorter survival time (Fig. 1C). These results indicated that the expression of m6A regulators was related to the development of ccRCC.

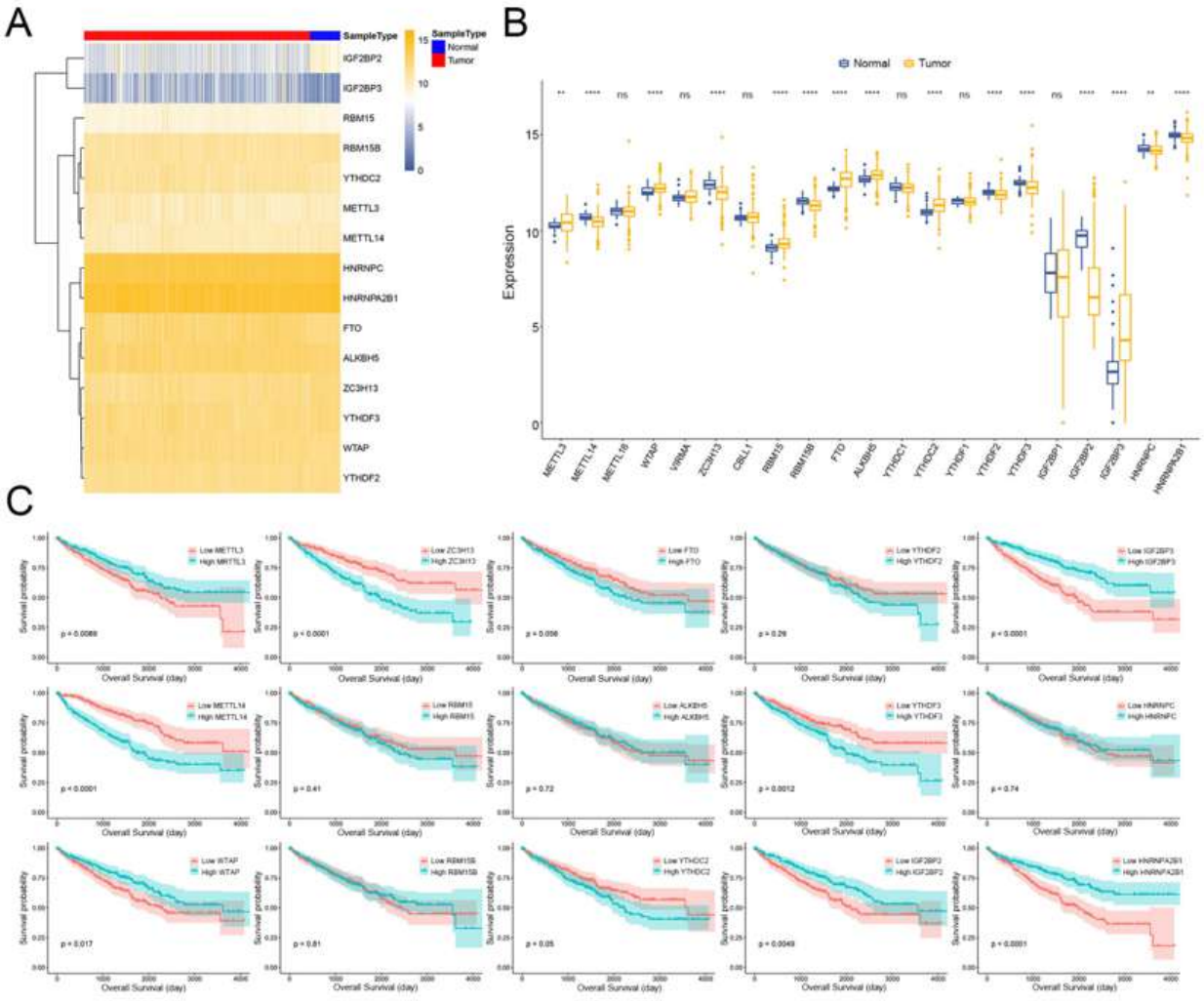

Fig. 1: Expression of m6A methylation regulators and OS analysis in ccRCC patients. Heatmap (A) and Vioplot (B) visualizing the expression levels of m6A methylation regulators among ccRCC and normal kidney tissues. Survival analyses for m6A methylation regulators with high or low expression by the Kaplan–Meier method (C)

Identification of two subtypes in ccRCC based on the expression of m6A regulators

Consensus clustering was then performed to classify ccRCC patients into clusters based on the expression of these 15 m6A regulators. Notably, K=2 was identified with optimal clustering (Fig. 2A-B). A total of 479 ccRCC patients were clustered into two subgroups, with 337 patients in cluster 1 and 142 patients in cluster 2. In addition, the PCA results clearly showed that there were two distinct distributions of the two clusters (Fig. 2C). Kaplan–Meier analysis showed that the patients of cluster 1 had a better OS than those of cluster 2 (Fig. 2D).

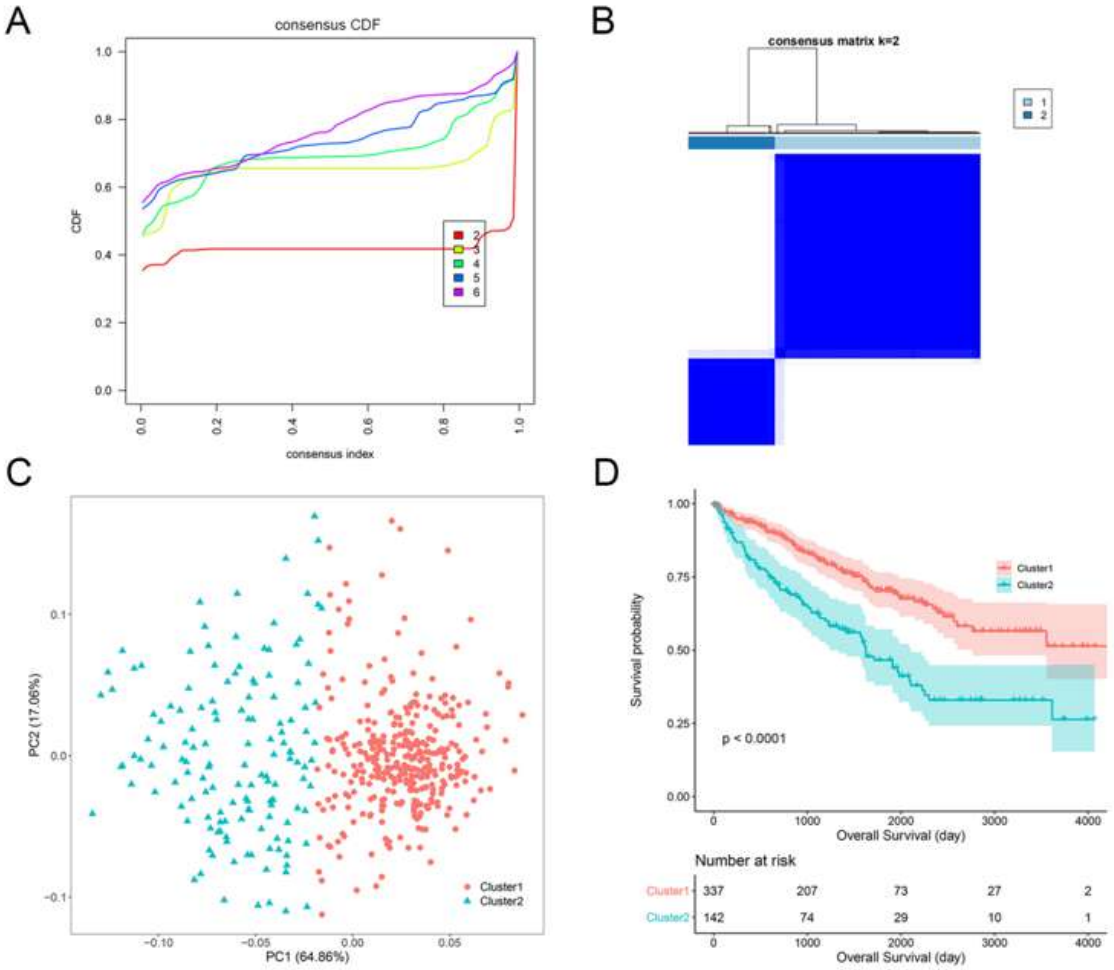

Fig. 2: Identification of two subgroups in ccRCC based on m6A regulator levels. (A) CDF visualization for k equals 2 to 6; (B) Consensus clustering matrix for k equals 2; PCA (C) and Kaplan–Meier (D) analysis of the two clusters

Biological function analysis of DEGs between cluster 1 and cluster 2

To better characterize the difference in biological processes involved in the development of ccRCC between the two clusters, we compared the expression of genes in the two clusters, and 4,429 DEGs were found (Fig. 3A). To obtain further insights into the biological characteristics of the DEGs, GO and KEGG pathway analyses were performed. GO analysis showed that the DEGs were enriched in immune-related functions, such as regulation of immune effector process, production of molecular mediator of immune response, immune response-activating cell surface receptor signaling pathway, lymphocyte mediated immunity, B cell mediated immunity, immunoglobulin mediated immune response and regulation of humoral immune response (Fig. 3B). Moreover, the results of the KEGG pathway analysis revealed several immune-related pathways, such as cytokine-cytokine receptor interaction, calcium signaling pathway, complement and coagulation cascades and bile secretion (Fig. 3C). These results demonstrated that m6A regulators were involved in the immunity of ccRCC patients.

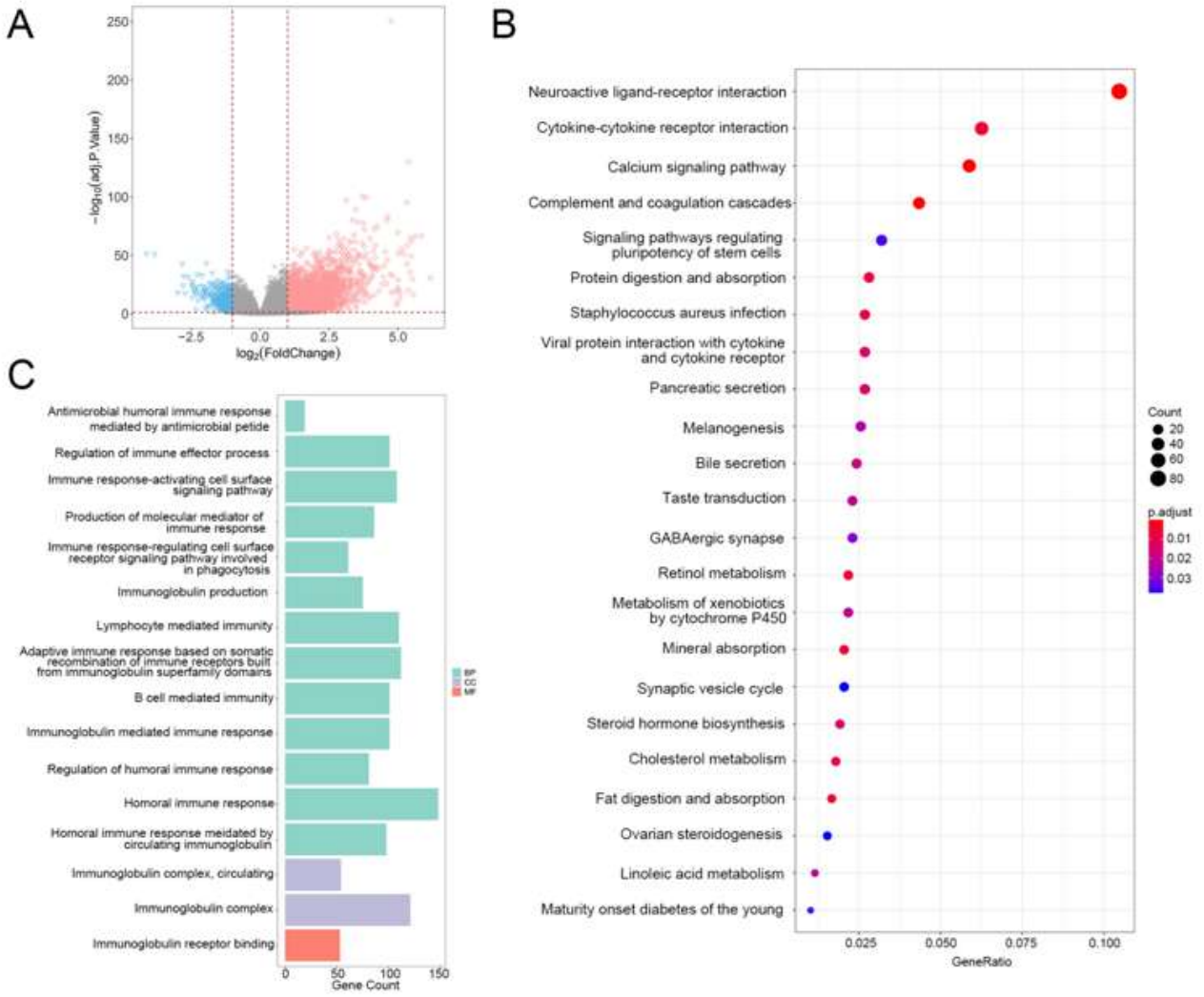

Fig. 3: Biological functional annotation of DEGs among the two clusters. Volcano map (A), GO (B) and KEGG (C) analyses of 4,429 DEGs

Identification of different TME characteristics between cluster 1 and cluster 2

We studied the TME characteristics of the two clusters to explore further the role of m6A regulators in ccRCC. The expression patterns of m6A regulators and TME features in cluster 1 and cluster 2 are shown by the heatmap (Fig. 4A). Specifically, the immune and ESTIMATE scores were remarkably higher in cluster 2 (P=0.0027 and P=0.018, respectively), and the stromal score of cluster 2 was relatively higher with no statistical significance compared to cluster 1 (P=0.8) (Fig. 4B-D), suggesting that m6A regulators were correlated with immune infiltration and tumor purity.

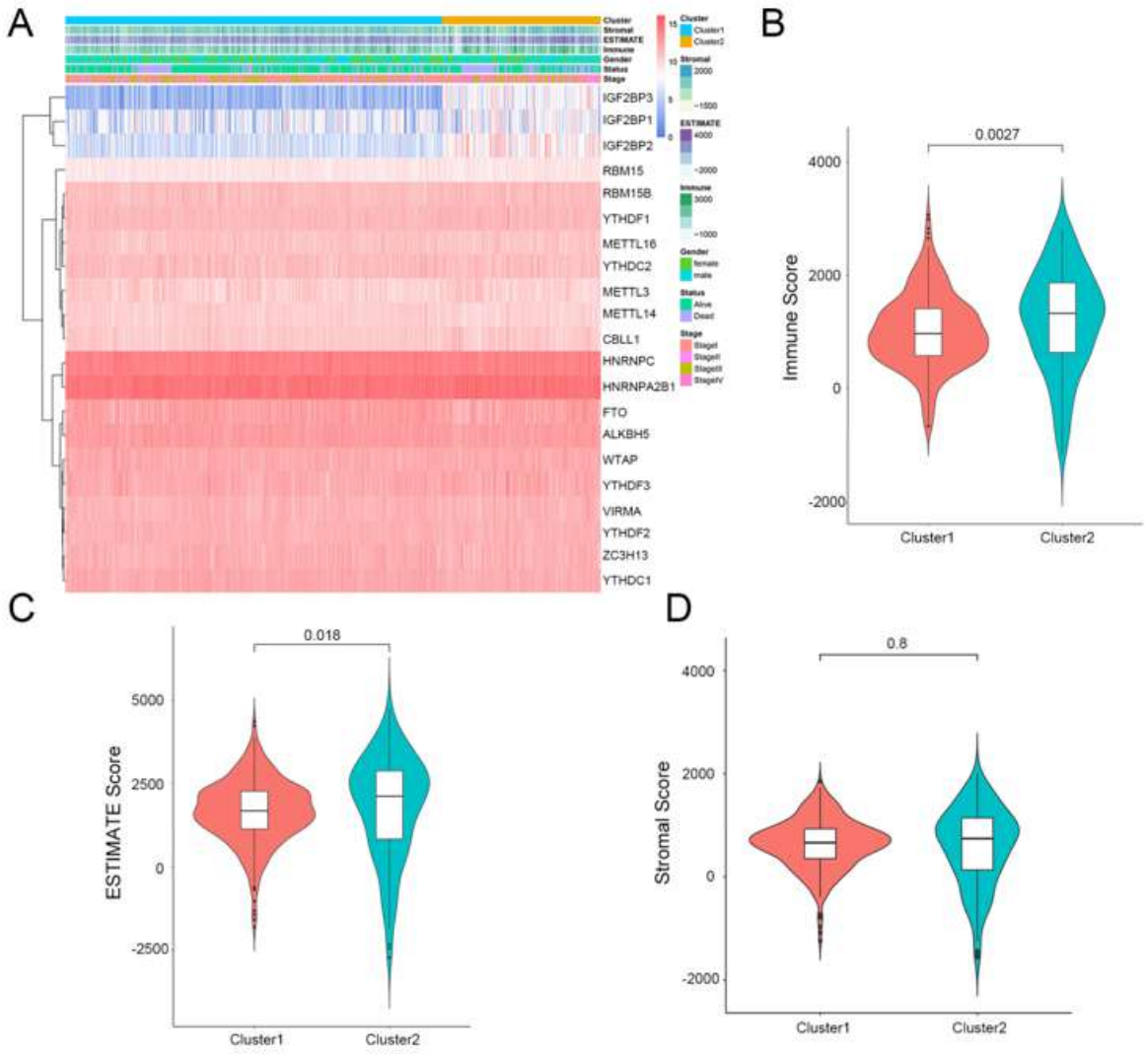

Fig. 4: Identification of different TME characteristics between the two clusters. (A) Heatmap visualizing m6A regulators and TME features between the two clusters. Comparison of the immune (B), ESTIMATE (C) and stromal (D) scores between cluster 1 and cluster 2

Identification of different immune landscapes between cluster 1 and cluster 2

Given the significant difference in the TME between the two clusters, we performed CIBERSORT analysis to estimate the immune infiltration of the two clusters. The distribution of immune cells was compared between cluster 1 and cluster 2 (Fig. 5) (Supplementary Fig. 1- not published). The abundances of 11 immune cell types were significantly different between the two clusters, with higher proportions of naive B cells, $CD4^+$ naive T cells, regulatory T cells, activated $CD4^+$ memory T cells and M0 macrophages and lower proportions of plasma cells, M1 macrophages, resting NK cells, monocytes, resting rest cells and neutrophils in cluster 2. Clustering subgroups based on the expression of m6A regulators were closely related to the immune microenvironment in ccRCC.

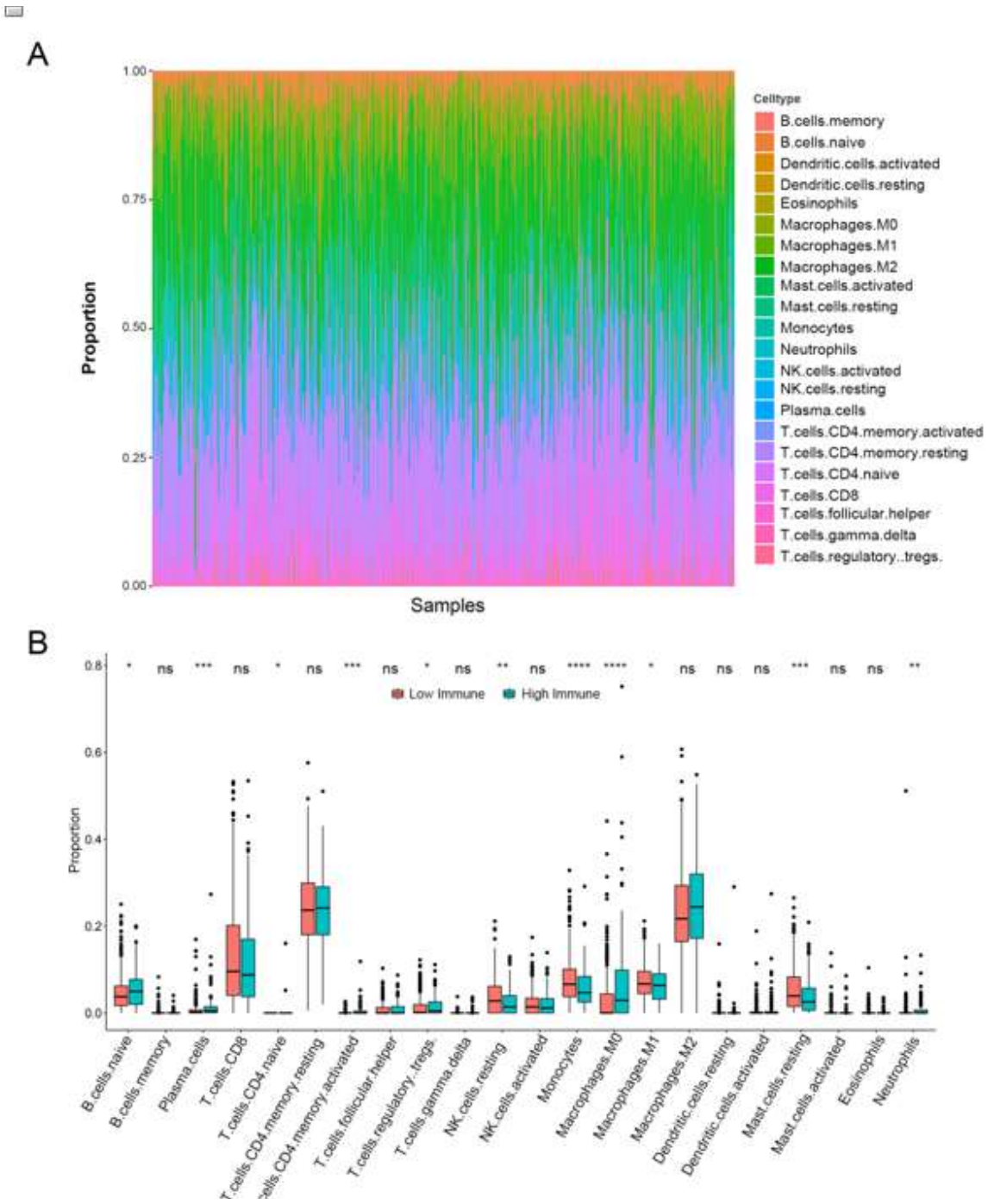

Fig. 5: Evaluation of immune cell infiltration between the two clusters. (A) The immune infiltration in each sample. (B) Comparison of 22 kinds of immune cells between the two clusters

Identification of different expression levels of HLA, immune checkpoint molecules and Th1/IFNγ pathway signatures between cluster 1 and cluster 2

We found that the expression of 12 HLA genes significantly differed between the two clusters, among which the expression of 11 HLA genes (P<0.05) (Fig. 6A). The 19 immune checkpoint molecules were remarkably differentially ex-

pressed between the two clusters, of which cluster 2 had higher expression of 17 immune checkpoints (P<0.05) (Fig. 6B). For the genes involved in the Th1/IFNγ pathway, the expression of IFNGR2, IFNγ and STAT1 was upregulated in cluster 2 (P<0.05), whereas the expression of JAK1 and JAK2 was upregulated in cluster 1 (P<0.05) (Fig. 6C). m6A regulators may affect the immune response and TME by regulating the expression of HLA, immune checkpoint molecules and Th1/IFNγ pathway gene signatures. As shown in Fig. 6D, cluster 2 presented a very poor response to anti-CTLA-4 therapy (Bonferroni corrected P=0.048). These results indicated that m6A regulators had a close relationship with therapeutic efficacy.

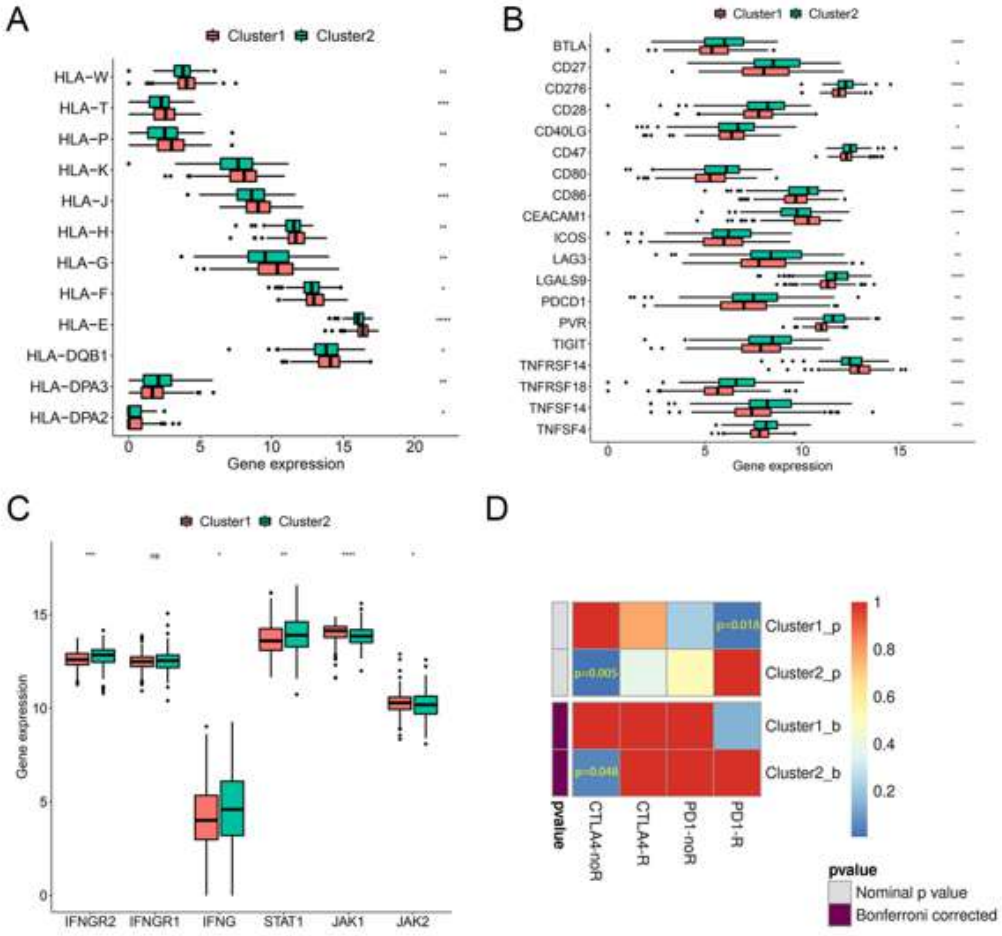

Fig. 6: The influence of m6A regulators on the immunotherapeutic response of ccRCC patients. Comparisons of HLAs (A), immune checkpoint levels (B) and the Th1/IFNγ pathway (C) between cluster 1 and cluster 2. Comparison of response to PD1 and CTLA4 immunotherapies (D). PD1-R means patients respond to PD1 treatment. PD1-noR means patients do not respond to PD1 treatment. In the vertical axis, p means nominal P value, and b means Bonferroni corrected P value

Discussion

Increasing evidence has demonstrated that m6A modification plays an indispensable role in inflammation, innate immunity and antitumor effects through interaction with different m6A regulators. Cancer research studies have shown that m6A regulator-mediated methylation is involved in tumorigenesis and angiogenesis, and this occurs partially through alteration of the TME.

However, the effect of m6A RNA methylation regulators on the TME of ccRCC remained unclear. In the current study, ccRCC patients could be divided into two clusters with distinct expression patterns of m6A regulators. Further bioinformatics revealed that patients in two clusters had significantly different overall survial, tumor microenvironment (immune infiltration, expressions of immune checkpoints, enrichment of immune-related pathways) and immunotherapeutic response, indicating that the interaction between m6A modification and tumor microenvironment is of great significance in the prognosis and treatment of ccRCC patients.

First, we compared the expression of 21 m6A regulators between ccRCC and normal kidney tissues and found that 15 m6A regulators had remarkably different expression levels, of which the expression abundance between ccRCC and normal kidney tissues was consistent with previous reports (11, 23). Among these dysregulated m6A regulators, we noticed that patients with higher expression of METTL3, IGF2BP3, WTAP, IGF2BP2 and HNRNPA2B1 had a better prognosis, whereas patients with higher expression of METTL14, ZC3H13, YHDF3 and YTHDC2 had a worse prognosis (Fig. 1C). Writers were regarded as the main m6A regulators and were related to patient OS in ccRCC (24). Our present study also confirmed the vital roles of the writers METTL3, METTL14, WTAP and ZC3H13 in predicting ccRCC patient OS. As members of the "reader" regulators, we observed that YTHDC2 and YTHDF3 were negatively correlated with ccRCC patient OS, while IGF2BP2, IGF2BP3 and HNRNPA2B1 were positively correlated, and these findings were similar to previous reports (25-28). Additionally, in other studies, erasers of FTO (Niu et al in breast cancer (29)) and ALKBH5 (Chen et al in hepatocellular carcinoma (30)) and readers of YTHDF2 (Li et al in Osteosarcoma (28), Zhang et al in lung adenocarcinoma (31)) and HNRNPC (Huang et al in gastric cancer (32)), Zhao et al in head and neck squamous cell carcinoma (33)) have been reported to be related to the OS of patients; however, in the current study, we found that these regulators exhibited no significant relationships with OS in ccRCC. Therefore, we speculated that the contributions of these m6A regulators to OS differed in distinct cancers, which may be due to different tumorigenesis mechanisms. More reliable experiments are needed to clarify this phenomenon.

Based on the expression of these 15 m6A methylation regulators, consensus clustering was applied to classify ccRCC patients into two subgroups, cluster 1 and cluster 2 (Fig. 2A). We found that cluster 1 had better OS than cluster 2 (Fig. 2D). Furthermore, when comparing the expression profiles of the two clusters, we found 4429 DEGs with different expression levels. Both GO and KEGG pathway enrichment analyses suggested that the DEGs participated in immune-related biological processes and signaling (Fig. 3B and C), which indicated that m6A regulators might be associated with immunity. The TME is closely associated with the immune response in ccRCC progression (17). Therefore, we next compared the TME and its related indicators (immune cell infiltration, HLA, immune checkpoint and Th1/IFNγ pathway gene expression), reported to be closely associated with immunity (34, 35), between the two clusters. Interestingly, we found that TME and its related indicators did differ between the two clusters, including abundance of stromal cells, immune cell infiltrates, expressions of HLAs and other checkpoint molecules, enrichment of immune-related pathways and immunotherapeutic responses.

In the current study, cluster 2, with shorter OS, had higher immune, stromal and ESTIMATE scores. Our results were consistent with the study of Xu et al (36), who divided their glioma samples into two subgroups and found that clusters with higher stroma ($P<0.05$), immune ($P<0.05$), and ESTIMATE scores ($P<0.05$) were associated with shorter OS. However, Liu et al found that patients with higher immune scores were significantly positively associated with OS time in endometrial carcinoma (37). The conflicting results may be caused by the diverse immune microenvironment and tumorigenesis mechanisms of different tumors.

According to our results, low HLA levels in ccRCC contributed to the inactivation of cytotoxic immune mechanisms, which in turn led to a worse OS in cluster 2 than in cluster 1. High-grade immune infiltration is usually accomplished with low levels of HLA-I-positive cancer cells. Based on our results, lower HLA-I levels in cluster 2 may contribute to higher immune scores (immune cell infiltration level of tumor tissue) than those in cluster 1.

Both the TME and immune checkpoints are associated with the therapeutic response (38). In the present study, we compared the expression of immune checkpoint molecules, the activation of which enables tumor cells to evade immune suppression and found that cluster 2 had relatively higher expression of immune checkpoints. Crucial immune checkpoints were mostly positively correlated with "$CD8^+$ T cells", "activated memory $CD4^+$ T cells" and "M1 macrophages" but negatively correlated with "resting memory $CD4^+$ T cells", "M0 macrophages" and "activated dendritic cells" (37). Similarly, higher expression of immune checkpoints in cluster 2 was also accompanied by abundant activated memory $CD4^+$ T cells, M0 macrophages, and lower M1 macrophages. Additionally, due to the differences in immune checkpoint molecules, we inferred that the immunotherapeutic response of cluster 1 and cluster 2 might be different. Therefore, we compared the responses of the two clusters to two commonly used immunotherapies, PD-1 and CTLA-4. Both clusters had a poor response to these two treatments, and cluster 2 had no significant response to CTLA-4 therapy. M6A regulators may influence the effect of immunotherapy, at least PD-1 and CTLA-4 treatment, in ccRCC.

Conclusion

M6A regulators are important participants in the development of ccRCC and are closely related to the TME. This study provides a new understanding of the role of m6A methylation in ccRCC and suggests that m6A regulators may affect the immune response and TME by regulating the expression of HLA, immune checkpoint molecules and Th1/IFNγ pathway gene signatures.

List of abbreviations

TME: Tumor microenvironment; ccRCC: Clear cell renal cell carcinoma; TCGA: The Cancer Genome Atlas; DEGs: Differentially expressed genes; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; HLA: Human leukocyte antigen; OS: Overall survival; m6A: N6-methyladenosine; RCC: Renal cell carcinoma; YTH: YT521-B homology.

Availability of data and materials

Supplementary materials may be requested by respected readers from corresponding author.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors declare that there is no conflict of interests.

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