The analysis of N6-methyladenosine regulators impacting the immune infiltration in clear cell renal cell carcinoma

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Research

Keywords: m6A, ccRCC, tumor immune microenvironment, IGF2BPs, HMGA2

Posted Date: November 23rd, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1063207/v1>

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Version of Record: A version of this preprint was published at Medical Oncology on January 29th, 2022.

See the published version at <https://doi.org/10.1007/s12032-021-01645-0>.

Abstract

Background: The tumor immune microenvironment (TIME) and N6-methyladenosine (m6A) are related to the progression of several types of cancer. Nevertheless, the impact of m6A on the tumor immune microenvironment of clear cell renal cell carcinoma (ccRCC) remains unclear.

Methods: This study used an unsupervised clustering algorithm to divide the samples into distinct subgroups. The single sample gene set enrichment analysis (ssGSEA) algorithm to estimate the tumor immune microenvironment. The correlation between m6A regulators and immune cells in different subgroups was calculated using Spearman analysis. At last, the relationship between *IGF2BP2* and *HMGA2* was validated in several datasets, including TCGA-KIRC, GEO, and HPA datasets.

Results: We found that m6A regulators were differently expressed in several clinical groups, such as grade and stage. Based on the expression of m6A regulators, we divided the samples into three subgroups. Then, the survival analysis for these three subgroups showed that the cluster 2 subgroup had poor overall survival (OS). Further, we found that *IGF2BP2* and *IGF2BP3* were essential components in the cluster 2 subgroup using the principal component analysis (PCA) algorithm. In addition, the expression of these two genes was significantly correlated with overall survival (OS), disease-specific survival (DSS), progression-free interval (PFI). At last, we found that *HMGA2* was significantly correlated with *IGF2BP2* in several datasets, which indicated that *HMGA2* is an essential role in affecting *IGF2BP2* regulating the TIME.

Conclusion: There is a close correlation between m6A regulators and TIME. Moreover, *IGF2BP2* is related to the progression of ccRCC and plays an essential role in affecting the tumor immune microenvironment.

Background

Kidney cancer is one of the most commonly diagnosed cancers in the globe (1). According to the World Health Organization statistics, there are more than 140,000 RCC-related deaths (2). Moreover, ccRCC is the most typical subtype of kidney cancer and contributes to most kidney cancer-related deaths (3,4). Thus, it is necessary to have a further understanding of ccRCC.

Numerous studies revealed that cancer development needs cancer cells and requires other components, like tumor immune cells and stromal cells (5). In recent studies, researchers have shown that the tumor immune microenvironment (TIME) strongly impacts the progression and prognosis of tumors. For instance, some studies showed that tumor-infiltrating immune cells are correlated with the prognosis of different tumors (6,7). Hereby, immunotherapy is a new potential cancer therapy strategy that stimulates and improves the immune system's natural ability to fight cancer cells (8). Moreover, immunotherapy based on immune checkpoint blockade (ICB, PD-1/L1, and CTLA-4) has already shown remarkable clinical benefit in a small number of patients with advanced tumors (9,10). Unfortunately, we have to face the fact that the satisfactory drug efficacy was limited to a small number of patients, and the overall

survival (OS) in advanced ccRCC is less than 30% (11,12). Meanwhile, numerous patients remain in no response or resistance to drugs based on immune checkpoint blockade (13). Therefore, an in-depth investigation of individual differences in the tumor immune microenvironment will contribute to precision immunotherapy.

The post-transcriptional regulation of the mammalian RNA contains large numbers of modified nucleosides, such as 5-methylcytidine (m5C), N6-methyladenosine (m6A), N7-methylguanosine (m7G), a portion of the 5' terminal cap. N6-methyladenosine (m6A) has been regarded as the most frequent internal modification in mRNA (14,15). m6A modification is a kind of dynamic reversible process in mammalian cells, which is regulated by methyltransferases, demethylases, and binding proteins, also known as “writers”, “erasers”, and “readers” (16). Recent studies have shown that m6A plays a critical role in regulating RNA transcription, processing, translation, and metabolism (17). Furthermore, emerging evidence suggests that m6A regulators are also closely associated with oncogenic or tumor-suppressive functions, including proliferation (18), tumorigenesis (19), metastasis (20), and other phenotypes of tumors. Meanwhile, researchers demonstrate that there is a close correlation between m6A modification and TIME (21,22). Han *et al.* discovered that the m6A molecular in tumor cells might impact dendritic cells' ability to cross-present tumor neoantigens to T lymphocytes (22). Yi *et al.* analyzed the m6A regulators of 1938 gastric cancer samples and revealed the m6A regulations played an essential role in TIME complexity of gastric cancer (23). Chong *et al.* analyzed 1307 colon carcinoma samples using multiple bioinformatics methods, finding that m6A modification correlated with the tumor microenvironment of colon carcinoma (24). However, until now, it has not been comprehensively clarified the prognostic effect of m6A modification in ccRCC and the potential roles of m6A modification in host anti-tumor immune response remain primarily unexplored (17).

This work utilized bioinformatics and high-throughput sequencing data to analyze the correlation between m6A regulators and immune cells in TIME. Further, we analyzed the correlation between m6A regulators and immune modulators, which indicated the m6A was associated with the TIME. In addition, we analyzed the correlation between gene expression and immune cells in TIME and found that *IGF2BP2* and *IGF2BP3* play an essential role in immune cells in TIME. Meanwhile, *HMGA2* was significantly related to *IGF2BP2* in the poor prognosis subgroup. These genes could be potential immunotherapeutic targets in ccRCC therapy.

Results

2.1 The expression of m6A regulators in various clinical characteristics

The workflow of this study is shown in **Figure S1**. Among the 503 patients with ccRCC, the expression of the 23 m6A regulators was discordant between tumor tissues and adjacent normal tissues (**Figure 1A, B**). The expression of “readers”, “writers”, and “erasers” were all significantly differently expressed in different clinical characters. *FMR1*, *HNRNPA2B1*, *IGF2BP2*, *LRPPRC*, *YTHDF2*, *YTHDF3*, *METTL14*,

RBM15B, and *ZC3H13* were downregulated considerably in tumors tissues, while *IGF2BP3*, *YTHDC2*, *ALKBH5*, *KIAA1429*, *METTL13*, *RBM15B*, and *WTAP* were significantly upregulated in tumor tissues (**Figure 1B**). Meanwhile, the m6A regulators were differently expressed across clinical grade and pathological stage (**Figure 1C, D**). The correlation analysis showed that most m6A regulators were related to each other, and FTO was significantly correlated with the most “writers” of m6A regulators (**Figure 1E**). The protein-protein internetwork (PPI) demonstrated that these m6A regulators were associated considerably in protein level (**Figure 1F**). To sum, these results implied that the m6A regulators

2.2 The distinguished subgroups obtained from cluster analysis

The unsupervised clustering result showed that the 503 patients could be categorized into three clusters with high stability when the cluster method was k-means and $k = 3$ using R package “ConsensusCluster” (**Figure 2A-C**). Then, the K-M survival analysis for m6A cluster subgroups showed that cluster 2 had a worse prognosis than the other two subgroups (**Figure 2D**), indicating that cluster 2 differed from cluster 1/3. Therefore, we analyzed the expression of m6A regulators in these three subgroups. 13 genes were significantly differentially expressed in subgroups (**Figure 2F**). Moreover, *IGF2BP1*, *IGF2BP2*, and *IGF2BP3* were highly expressed in cluster 2 (**Figure 2E**). This result indicated that the subgroups screened via m6A regulators expression were closely associated with the progression and prognosis of ccRCC.

To further analyze cluster 2, we use the PCA algorithm to find the vital components in cluster 2. **Figure 3A** showed that the result of each principal component contributes to the distribution, and dimension 1 contributes to the distribution most extensive. **Figure 3B** showed a two-dimensional image based on the principal component classification. We found that the difference between cluster 2 and cluster 1/3 was mainly in the principal component 2 (PC2), so we primarily analyzed the vital factors in principal component 2 (PC2). We found that *IGF2BP2* and *IGF2BP3* contributed to the top two in the principal component 2 (PC2) (**Figure C, D**). These results indicated that *IGF2BP2* and *IGF2BP3* were important roles in cluster 2.

2.3 The m6A cluster-related modules and genes

Based on the above analysis, the m6A cluster 2 and *IGF2BPs* attracted our attention. We used the TCGA-KIRC dataset to construct the gene co-expression network to screen m6A cluster-related modules using WGCNA tool. We calculated the correlation coefficient of each gene with *IGF2BP2* and *IGF2BP3*, then these genes with P -value < 0.05 were included in the WGCNA. The scale-free R^2 was set at 0.85, and the soft threshold was set at 7 (**Figure 4A, B**). The selected genes were recognized as seven modules (**Figure 4C**). As shown in **Figure 4D, E**, the result demonstrated that the brown module was significantly correlated with gene significance in cluster 2 ($\text{cor} = 0.83$, $P < 0.001$). To further understand the module genes, we utilized GO analysis to obtain an insight into the biological activities of 23 RNA-methylation regulators

and cluster-related genes. GO analysis demonstrated that the result of enrichment was related to nuclear activation (**Figure 4F**). The GSEA showed that these cluster 2 associated genes were significantly associated with CD8+ T cell infiltration (**Figure 4G**). These results suggested that m6A methylation regulators, especially *IGF2BP2* and *IGF2BP3*, have a close relationship to immune cells and TIME.

2.4 The landscape of immune infiltration cells in ccRCC

The above analysis demonstrated that m6A cluster 2 is related to the immunological response. Consequently, we focused our research on the characteristics of TIME cell infiltration. The immune infiltration of ccRCC patients was obtained from ssGSEA algorithm. The correlation between immune cells infiltration and clinical traits demonstrated that tumor immune cells are significantly related to the progression of ccRCC. The majority of immune cell types are significantly differently infiltrated in tumor samples and adjacent normal samples (**Figure 5A**). In addition, the immune cells were differently infiltrated in different clinical grades and pathological stages (**Figure 5B, C**). These results demonstrated that tumor immune infiltrating cells were associated with clinical characters of ccRCC, and tumor immune cells can perhaps affect the progression of ccRCC.

2.5 The relationship between m6A regulators subgroups and immune infiltration cells

Based on the above analysis, the association between immune infiltration cells and m6A regulators is necessary to analyze. Our study revealed that cluster 2 was significantly highly exhibited CD4+ T cell, CD8+ T cell, MDSC, T follicular helper cell, Th1, and Th2 cells ($P < 0.05$) (**Figure 6A**). This result further showed that the m6A cluster was closely correlated with immune cell infiltration, especially cluster 2 strongly associated with several T cells. To further analyze the correlation between m6A regulators and the immune microenvironment, we used the ESTIMATE algorithm to calculate the ESTIMATE score, immune score, and stromal score for each sample, assessing the purity of tumor samples. When comparing the purity of tumor samples in three m6A regulators clusters, we found out the cluster 2 exhibited a high ESTIMATE score ($P < 0.001$) (**Figure 6B**) and immune scores ($P < 0.001$) (**Figure 6C**), while cluster 2 exhibited no significant difference in the stromal score ($P = 0.075$) (**Figure 6D**). This result revealed a difference in immune infiltration between m6A cluster 2 and cluster 1/3, corroborating the previous findings.

2.6 The relationship between m6A regulators subgroups and immunomodulator agonists

Antigen-presenting molecular and immunomodulators play an essential role in tumorigenesis. Therefore, we explore the expression of antigen-presenting genes and immunomodulatory genes in m6A cluster

subgroups. The result showed that several antigen-presenting genes were significantly differentially expressed in m6A cluster subgroups. Compared with cluster 1/3, *HLA-DBM*, *HLA-DOB*, *HLA-DPB2*, *HLA-DRA*, and *HLA-DRB6* were highly expressed in cluster 2 (**Figure 6E**). Moreover, the expression of these antigen-presenting genes is consistent in cluster 1 and cluster 3, suggesting that cluster 2 has different immune characters compared with cluster 1/3. Previous studies have revealed that immune checkpoint was significantly correlated with the progression and prognosis of various cancers. We accessed the expression of several immunomodulator agonists genes between tumor samples and adjacent normal samples. The results showed that these immunomodulator agonist genes were differently expressed in tumor and normal samples (**Figure S2**). Further, we analyzed the correlation between m6A clusters and immunomodulator agonists genes. The result showed that the *LAG3*, *TIGIT*, *CTLA4*, *PD1*, *CD27*, and *PDL2* were significantly highly expressed in m6A cluster 2 (**Figure 6F**). In summary, m6A cluster 2 is closely related to immunomodulatory factors in ccRCC, and cluster 2 perhaps has a better immune response than cluster 1/3.

2.7 The role of m6A regulators in the progression and prognosis of ccRCC

The above analyses showed that the m6A regulators were highly correlated with the TIME cells, and some tumor-infiltrating immune cells were associated with the prognosis of patients with ccRCC. Therefore, we explored the role of m6A regulators in the progression and prognosis of ccRCC. The univariate Cox analysis for 23 m6A regulators found that 16 genes were significant prognostic factors (**Figure 7A**). *ELAVL1*, *HNRNPA2B1*, *HNRNPC*, *IGF2BP1*, *IGF2BP2*, *IGF2BP3*, *YTHDF1*, *METTL3*, and *MTAP* were adverse prognostic factors. While *LRPPRC*, *YTHDC1*, *YTHDF1*, *FTO*, *CBLL1*, *KIAA1429*, *METTL14*, and *ZC3H13* were favorable prognostic factors. Furthermore, the risk factors have relatively consistent expression, and the correlation analysis among these significant prognostic factors was consistent with the univariate Cox analysis (**Figure 7B**). Then the K-M survival analysis for *IGF2BP2* and *IGF2BP3* showed that these two genes were significantly correlated with the OS, DSS, and PFI of patients with ccRCC (**Figure 7C-H**), suggesting that these two m6A regulators play a vital role in the prognosis of ccRCC.

2.8 Mutation and copy number variation in m6A cluster-related subgroups

Next, we analyzed the mutation profiles and CNV of 23 m6A regulators in all samples and different m6A cluster subgroups. The result of CNV in all samples revealed that the most amplification is *YTHDC2*, and the highest frequency of CNV deletion is *RBM15B* (**Figure S3A**). The result of the mutation in all samples indicated that the most amplification of m6A genes is *VHL* (**Figure S3B**). Interestingly, we found that CNV and TMB in m6A cluster subgroups were different from all samples, and the result of these top highest mutation genes has some difference in different m6A cluster subgroups (**Figure 8A-C**). The mutation frequency of *VHL* gene was 54%, 41%, 41% in Cluster 1, 2, 3, respectively. The mutation of *PBRM1* (37%,

45%, 43%), SETD2 (12%, 29%, < 4%), *BAP1* (5%, 20%, 12%), *TTN* (12%, 20%, 14%) was significantly higher in the cluster 2, suggesting that the mutation in cluster 2 is different from the cluster 1/3. These different mutations among m6A cluster subgroups may lead to different immune responses.

The CNV analysis of three m6A cluster subgroups showed that the highest CNV frequency of m6A genes was *IGF2BP3* in cluster 2 (**Figure 8E**), higher than the CNV frequency in cluster 1/3 (**Figure 8D, F**). Meanwhile, the CNV frequency of *IGF2BP2* was about 40%, which is significantly higher than that in cluster 1/3. The result of CNV analysis indicated that CNV of m6A genes in cluster 2 might lead to different mutations among three groups. Further, these CNV and mutation differences among the m6A cluster subgroup influenced the immune infiltration.

2.9 The association between m6A regulators and immune cells

Based on the above analysis, cluster 2 has a close relationship to immune cells and immunomodulators. Specifically, *IGF2BP2* and *IGF2BP3* were significantly highly expressed in cluster 2 and worked as vital components in cluster 2. Therefore, we analyzed the association of these two genes with immune cells. **Figure 9A, E** showed that these two genes are closely related to several tumor-infiltrating immune cells in all samples, inferred that these two genes were associated with the immune response. To further explore the mechanism of these two genes in affecting immune cells and their role in different cluster subgroups, we analyzed the relationship between these two genes and immune cells in three m6A cluster subgroups, respectively. In cluster 1, *IGF2BP2* and *IGF2BP3* were significantly associated with several types of T cells (**Figure 9B, F**). Interestingly, we found that *IGF2BP2* was only positively correlated with activated CD4+ T cell in cluster 2 (**Figure 9C**), while *IGF2BP3* was positively correlated with the activated CD4+ T cell and central CD8+ T cell (**Figure 9G**). The relationship found in cluster 2 was significantly different from cluster 1/3 (**Figure 9D, H**).

To further understand the role of these two genes, we used univariate Cox analysis to explore the role of immune cells in the prognosis of patients with ccRCC. As was shown in **Figure 9I**, ten types of immune cells were significantly correlated with the OS of patients with ccRCC. In these immune cells, activated CD4+ was a risk factor in prognosis, which was validated in K-M survival analysis (**Figure 9J**). The correlation between *IGF2BPs* and T cells indicated that *IGF2BPs* might interfere and influence the infiltration of activated CD4+ T cells and CD8+ T cells in ccRCC, which further influence the progression of cancer cells.

2.10 Screening the genes related to IGF2BP2 in cluster 2

Based on the above analysis, *IGF2BPs* are crucial components of cluster 2 closely related to TIME. To further analyze the mechanism of *IGF2BPs* in affecting immune infiltration, we analyzed the expression of these two genes in ccRCC in protein level and found that IGF2BP3 was negative in IHC of ccRCC

(**Figure S4A**). Therefore, we focused on analyzing the *IGF2BP2* and related genes. And we found that *HMGA2* was most closely related to *IGF2BP2* in cluster 2 ($R = 0.5901$, $P < 0.001$) (**Figure 10A**). According to published literature, aberrant alterations of the *HMGA2* have a relationship with the occurrence of several types of tumors (42,43), which means *HMGA2* potentially is an oncogene. In our study, we found that the expression of *HMGA2* was different in tumor tissue and normal tissue (**Figure 10B**), and *HMGA2* was significantly higher expressed in cluster 2 subgroup than other two cluster subgroups (**Figure 10C**), which indicated that *HMGA2* affected the tumor progression in cluster 2. To validate the correlation between *IGF2BP2* and *HMGA2*, we conducted the correlation analysis in GSE33689 and GSE53757. The results showed that *HMGA2* was significantly related to *IGF2BP2* in GSE33689 ($R = 0.5695$, $P = 0.0013$) (**Figure 10D**) and in GSE53757 ($R = 0.2322$, $P = 0.0497$) (**Figure 10E**). These external validations confirmed the relationship between *HMGA2* and *IGF2BP2*.

To further analyze the relationship between *HMGA2* and *IGF2BP2*, we downloaded the IHC of *HMGA2* and *IGF2BP2* in renal cancer from HPA. Moreover, we found that *HMGA2* is expressed mainly in the nucleus (**Figure 10F, Figure S4B**), *IGF2BP2* is principally expressed in the cytoplasm and cell membrane (**Figure 10G, Figure S4B**). Meanwhile, we found that *HMGA2* and *IGF2BP2* have corresponding expressions in the same patients, confirming a specific positive correlation between *HMGA2* and *IGF2BP2*. Based on the expression location of these two genes and the relationship between these two genes, we speculated that *HMGA2* regulates the expression of *IGF2BP2* in the nucleus, then *IGF2BP2* affects the ccRCC tumor cells proliferation.

Discussion

Kidney cancer is one of the most diagnosed cancer diseases, and ccRCC accounts for the majority of kidney cancer (1,44). The traditional treatment for kidney cancer is conducting surgery to remove the tumor node. Nowadays, many studies have shown that kidney cancer is associated with genetic alteration (45). Therefore, understanding kidney cancer at the molecular genetic level will be more beneficial to treating kidney cancer. N6-methyladenosine (m6A) modification is the most commonly encountered alteration in eukaryotic mRNA. Although Numerous studies have shown that m6A plays an indispensable role in the immune response and anti-tumor effect through methylation (21–23), the interaction between the tumor immune microenvironment (TIME) and m6A regulators in ccRCC have not been comprehensively understood. Therefore, it is essential to identify the role of m6A in TIME of ccRCC, which will be beneficial to develop novel targets for immunotherapy strategy.

In this work, we found that most m6A genes were differently expressed in ccRCC compared with the normal tissue of the kidney, and most m6A genes were significantly differentially expressed in clinical characters groups, suggesting that these m6A regulators played a crucial role in the tumor progression. In several previous studies, researchers found that the expression level of m6A regulators was associated with the progression of tumors, and these m6A regulators can be oncogene or tumor suppressors (14). Ma *et al.* showed that the low expression level of *METTL14* has a worse prognosis in liver cancer patients (46). While, as shown in the studies of Chen *et al.*, *METTL3* has a positive correlation with

progress liver cancer progression, and *METTL3* promotes the tumor progression via YTHDF2-dependent post-transcriptional silencing of *SOCS2* (47). Although *METTL4* and *METTL3* were m6A “readers”, the effect of these m6A readers on tumor progression was different, indicating that the complex mechanism of the m6A regulating process. Therefore, our study found that m6A regulators were differently expressed in tumor samples and normal samples, which was consistent with the previous studies. Moreover, the role of m6A in ccRCC deserved to be further analyzed.

We divided the samples into three subgroups via unsupervised clustering based on the expression of m6A regulators. Meanwhile, we calculated the abundance ratio of tumor-infiltrating immune cells via ssGSEA algorithm. Then, we found that the relative abundance of immune cells was distinctly distributed among these three subgroups. Especially in cluster 2, T cells were highly infiltrated compared with the other two groups. Interestingly, the cluster 2 subgroup has worse OS than cluster 1/3, indicating that the different distribution of immune cells contributes to the worse prognosis. The functional enrichment analysis showed that the genes correlated with *IGF2BPs* in cluster 2 were enriched in some immune response and regulating T cells, suggesting that T cells in TIME played a crucial role in the progression of ccRCC. To further comprehend the relationship between m6A regulators and TIME, we analyzed the correlation between the m6A regulators and immune modulators. This procedure indicated that the immune modulators were differently expressed in m6A cluster groups, indicating that the m6A regulators perhaps affected the immune response in tumor progression. Next, the *IGF2BP2* and *IGF2BP3* were identified as essential genes in cluster 2, and these two genes were significantly correlated with activated CD4⁺ T cells and central memory CD8⁺ T cells compared with the other two clusters. Therefore, the alteration of these two genes may affect the correlated immune cells to influence the progression of ccRCC.

We learn from previous articles that m6A molecular may have a regulatory effect on immune cell infiltration. Han *et al.* revealed that YTHDF1 could affect the tumor antigen presentation ability and immune recognition ability of DCs to influence the tumor immune microenvironment (22). This effect of m6A regulators was also observed in several studies (23,24). Zhang *et al.* found that after knocking down *METTL14/YTHDF1* in gastric cell lines, the transcript levels of IFN- α/β were downregulated (19). IFN- α/β are critical for tumor cell suppression and anti-tumor immune stimulation through promoting and balancing the anti-inflammatory response (48). Therefore, m6A readers, *METTL14*, could regulate the immune microenvironment of the tumor cell to affect the progression of gastric cancer. As shown in these studies, the effect of m6A regulators on immune cells infiltration in the tumor microenvironment is a crucial factor that cannot be ignored in the progression of the tumor.

Our study found that high expression of *IGF2BP* family genes (*IGF2BP2* and *IGF2BP3*) was significantly correlated with the poor OS, DPI, and PFI of ccRCC patients. Meanwhile, the expression of *IGF2BPs* was positively related to the activated CD4⁺ T cells and central memory CD8⁺ T cells in cluster 2. Further, our study found that *IGF2BP2* is closely correlated with *HMGA2* in cluster 2, and *HMGA2* and *IGF2BP2* have corresponding expressions in the patients. In previous studies, Jan R. *et al.* found that *IGF2BP2* was inhibited in *HMGA2*-null mice, suggesting that *HMGA2* regulated *IGF2BP2* in mice (49). In our research,

the HMGA2 was highly expressed in the nucleus, which indicated that *HMGA2* could also regulate the *IGF2BP2* in ccRCC. Zhou *et al.* have revealed that the *IGF2BPs* enhance the stability and translation of their target mRNAs (50). Some studies also showed that IGF2BPs proteins post-transcriptionally regulate the stability of target mRNAs to affect the progression of tumor cells, in which the *MYC* is the essential target (51,52). Huang *et al.* have revealed that IGF2BPs regulate the *MYC* expression by binding to *MYC* mRNA and affect the cancer cell proliferation (52). Our research can infer that m6A regulators can impact the ccRCC tumor cell by regulating the HMGA2 expression. Therefore, take our research sum, *IGF2BP2* and *IGF2BP3* could affect the tumor microenvironment through multiple functional pathways, and these two genes and their correlated genes could be potential immunotherapy targets in ccRCC patients.

Inevitably, there are several limitations of this study that are supposed to discuss. First, the gene expression data were extracted from the TCGA and GEO databases, in which most of the patients were white people. Therefore, the data will exert some selection of bias of the population. Secondly, we comprehensively analyzed the correlation between m6A regulators and TIME; however, the mechanism of how m6A molecular affected the immune modulators and immune cells did not clarify. The *vitro* or *vivo* experiments could be conducted to analyze further how m6A affects the ccRCC.

Conclusion

In conclusion, our work demonstrated a close relationship between m6A regulators and immune infiltrating cells in ccRCC. The difference of m6A modification subgroups has some effect on the regulating tumor microenvironment. Further, *IGF2BP2* and *IGF2BP3* are essential m6A regulators that cannot be neglected in affecting the tumor immune microenvironment of ccRCC.

Materials And Methods

5.1 Extracting the data

The Cancer Genome Atlas: the gene expression and the clinical data of TCGA-KIRC (count data) were obtained from the UCSC Xena (<https://xenabrowser.net/datapages/>), which consist of 534 tumor samples and 73 adjacent normal tissue samples. The mutation of genes and copy number variation (CNV) were obtained from The Cancer Genome Atlas (TCGA) using the GDC-client tool.

Gene Expression Omnibus: two ccRCC cohorts (GSE36895 and GSE53757) were enrolled and downloaded from Gene Expression Omnibus (GEO, <https://www.ncbi.nlm.nih.gov/geo/>).

GSE36895 included 29 ccRCC tumor samples and 23 normal tissue samples (25).

GSE53757 included 72 ccRCC tumor samples and 72 normal tissue samples (26).

Human Protein Atlas (HPA): the Immunohistochemistry (IHC) of selected genes were obtained from Human Protein Atlas (HPA, <https://www.proteinatlas.org/>) (27).

5.2 Collecting the m6A regulators

In previously published studies (15,16,28), we collected 23 m6A RNA methylation regulators for further analysis. These genes included three types of m6A regulators, including “writers” (*CBLL1*, *KIAA1429*, *METTL14*, *METTL3*, *RBM15*, *RBM15B*, *WTAP*, *ZC3H13*), “readers” (*ELAVL1*, *FMR1*, *HNRNPA2B1*, *HNRNPC*, *IGF2BP1*, *IGF2BP2*, *IGF2BP3*, *LRPPRC*, *YTHDC1*, *YTHDC2*, *YTHDF1*, *YTHDF2*, *YTHDF3*), and “erasers” (*ALKBH5*, *FTO*). The information of these genes is shown in **Table S1**.

5.3 Bioinformatics analysis

The immune cells infiltration of samples was calculated via single sample gene set enrichment analysis (ssGSEA) using the R package “GSVA” (29). Then the relative abundance of each immune cell was normalized to -1 to 1 via the function “scale” of R. To have further understanding of the biological function of genes related to m6A regulators, weighted correlation network analysis (WGCNA) was conducted to obtain the core module associated with m6A subgroups using R package “WGCNA” (30). Gene Ontology (GO) analysis was undertaken to investigate the possible function of the m6A associated genes using the R package “clusterProfiler” (31). Further, gene set enrichment analysis (GSEA) with hall marker gene set was performed to understand the biological process involved in different m6A cluster subgroups using R package “clusterProfiler”. R package “pheatmap” was conducted to visualize the expression of m6A genes in different groups (32). The STRING database is a powerful function for analyzing protein interaction. We utilized the “STRING” app to calculate and visualize the interaction network of m6A regulators (33). The gene expression relationship was calculated by R package “corrplot” using Spearman correlation analysis (34). To estimate the tumor microenvironment, we utilized R package “estimate” to assess the stromal cells and immune cells of each sample (35), by which the immune scores and stromal scores were obtained. In this study, we used R package “survminer” to determine the best cutoff (36) and used R package “survival” to conduct K-M survival analysis (37). The TMB of each sample was calculated and visualized via R package “maftool”(38).

5.4 Machine learning analysis

Unsupervised clustering analysis was conducted to detect distinct m6A modification patterns based on m6A regulators gene expression. In this process, we utilized R package “ConsensusClusterPlus” to classify the samples based on the k-means method (39). Principal component analysis (PCA) is an algorithm to reduce the dimensionality of multivariate data to two or three that can be visualized graphically with minimal loss of information. Furthermore, PCA also can detect the essential components in multivariate data. We utilized R package “factoextra” (40) and “FactoMineR” (41) to calculate the principle factors in m6A regulators related subgroups.

5.5 Statical analysis

This study used R software (version 4.0.3; The R Foundation for Statistical Computing) for data analysis and statistics. The expression of m6A genes in different groups was compared via Wilcox test. K-M survival analysis and log-rank tests analyzed the OS, DSS, PFI in different subgroups. Hazard ratio (HR) was generated via univariate Cox proportional hazards regression when examining the role of immune cells and m6A genes in OS. The correlation between m6A genes and tumor-infiltrating immune cells was evaluated by Spearman correlation. All statistical tests were two-sided. $P < 0.05$ was regarded as statically.

Abbreviations

ccRCC: clear cell renal cell carcinoma;

m6A: N6-methyladenosine;

ssGSEA: single sample gene set enrichment analysis;

WGCNA: weighted gene co-expression network analysis;

TMB: tumor mutation burden;

CNV: copy number variation;

PCA: principal component analysis;

OS: overall survival;

DSS: disease-specific survival;

PFI: progression-free interval;

TIME: tumor immune microenvironment;

TCGA: The Cancer Genome Atlas;

GEO: Gene Expression Omnibus;

HPA: Human Protein Atlas;

GO: Gene ontology;

GSEA: gene set enrichment analysis;

HR: hazard ratio;

PPI: protein-protein internetwork

Declarations

Ethics approval and consent to participate

All data used in this work are obtained from public databases and are publicly available. This work did not contain experiments on humans or animals; therefore, no ethical approval was obtained. The patients involved in the public database have obtained ethical approval. Our study is based on open-source data, so there are no ethical issues and other conflicts of interest.

Consent for publication

The UCSC Xena is a public database and patients included in the database have provided informed consent for the use of their data and for publication.

Data availability statement

All high-throughput sequencing data and clinical data can be obtained from UCSC Xena (<https://xenabrowser.net/datapages/>). The data of GSE36895 and GSE53757 can be obtained from Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>). The images of IHC were obtained from Human Protein Atlas (HPA) (<https://www.proteinatlas.org/>).

Author contribution

The study conception and design were performed by JL, JT. Material preparation, data collection, and analysis were performed by JL, JC, RD. The first draft of the manuscript was written by JL, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported by (1) Science and Technology Project of Chengguan District, Lanzhou City, Gansu Province Science and Technology Bureau (Project number: 2017KJGG0052); (2) Fundamental Research Funds for the Central Universities (Project number: 561219007); (3) Cuiying Graduate Supervisor Applicant Training Program of Lanzhou University Second Hospital (Project number: 201704); (4) Lanzhou City Talent Innovation and Entrepreneurship Project (Project number: 2019-RC-37); (5) Gansu Provincial Department of Education: Outstanding Postgraduate "Innovation Star" Project (Project number: 2021CXZX-154).

Acknowledgment

The information of this study is obtained by UCSC Xena, Gene Expression Omnibus (GEO), and Human Protein Atlas (HPA). We are grateful to them for the source of data used in our study.

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Figures

Figure 1

Differential expression of m6A regulators in different groups, (A) heatmap visualizing the expression levels of m6A regulators in each sample; (B) the boxplot for m6A regulators expressed between tumor tissues and adjacent normal tissues; (C) the boxplot for m6A regulators expressed in different grades; (D)

expression profiles of 23 m6A genes among three cluster subgroups in the TCGA dataset, (C) Variables-PCA, (D) Contribution of variables to Dim-2.

Figure 4

Identification of the core modules related to the m6A cluster via WGCNA. (A) The scale-free topology model fit index was analyzed for various soft threshold powers (β), and the scale-free R2 was set at 0.85. (B) Analysis of the mean connectivity for various soft threshold powers and the soft threshold was set at

7. (C) A cluster dendrogram was established to detect co-expression gene modules with corresponding color assignments in these genes related to IGF2BPs. The gray module refers to no co-expression between the genes. (D) Heatmap showing the correlation between six modules and three m6A cluster subgroups. (E) The scatterplot of gene significance for cluster 2 subgroup and module membership in the brown module.

Figure 5

The infiltrating levels of immune cells in cluster1, 2, 3 in different clinical characters. (A) The box plot of the 28 immune cell types in tumor tissues and adjacent normal tissues. The box plot of the 28 immune cell types in different stages (B) and stages (C).

Figure 6

The immune characters in m6A cluster subgroup. (A) The box plot of significantly different infiltrating immune cells in three m6A cluster subgroups. (B-D) The difference in ESTIMATE scores, immune scores,

and stroma scores in three m6A cluster subgroups. (E) The box plot of different antigen-presenting molecular in three m6A cluster subgroups. (F) The box plot of different immunomodulators in three m6A cluster subgroups.

Figure 7

(A) The forest plot for univariate Cox regression analysis of m6A regulators. (B) Correlations between m6A genes (with P < 0.05 in univariate Cox analysis) in TCGA. Circle size represents P-value calculated by

the log-rank test, which ranged from 0.1 to 0.0001. (C-E) K-M survival curves of high expression and low expression of IGF2BP2 samples for OS, DSS, and PFI analysis. (F-H) K-M survival curves of high expression and low expression of IGF2BP3 samples for OS, DSS, and PFI analysis.

Figure 8

The mutation frequency of top genes and CNV analysis in three m6A cluster subgroups. (A-C) The mutation frequency of top genes in three m6A cluster subgroups. Column presents individual patients. The upper bar plot represents TMB. The number on the right represents the mutation frequency in each gene. The right bar plot represents the proportion of each variant type. (D-F) The CNV frequency of 23 m6A genes in three subgroups. The height of the column represents the frequency, and the color represents gains or losses. Gains: red; Losses: green. CNV: copy number variation; TMB: tumor mutation burden.

Figure 9

The correlation analysis between IGF2BPs and immune cells. (A-D) The correlation between tumor-infiltrating immune cells and IGF2BP2 in all samples, cluster 1, 2, and 3, respectively. (E-H) The correlation between tumor-infiltrating immune cells and IGF2BP3 in all samples, cluster 1, 2, and 3, respectively. The dot size represents the coefficient of correlation; the dot color represents the P-value of correlation. The correlations with $P < 0.05$ were displayed in the plot. (I) The forest plot for univariate Cox regression

analysis of immune cells for OS (with $P < 0.05$). (J) K-M survival curves of high and low infiltration level of activated CD4 T cell for OS analysis.

Figure 10

The relationship between IGF2BP2 and HMGA2. (A) The correlation between IGF2BP2 and HMGA2 in TCGA-KIRC dataset. (B) The box plot for the expression of HMGA2 in tumor samples and adjacent normal tissue in ccRCC. (C) The box plot for the expression of HMGA2 in three m6A cluster subgroups. (D-E) The correlation between IGF2BP2 and HMGA2 in GSE36895 dataset and GSE53757 dataset. (F) The IHC of HMGA2 in renal cancer. (G) The IHC of IGF2BP2 in renal cancer.

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