Research Square

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Identification of highly conserved alternative splicing and RBP regulators contributing to immune microenvironment regulation in clear-cell renal cell carcinoma patients

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Research Article

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Abstract

Background

Clear cell renal cell carcinoma (ccRCC) is a common renal malignant tumor, and its abnormal immune microenvironment is closely related to tumor progression, but the regulatory mechanism of alternative splicing and RNA binding protein (RBP) in this process is not clear. The purpose of this study was to investigate the effects of abnormal alternative splicing events on the immune microenvironment of ccRCC, and to identify the RBPs that mediate these events.

Methods

The RNA sequencing data in TCGA database were used to identify the differentially regulated alternative splicing events (RASE) between ccRCC and adjacent tissues by SUVA process. CIBERPORT algorithm was used to evaluate the degree of immune cell infiltration, screen the differentially expressed RBP, and construct the RBP-RASE-immune cell co regulatory network. Finally, immunohistochemistry was used to verify the expression of key RBP in tumor tissues.

Results

a total of 744 highly conserved rases in ccrcc were identified, of which 22 were significantly associated with immune cell infiltration, involving T cells, natural killer cells, macrophages and other immune groups. By overlapping the differentially expressed genes with the known RBP list, 225 differentially expressed RBPs were screened out, and the regulatory network was constructed. Four key RBPs (DDX47, RNASE2, RPL36, RPL36A) were identified, and their high expression was associated with poor prognosis. Immunohistochemistry showed that the expressions of RNASE2, RPL36 and RPL36A in ccRCC tumor tissues were significantly higher than those in adjacent tissues.

Conclusion

In this study, the RBP-RNASE-immune cell regulatory network was constructed systematically in ccRCC for the first time, revealing the potential mechanism of abnormal alternative splicing affecting tumor immune microenvironment through RBP, and providing a new molecular target for immunotherapy of ccRCC.

Introduction

Clear cell renal cell carcinoma is the most frequent tumor of the kidney tumors, originating from renal tubular epithelial cells, with 60%-70% being clear cell renal cell carcinoma[1]. Clinically, ccRCC is one of the most aggressive types and one third of patients will present with metastatic renal cell carcinoma[2].

The occurrence and progress of tumors are closely related to the tumor microenvironment, moreover, immune cells play a crucial part of tumor microenvironment, which have been discovered to correlate with prognosis and response to therapy[3, 4]. Nowadays, surgical resection remains the preferred treatment option for ccRCC. However, for advanced patients, chemotherapy, radiotherapy and even targeted therapies are often experienced with resistance, resulting in poor clinical outcome[5]. In recent decades, more and more researches show that tumor microenvironment, especially immune microenvironment, plays a key role in the development of tumors. Some studies demonstrated that ccRCC is one of the most immunoinvasive tumors in pan cancer comparison, and the tumor microenvironment may affect the response to systematic treatment[6, 7]. Although recent studies have identified some molecular targets related to immune cell infiltration, the specific molecular mechanisms underlying changes in the immune microenvironment during the occurrence and development of ccRCC tumors are still unclear.

Pre-mRNA splicing is an fundamrntal step in the expression of most eukaryotic genes[8]. Alternative splicing gets rid of introns from pre-mRNAs and connects the rest of exons to produce mature mRNA, which contributes different isoforms and diversify protein expression, and about 94% of gene generate alternatively spliced transcripts may produce different protein isoforms with diverse functions[9]. AS has emerged as a decisive event in tumor development and microenvironment formation[10].AS is thought to be involved in regulating the differentiation and activation of immune cells, moreover abnormal AS is closely associated to tumor progression and immunotherapy[11]. In 2010, Piekielko-Witkowska et al. discovered some partially regulated splicing factors, which may possible lead to impaired AS of genes regulating tumor growth[12]. In 2013, Zhao, Q et al. identified 113 top candidate alternatively spliced genes in ccRCC and FGFR2-IIIc may be an early noticeable detection biomarker and therapeutic target[13]. With the continuous improvement of public databases, more and more aberrant AS events of ccRCC have been reported. Although some researches on ccRCC have focused on this field, but still lacking. A growing number of studies have shown that AS plays an indispensable role in the prognosis and remodeling of tumor immune microenvironment in ccRCC, the correlation between splice factor expression levels may serve as a marker of carcinogenesis and may become a therapeutic target for ccRCC patients[12, 14]. However, no studies have systematically analyzed the AS that regulated the tumor immune microenvironment in ccRCC.

RNA binding proteins interact with RNA by recognizing sequence motifs, structures and their combinations to regulate condition-specific alternative splicing[15]. The specific RBPs are finely regulated at their localizations, expression levels, their splicing, mRNA stability and translation during tissue development and cell differentiation[16]. Therefore, an increasing amount of data suggests that changes in RBP expression and RBP dysfunction are associated with the progression of various diseases, including cancer and immune disorders. In 2017, Jiang, J et al. found an essential RBP, PTBP1, plays a tumorigenic role in ccRCC by mediating PKM2 alternative splicing[17]. In 2023, Pan,Y et al. discovered a multifunctional RNA binding protein, HnRNPC, can inhibit the expression of TP53INP2 after transcription through alternative splicing, thereby affecting the drug resistance of Sunitinib[18]. Still,

there is no relevant research on the impact of RBP and related alternative splicing factors on the immune microenvironment of ccRCC.

To explore the effects of highly conservative alternative splicing and RBP regulatory factors on the immune microenvironment of ccRCC, we utilized the TCGA database to perform AS analysis to acquire RAS events which have significant differences between cancer and adjacent caner tissue, and constructed an RBP-RAS-immune cell network, and we found some RBPs which closely related to tumor progression as potential molecular targets. These RBPs can affect the immune microenvironment of ccRCC probably by regulating related alternative splicing events, thereby playing an essential role in the occurrence and development of tumors, and providing significant clues for the immunotherapy of ccRCC.

Materials and methods

Retrieval and process of public data

The gene expression profile, RNA-seq profile and clinical information of TCGA KIRC project data were obtained from the UCSC XENA database, 377 tumors and 72 normal samples were screened. Splice junction data in BED format from TCGA were acquired from Genomic Data Commons Data Portal (GDC) for alternative splicing identification.

Alternative splicing analysis

The alternative splicing events (ASEs) and regulated alternative splicing events (RASEs) among different groups were defined and quantified by utilizing the SUVA (v2.0) (Splicing site Usage Variation Analysis) pipeline. The proportion of reads with a SUVA AS event (pSAR) was calculated for each AS event.

Cell-type quantification

The CIBERSORT algorithm was used with the default parameter for estimating immune cell fractions. Decomposing the RNA seq data of TCGA, the relative proportions of 18 types of cells were identified in samples of renal clear cell carcinoma and adjacent tumors, respectively.

Identification of differentially expressed RBPs between tumor and normal samples

The software DEseq2 (v. 1.30.1), which is specifically used to analyze the differential expression of genes between cancer and adjacent tissues, was applied to screen out the raw count data for DEGs. The fold change (FC ≥ 2 or ≤ 0.5) and false discovery rate (FDR ≤ 0.05) were set as the threshold to comfirm whether a gene was differentially expressed. Then expression profiles of differentially expressed RBPs were filtered out from all DEGs according to a catalog of 2,141 RBPs retrieved from four previous reports[19, 20].

Co-expression analysis

Co-expression analysis was performed for all differentially expressed RBP and RAS (pSAR ≥ 50%). Meanwhile, differentially expressed RBP and RAS was calculated by using Pearson correlation coefficient, and DERBP-RAS relationship pairs which are satisfying absolute value of correlation coefficient ≥ 0.15 and P value ≤ 0.05 were screened out. And then co-expression analysis was performed for RAS (pSAR ≥ 50%) and immune cells. Meanwhile, RAS and immune cells was calculated, and RAS-immune cells relationship pairs satisfying absolute value of correlation coefficient ≥ 0.15 and P value ≤ 0.05 were screened out.

Functional enrichment analysis

Gene Ontology (GO) terms and KEGG pathways were identified using KOBAS 2.0 server to analyze the associated pathway, biological process and molecular function of ASEs, DEGs and RBPs. Hypergeometric test and Benjamini-Hochberg FDR controlling procedure were utilized to define the enrichment of each term. A p value < 0.05 was set down as the cutoff criterion.

Immunohistochemistry

For immunohistochemical analysis, biopsy specimens were fixed in 10% neutral-buffered formalin and routinely processed. After the pathologic evaluation, H&E-stained sections were marked by the pathologist to identify lesion sites. Immunohistochemistry was carried out DDX 47 (Catalog Number: 17795-1-AP), RNASE2 (Catalog Number: 18172-1-AP), RPL36 (Catalog Number: 15145-1-AP), RPL36AL (Catalog Number: 15535-1-AP) with Roche Benchmark XT Staining Instrument. All antibodies were obtained from Wuhan Sanying Co. Led. Qupath 4.3 and Openslide are used to complete the format analysis. Using deep learning models (such as u-net) combined with manual correction, accurate labeling of organizational areas is achieved. In immunohistochemical quantitative analysis, after repeated parameter optimization, AI automatically completed the total cell and positive cell count, and calculated the positive rate, H-score and other indicators. After ensuring the reliability of data through spot check and comparison, batch output the analysis results and collect representative images.

Result

Identification of cancer-associated AS events between tumor and normal samples in TCGA ccRCC dataset.

According to the RNA-seq data, all AS events were detected by SUVA (Figure S1A). We identified the regulatory AS (RAS) events with statistical meaning between tumor and normal samples, more than 900 events are concentrated in alt3p and alt5p events (Fig. 1A). Splice junction (SJ) composing RAS events was annotated to classical AS event types, and A5SS, cassetteExon and 5pMXE is the most frequent events (Fig. 1B). Like the tumorigenesis process, most RAS events are complex events which indicate SJs participated in two or more different classical splicing events (Fig. 1C). The studies above show that alternative splicing regulation in ccRCC is a complex process. To identify more dominant RAS events, we extract 744 splicing events that dominated transcripts from RAS events (pSAR > 50%) for subsequent

analysis (Fig. 1D). Principal component analysis (PCA) based on splicing ratio of RAS with pSAR > 50%. In Fig. 1E, the groups were clearly clustered in distinct regions, suggesting that the splicing pattern of cancer tissue has higher heterogeneity. Based on RAS events with pSAR ≥ 50%, we calculate a comprehensive heatmap visualizing the relation between caner and paracancerous tissues (Fig. 1F).These splicing events are highly conserved in ccRCC which are worthy to intensive study as a potential therapeutic target. To better understand the potential functions of the identified splicing events, we performed GO function and KEGG pathway enrichment analysis. Figure S1B shows the most enriched GO terms including biological processes, cellular components and molecular functions. In top 10 most enriched KEGG pathway, it is found that these RAS events are mainly enriched in some cancer-related pathways, such as Rap1 signaling pathway, Ras signaling pathway, regulation of actin cytoskeleton andEGFR tyrosine kinase inhibitor resistance, suggesting that these genes may be closely related to ccRCC development (Fig. 1G), andthese pathways and RAS events have been confirmed to be associated with immune infiltration in some tumors[21]. We select several important RAS events from Rap1 signaling pathway, such as FGFR1OP2, CTNND1 and FGFR1, which have a significant splicing ratio and survival probability (Fig. 1H). Altogether, these result showed that the RAS events with pSAR ≥ 50% we screened may play a crucial role in the progress of ccRCC and may affect the tumor immune microenvironment.

Diversity of immune microenvironment characteristics in CCRCC and construction of co-disturbed network between immune cells and RAS

CIBERSORT analysis tool is an excellent bioinformatic technology, which can evaluate the cell composition of complex tissues based on gene expression data. Using the CIBERSORT to calculate the expression content of 18 kinds of immune cells in ccRCC and paracancerous tissues from RNA-Seq gene expression data (Figure S2A). We found that 16 cell clusters have statistically significant (Fig. 2A and Figure S2B). It had a higher proportion of CD8 + T cells, activated CD4 memory T cells, follicular helper T cells, regulatory T cells, gamma delta T cells, resting NK cells, M0 macrophages and M1 macrophages, whereas a low amount of native B cells, resting CD4 memory T cells, activated NK cells, monocytes, resting dendritic cells, activated dendritic cells, resting mast cells, neutrophils. Principal component analysis (PCA) was performed for the analysis of these different immune cell groups in cancer and adjacent tissue. In the PCA plots, the spots of cancer and adjacent tissue dispersed widely, which indicates that discrimination can be quite successful (Fig. 2B). Figure 2C shows the ratio of the average proportion of different cells between cancer and the adjacent tissue, in which the up-regulated cells in the cancer tissue are mainly T cells, resting NK cells and macrophages; the down-regulated cells are mainly native B cells, activated dendritic cells and activated NK cells. In order to investigate the role of RAS events in immune infiltration, the correlation analysis between each immune microenvironment infiltration cell type and RASs was carried out, and a total of 22 RASs were obtained that were significantly related to the type of infiltrating cells in the immune microenvironment (Fig. 2D). We selected nine RASs related to the immune microenvironment and displayed their splitting ratio values (Fig. 2E). In brief, the immune microenvironment has dramatic changes in ccRCC, and our research

results also confirm that different immune cell infiltration plays an important role in the development of tumors, which is consistent with other researches[22].

RNA binding proteins are involved in the regulation of immune-related RAS

RNA binding protein shows strong selectivity of sequence or structural when binding to target RNA, and is a known regulator of RNA splicing[23]. Based on the above results, we hope to identify the RBPs involved in the regulation of immune related RAS. Firstly, a total of 225 DE-RBPs were identified by overlapping between the differentially expressed genes and 2141 RBP genes reported (Fig. 3A) and displayed the expression levels of all DE-RBPs in heatmap (Fig. 3B), which can intuitively demonstrate the differential expression of DE-RBP between tumor and normal tissue. Then we performed GO function and KEGG pathway enrichment analysis of all DE-RBPs. GO analysis results revealed that these DE-RBPs are not only related to RNA regulation, such as RNA splicing, mRNA processing, RNA phosphodiester bond hydrolysis and RNA catabolic process, but also enriched in biological pathways such as virus defense response and innate immune response (Fig. 3C). According to the KEGG pathway analysis, DE-RBPs are enriched in carbon metabolism, biosynthesis of amino acids, ribosome and et al (Figure S3A). Whereupon utilizing 377 tumor and 72 normal samples from TCGA ccRCC, co-expression analysis of these DE-RBPs and RAS evens was carried out, and the relationship network of RAS-immune cells was analyzed and displayed, and the co-disturbed network of RBP-RAS-immune cells was constructed. The identified immune related RAS events and upstream differential RBPs are highlighted (Fig. 3D). The KEGG pathway analysis of DE-RBPs co-expressed RAS and cell genes were showed in Figure S3B and put the important pathways of DE-RBPs regulated RAS in the co-disturbed network (Figure S3C). We screen four potential RBPs, DDX47, RPL36, RPL36A and RNASE2, in the co-disturbed network which have statistical significance and analysis their expression and prognosis. In Fig. 3E and 3F, four potential RBPs are highly expressed in ccRCC and related to poor overall survival.

The selected RBPs were highly expressed in ccRCC compared with adjacent normal tissues

In order to further verify the differential expression of RBPs in renal cell carcinoma, we performed immunohistochemical verification on paraffin samples. We selected cancer and paracancerous paraffin samples from five patients with ccRCC. The samples of these five patients were reviewed by three experienced pathologists. The WHO/ISUP classification, age and gender were different. In the pre experiment, we found that the DDX47 antibody used was not suitable for the immunohistochemical experiment, and there was no DDX47 antibody available on the market for immunohistochemistry, so we suspended the DDX47 immunohistochemical experiment. The expression of the other three RBPs (RNASE2, RPL36, RPL36A) in ccrcc was higher than that in adjacent tissues (Fig. 4), and had statistical significance (Fig. 5).

Discussion

Clear cell renal cell carcinoma is the most common type of renal cell carcinoma, approximately 70% of all adult epithelial renal tumors removed by surgery[24]. Present studies indicate that blocking immune

checkpoints is a new strategy for treatment of ccRCC, however, due to intratumoral heterogeneity and immunosuppressive tumor environment, the efficacy of this treatment is not ideal[9]. As a major posttranscriptional mechanism, numerous studies have shown that AS has a preponderant role in cancer development, especially in the immune microenvironment, and as a new promising cell therapy, many AS that cause cancer may reversed through corresponding targeted treatment[25, 26]. In our study, we identified the RAS events that dominate the transcripts (pSAR > 50%) between ccRCC and adjacent tissues, and the occurrence of these RAS events may be closely related to the prognosis of ccRCC patients. Besides, we analyzed the proportion change of immune cells and establishing a co-regulatory network with immune cell and RAS events in ccRCC for the first time. Due to RBP as a largely controlled factor regulating post transcriptional regulation by influencing AS and each RBP specificity affects a subset of its RNA target genes[16, 27]. We constructed a regulatory network between RBP and immune infiltration associated RAS, this is also the first time that RBP has been linked to immune-related RAS events, providing a new idea for immunotherapy targets of ccRCC.

In our study, we found that the regulation of splicing events are complex and splicing patterns are heterogeneous in ccRCC. Moreover, 744 RAS events (pSAR > 50%) were screened using TCGA database, which are mainly enriched in many pathways associating with cancer process after enrichment analysis. Especially Rap1 signaling pathway, as a key regulator in cell-cell junction formation, it regulates MAP kinase activity in a manner highly dependent on the context of cell types, playing a vital role in the development of malignant tumors, such as lung cancer, gliomas, hepatocellular carcinoma, and is a potential molecular target of malignant tumors[28, 29]. RAP1 plays a major role in regulating the function of integrins and other adhesion molecules, and also regulating the activity of MAPK, NF-κB, Notch signaling pathway and et. al[30, 31]. At present, RAP1 has been shown to be associated with tumor development and prognosis in ccRCC. However, the research of specific mechanism, especially the role of AS, is insufficient. We picked out some important RAS events in RAP1 signaling pathway which associated with the prognosis of ccRCC. There are some studies indicated that high expression of FGFR1 is positively correlated with the mRNA expression of HIF-1α, HIF-2α genes, participate angiogenesis, tumor growth and metastasis in ccRCC[32, 33]. At present, many researches on FGFR1 alternative splicing isoforms, FGFR 1α and FGFR1β, have been carried out in breast cancer and bladder cancer, they identified that these isoforms play a key role during tumor progression[34, 35]. Different splicing ratio will lead to different splicing factor isoforms, which can enhance the flexibility of the spliceosome composition and the splicing process itself[36]. Some of the RAS events with lower AS ratio we found can be linked to FGFR1, FGFR3 and PDGFA, leading to a better prognosis in ccRCC patients. Few studies have shown that there are differences in the expression of FGFR3 and PDGFA in ccRCC compared to adjacent tissues, and may make a difference on target therapeutic efficacy, but the specific studies and their role in RAP1 signaling pathway are limited[37, 38]. As early as 2003, FGFR3 splicing isoform, FGFR3IIIS, has been found to contribute to the development of a malignant phenotype[39]. And then, FGFR3 splice variant, FGFR3 Delta8-10, has been identified to induce proliferation in urothelial carcinoma cell line[40]. Currently, some studies on FGFR3 AS events have been conducted in prostate cancer and ependymoma, but there is no specific study in ccRCC yet. PDFGA has

been studied in many tumors, but there is still lack of relevant research in ccRCC, and haven't studies on its splicing variants. We also discovered some RAS events with higher AS ratio, such as CTNND1 and FGFR1OP2, have a poor prognosis in ccRCC patients. According to current research, CTNND1 and FGFR1OP2 have been studied in multiple tumors but not in ccRCC. CTNND1 AS variants has been noticed in breast cancer[41, 42]. FGFR1OP2 and its splicing isoforms were still haven't relevant studies on human cancer.

AS events can produce multiple isoforms of mRNA and diversify the repertoire of the transcriptome and proteome, which is utilized by the immune reprogramming system, immune cells employ AS to ensure high transcriptional diversity and regulator gene expression[25, 43]. With the success of targeted therapy in multiple tumor treatments, cccRCC has so far been a lack of reliable targets, and more and more studies are considering immunotherapy as a promising approach to treating. In 2022, Zhong, W et al. analyzed the potential functions in immunotherapy of AS and alternative polyadenylation[44]. Wang, Z et al. explored the association between immune infiltration cells, key immune checkpoints and AS-based prognostic signature[9]. In recent years, some research teams have focus on the role of CD8 positive T cell infiltration in ccRCC[45, 46]. At present, there are many studies on immune infiltration in ccRCC, but there are a few researches on the combination of AS and immune infiltration. We used CIBERSORT technology to identify 22 RAS events significantly related to the type of infiltrating cells in the immune microenvironment from 744 selected RAS events, involving 16 cell groups, the previous screened RAS of FGFR1 is also included. Activating FGFR1 can induce the expression of PD-L1 in K-ras- driven cancers in lung squamous cell carcinoma[47]. Moreover, many studies shows that the abnormal expression of FGFR1 can alter the proportion of immune infiltrating cells in the tumor microenvironment and promote tumor development[48, 49]. The EPB41 and EPB41L1 encode an impressively complex array of structural adaptor proteins and tissue-specific alternative pre-mRNA[50]. EPB41 could regulate B cell expression by inhibiting the canonical NF-κB signaling pathway, or regulate T cell activation[51, 52]. ITPKB alternative splicing isoform has been found in lymphoma patient[53]. Furthermore, ITPKB has been discovered that could alter T cell activity and implicate in B- and T-cell development in the occurrence of disease[54, 55]. SYNE2 has been identified as a CD8 + T cell related biomarker in ovarian cancer and its related AS events has been found in prostate cancer and non-small cell lung cancer[56, 57].In our study, These 22 RAS events may change the immune microenvironment by affecting the activity of related immune cells, thus playing a significant role in ccRCC progression.

Sebestyen et al. systematically analyzed the gene expression patterns of RBPs in eleven solid tumors, and found the RBP related genes have opposite expression pattern in renal cell carcinoma, including ccRCC, chromophobe renal cell carcinoma and papillary renal cell carcinoma[58]. In our research, we coincided the differentially expressed genes between ccRCC and para-cancerous tissues with the results, we constructed an RBP-RAS-immune cell network and highlight the identified immune related RAS events and its upstream RBPs. Firstly, DDX47 is a member of the DEAD box protein family and implicated in translation initiation, nuclear and mitochondrial splicing, and have two AS transcript variants encoding distinct isoforms[59]. Currently, B. Singh et al. have been found that rs34330 as a significant expression quantitative trait locus, especially in immune cells, affecting nearby genes,

including DDX47, which is involved in innate immunity[60]. In 2021, Qin et al. discovered 125 differently RBPs between renal cell carcinoma and normal tissue by using TCGA database, including DDX47 as a prognosis-related RBPs[61]. Meanwhile, Wu et al. also found DDX47 would be a prognosis-related RBPs in ccRCC[27]. At present, there is no research on DDX47 and its splicing variants in tumors, nor on its impact on tumor microenvironment. In our study, DDX47 is highly expressed in ccRCC and produces different RNA isoforms by affecting dendritic cells related RAS, leading to a poor prognosis. Then, RNASE2 is a subset of the ribonuclease A superfamily, belonging to the pancreatic ribonuclease family. In ccRCC, some studies have demonstrated that RNASE2 is a robust prognostic signature and associated with immune infiltration[62, 63]. Unfortunately, there is no study on RNASE2 alternative splicing events in tumors. In our study, RNASE2 also highly expressed in ccRCC, affected gamma delta T cells by producing abnormal RASE splicing isoforms. As for RPL36, the protein belongs to the L36E family of ribosomal proteins, existing transcript variants derived from AS. Shen et al. discovered RPL36 play a significant role during the development of cisplatin resistance in human cancer cells[64]. In 2021, Cao, X et al. found that RPL36 transcript variant 2 affects the translation process of downstream Alt-RPL36, thereby regulating the state of PI3K- ALK- mTOR signaling pathway[65]. Although, some researchers have found that RPL36 may be abnormally expressed in breast cancer, prostate cancer and hepatocellular carcinoma, there is still a lack of research on the impact of RPL36 and its splicing variants on the tumor immune microenvironment[66–68]. Our study found that RPL36 may regulate NK cell activated related RAS event. About RPL36A, belonging to the L44E family of ribosomal protein, naturally occurring read-through transcription occurs between this locus and the heterogeneous nuclear ribonucleoprotein H2 gene. Some studies have shown abnormal expression of RPL36A and its correlation with patients' prognosis[27, 63, 69]. But these studies have not linked RPL36A to related RAS events and immune cells. We discovered RPL36A could regulate NK cells activated RAS event.

This study constructed the network based on the Cancer Genome Atlas (TCGA) database and verified the expression of RBPs in paraffin samples of ccRCC and corresponding paracancerous tissues, but lacked further verification at the cellular and animal levels Moreover, the exact molecular mechanisms and biological function of these RBPs involved immune cell related RASE need to be further explored.

Conclusion

In summary, our study constructed an RBP-RAS-immune cells network for the first time and screened out four key RBPs, DDX47, RNASE2, RPL36 and RPL36A, which highly expression in ccRCC not only affects the prognosis but also changes the tumor immune microenvironment. This RBPs may be potentially used as new molecular intervention targets especially in immunotherapy of ccRCC.

List of abbreviations

Clear cell renal cell carcinoma (ccRCC)

Abnormal alternative splicing events (ASEs)

Regulated alternative splicing events (RASEs)

RNA-binding proteins (RBPs)

Differentially expressed genes (DE)

Splice junction (SJ)

Principal component analysis (PCA)

Immunohistochemistry (IHC)

Declarations

Clinical Trial Number

Clinical trial number: not applicable.

Ethics approval and consent to participate

This study was approved by the ethics committee of People's Hospital of Xinjiang Uygur Autonomous Region. We certify that the study was performed in accordance with the 1964 declaration of HELSINKI and later amendments. Informed consent was waived by our Institutional Review Board.

Consent for publication

Written informed consent was obtained from all patients whose tissue specimens were used for immunohistochemical analysis in this study. The patients have been informed that the results of this study, including representative histopathological images (e.g., Figures 4), will be submitted for publication in a peer-reviewed journal. All patients provided their consent for the publication of these images and any associated clinical data, with the understanding that their anonymity will be strictly maintained. A copy of the written consent is available for review by the journal's editorial office.

Availability of data and materials

The TCGA KIRC project data, including gene expression profile (normalized FPKM expression level data and normalized HTSeq - Counts data) and clinical information were downloaded from the UCSC XENA database (https://xenabrowser.net/datapages/), 377 tumors and 72 normal samples were screened. Splice junction data in BED format from TCGA were downloaded from Genomic Data Commons Data Portal (GDC) for alternative splicing identification. 377 tumors and 72 normal samples were screened. (More information on the GDC pipeline used to generate this data: https://docs.gdc.cancer.gov/Data/Bioinformatics_Pipelines/Expression_mRNA_Pipeline).

Data Availability Statement

The datasets analyzed in this study are publicly available from the following sources:

(1) The TCGA KIRC gene expression and clinical data were obtained from the UCSC XENA database (https://xenabrowser.net/datapages/);

(2) The splice junction data in BED format were downloaded from the GDC Data Portal (https://portal.gdc.cancer.gov/).

All data used in this study are publicly accessible, and no newly generated datasets requiring deposition were produced.

Competing interests

Competing interests: The authors declare there are no competing interests.

Authors' contributions

All authors conceived this research. Mei Cui and Hongrun Zhou obtained and analyzed the data. Yongli Xiao and Yaoyuan Shen were responsible for proofreading and drawing figures. Chun Wang reviewed and edited the paper. Chun Wang had final responsibility for the decision to submit for publication. All authors have read and approved the final manuscript.

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Figures

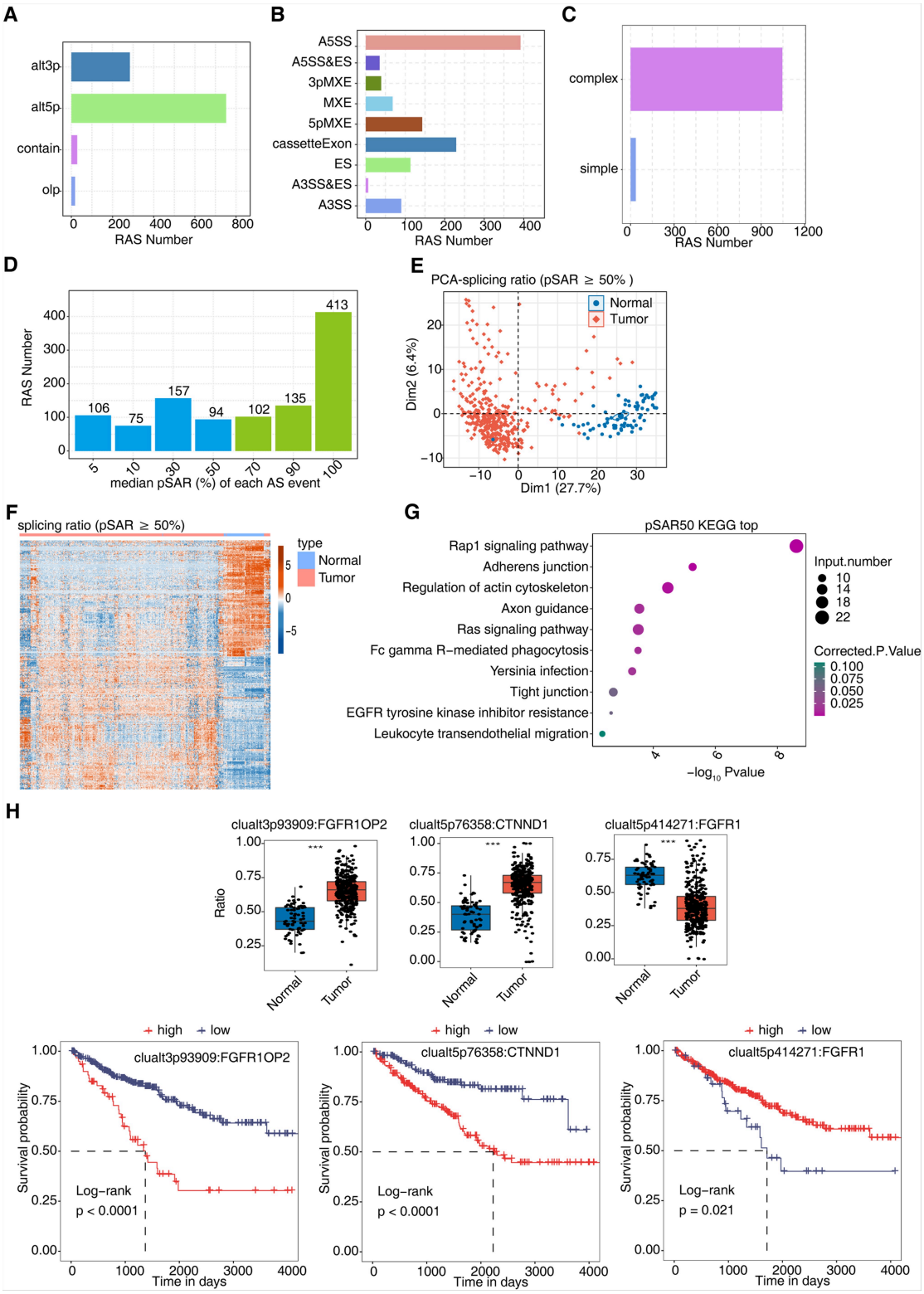


Figure 1

The highly conserved regulatory AS events (RAS) of the groups

A. Barplot showing number of regulatory AS detected by SUVA in the group.

B. Splice junction constituting RAS events detected by SUVA was annotated to classical AS event types. And the number of each classical AS event types were showed with barplot

C. Barplot showing number of SUVA RAS events which contains SJs involved in

two or more different classical splicing events (complex) or in same classical splicing event

(simple).

D. Barplot showing RAS number with different pSAR. RAS which pSAR (Reads proportion of SUVA AS event) ≥ 50% were labeled.

E. Principal component analysis (PCA) based on RAS of pSAR ≥ 50%. The ellipse for each group is the confidence ellipse.

F. Heatmap presenting 5 classes of RAS(pSAR ≥ 50%)

G. The top 10 most enriched KEGG pathway were illustrated for RAS(pSAR ≥ 50%) genes of the group Tumor vs Normal. The colour scale shows the row-scaled significance (-log10 corrected P value) of the terms.

H. Boxplot showing splicing ratio of the important RASEs .*P < 0.001, **P < 0.001, ***P < 0.001. And The survival curves regulated by the important RASEs.

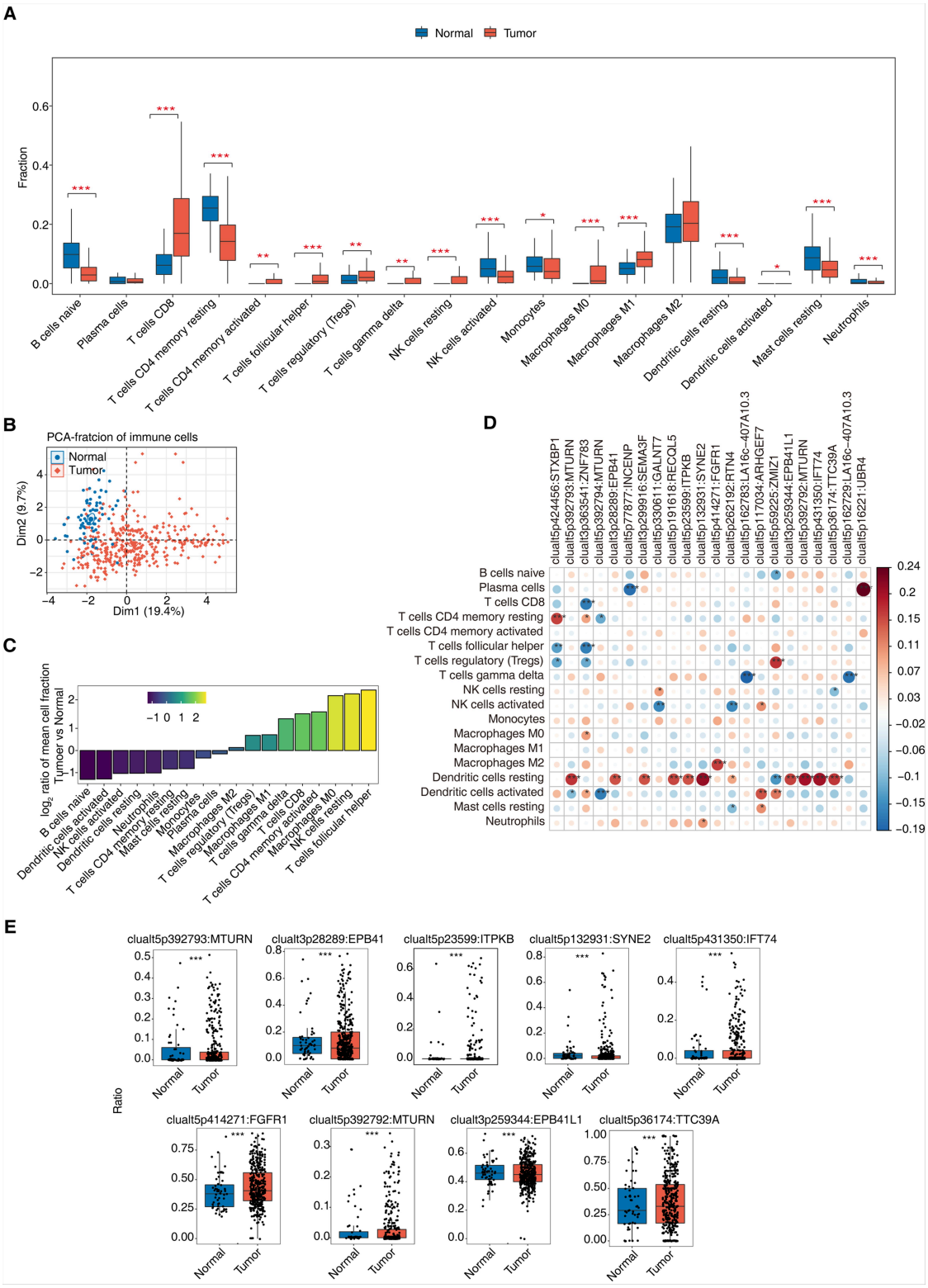


Figure 2

Diversity of immune microenvironment characteristics between Tumor and Normal samples from KIRC patients.

A. Boxplot showing fraction of each cell type in each group. The significant difference in the immune cell fractions between tumor and normal samples was calculated using the Student's t test.*: P value ≤ 0.05,

: P value ≤ 0.01,*: P value ≤ 0.001.

B. Principal component analysis (PCA) based on Fractions of different cells of all expressed

genes. The ellipse for each group is the confidence ellipse.

C. Tumor relative to normal group rank ordered based on decreasing values of the relative frequency ratio at tumor versus normal

D. The dot-plot demonstrated the correlations between each immune microenvironment infiltration cell type and the RASEs. Different color indicate correlation of immunocyte-RASEs and significant ones were labeled with star. *: P <=0.05; **: P<= 0.01; ***: P <= 0.001

Boxplot showing splicing ratio of the important RASEs .*P < 0.001, **P < 0.001, ***P < 0.001.

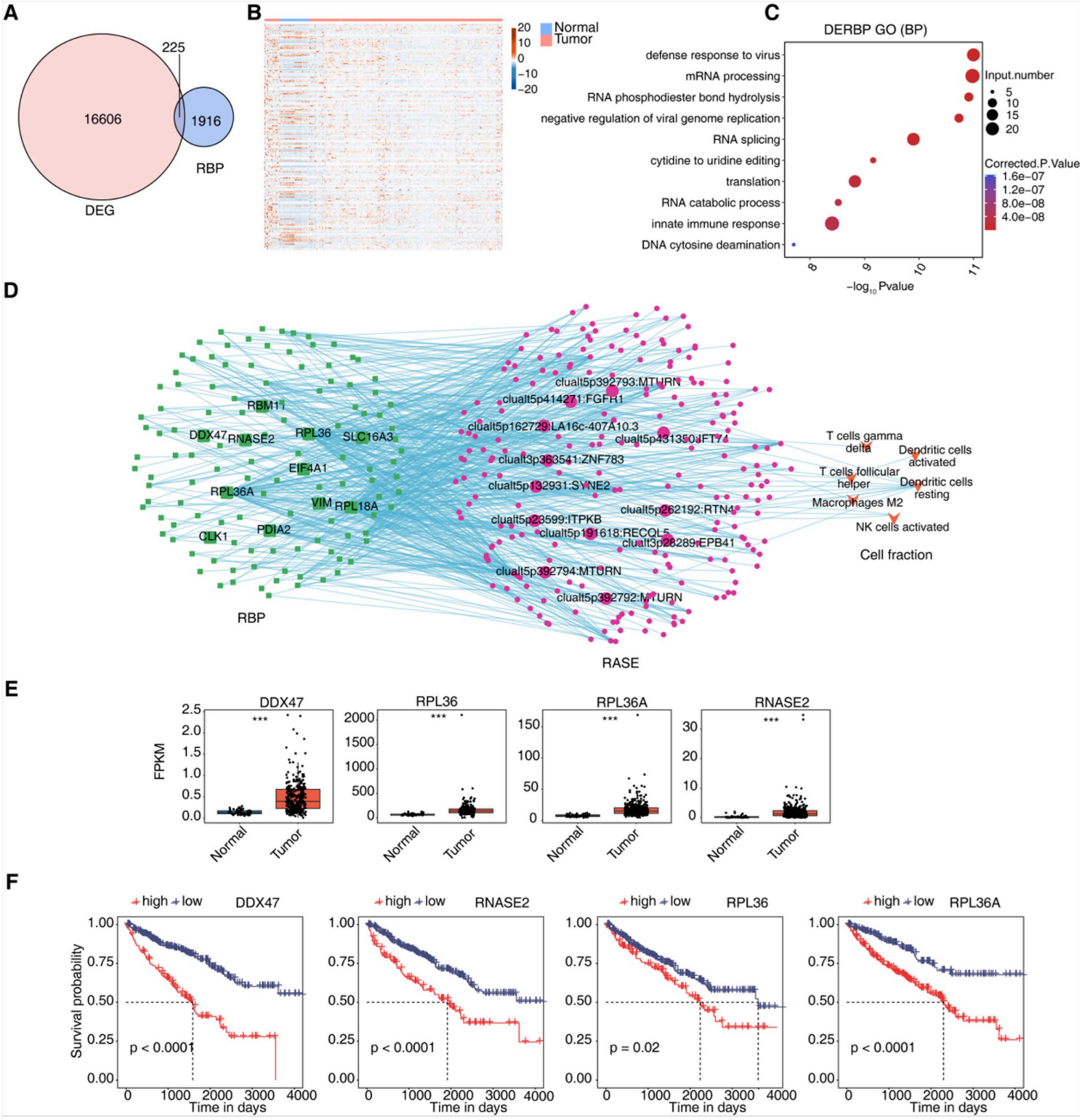


Figure 3

Identification of differentially expressed RBPs co-disturbed which immune related KIRC-RAS.

A. Venn diagram showing the overlap genes number of RBPs and DEGs.

B. Hierarchical clustering heat map showing expression levels of all DERBPs.

C. Network showing the important pathways of RAS and cell fraction regulated by DERBP.

D. The survival curves regulated by the importanct RBPs

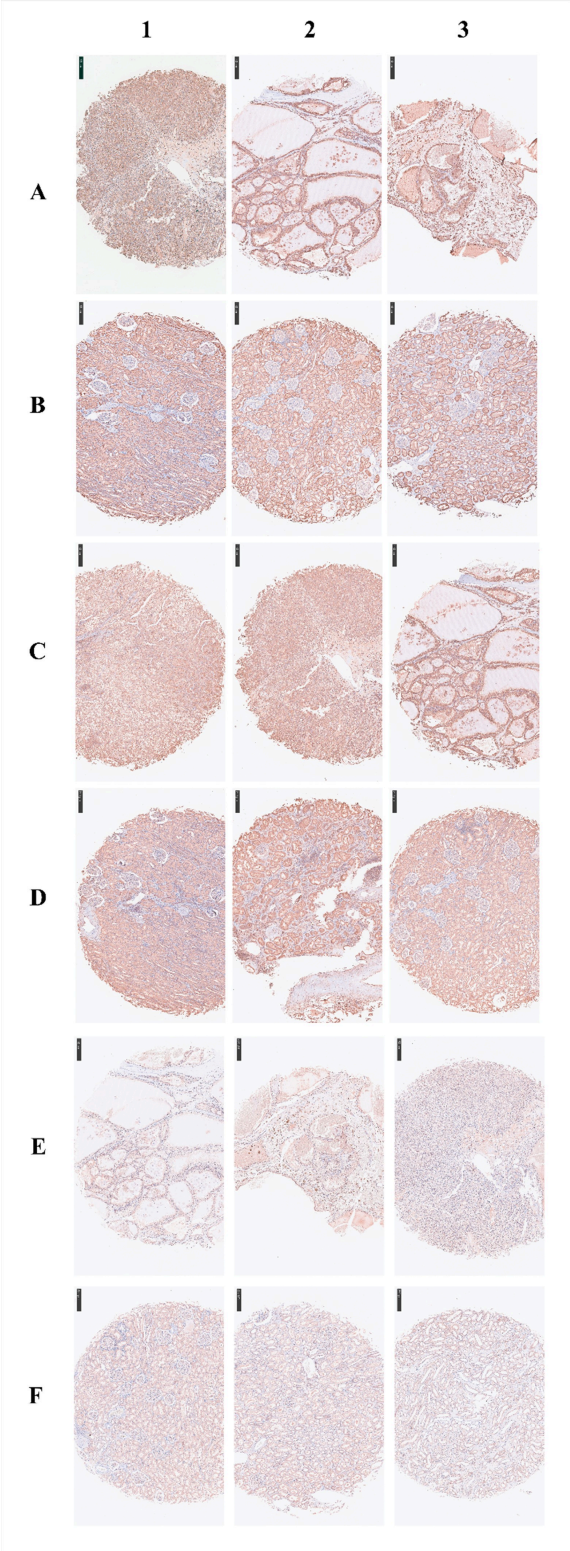


Figure 4

The expression of RBPs screened by immunohistochemistry in ccrcc and adjacent tissues

A. Expression of RNASE2 in cccRCC tissues.

B. Expression of RNASE2 in corresponding paracancerous tissues.

C. Expression of RPL36 in cccRCC tissues.

D. Expression of RPL36 in corresponding paracancerous tissues.

E. Expression of RPL36A in cccRCC tissues.

F. Expression of RPL36A in corresponding paracancerous tissues.

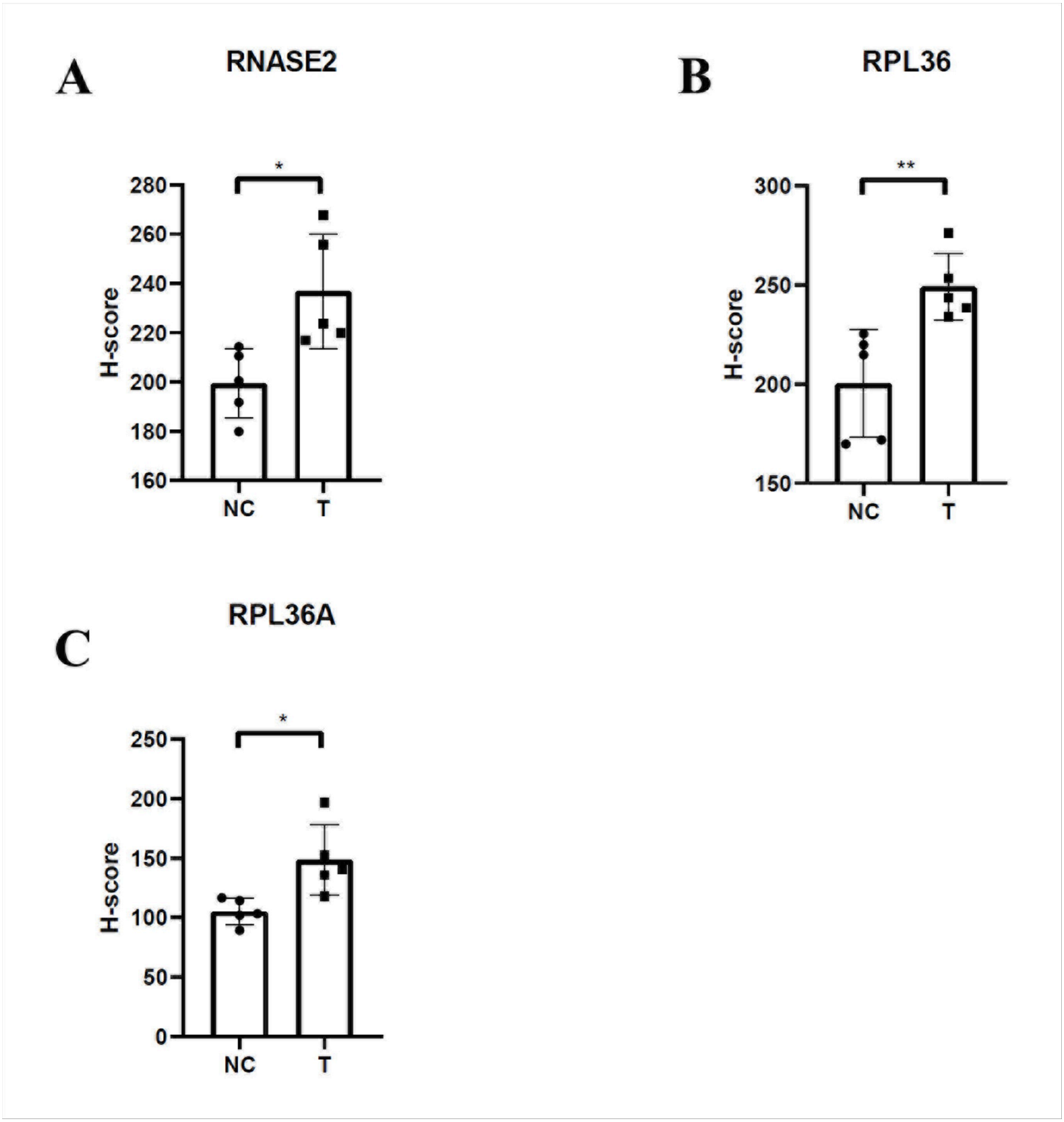


Figure 5

The expression of RBPs in tumor was higher than that in adjacent tissues.

A. The histogram showed that the expression of RNASE2 in tumor was higher than that in adjacent tissues.

B. The histogram showed that the expression of RPL36 in tumor was higher than that in adjacent tissues.

C. The histogram showed that the expression of RPL36A in tumor was higher than that in adjacent tissues.

*P < 0.001, **P < 0.001, ***P < 0.001.

Supplementary Files

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