Immunophenotyping analysis of renal cell carcinoma identified APOC1 as an immune-related biomarker and therapeutic target

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Abstract

Background: Renal cell carcinoma (RCC), the most lethal common kidney cancer, accounts for 2–3% of all cancers in adults with no effective diagnostic biomarker and therapeutic target till now.

Methods: In this study, the singularly expressed transcriptomics and immune infiltration phenotype of RCC had been clarified through systematic analysis of the RCC data set in TCGA. The abnormally expressed immune-related markers of RCC were analyzed via the above analysis. Using overexpression and knockdown assay, combined with migration, invasion, colony formation, cell cycle, immunohistochemistry, western blotting method experiments etc., the function and mechanism of immune-related marker were studied on RCC cell lines and nude mice.

Results: Interestingly, we found that apolipoprotein C1 (APOC1) was dramatically overexpressed and correlated with RCC immunophenotype. The level of APOC1 in the serum of RCC patients was significantly increased with development of RCC and the expression level of APOC1 was related to the overall survival and disease-free survival of the patients. Knockdown of APOC1 expression inhibited the migration and invasion of RCC cells, which might be mediated by TGF- β , MMP3, and E-cadherin. Overexpression of APOC1 promoted the growth of RCC cells in medium, 3D Matrigel, and nude mice. Overexpression of APOC1 promoted the transition from G1 to S phase, while silencing of APOC1 arrested cycle of RCC cells at G2/M phase via FOXO1/P27/Cyclin D1 and P53/GADD45A/cdc2/Cyclin B1 pathway.

Conclusions: Together, these data indicate that APOC1 was critical for mitotic progression, metastasis, and immune infiltration of RCC and may be an immune-related biomarker and therapeutic target for renal cell carcinoma.

Introduction

Renal cell carcinoma (RCC), the most lethal common kidney cancer, accounts for more than 100,000 deaths each year, and the incidence has steadily increased in recent decades^[1, 2]. Surgical resection is the main way of treatment^[3]. However, more than 40% patients with RCC develop metastases after radical nephrectomy, and the 10-year cancer-specific survival rate is dismal^[2, 4]. Unfortunately, there are no effective diagnostic biomarker and therapeutic target till now^[5]. It is, therefore, of necessity to find novel and significant targets or biomarkers for early diagnosis and treatment of RCC patients.

In recent years, immunotherapy with immune checkpoint inhibitors has become an essential pillar for cancer treatment and now represents the standard of care for several cancer types, including RCC^[6, 7]. However, several immune checkpoint inhibitors targeting programmed cell death protein 1 (PD1) and its ligands PD-L1 currently the most well-established approach, are only beneficial in ~20% of patients^[8]. Therefore, it is of great importance to discover a novel immunotherapy target. The composition of tumor-infiltrating immune cells can be characterized from bulk tumor RNA-seq data using computational

approaches based on a set of immune-specific marker genes or expression signatures^[9, 10]. It is an effective means to make use of gene expression data to define molecular immune signatures and evaluate immune infiltration level, which might help to discover new immunotherapy targets^[11].

Apolipoprotein is the protein component of lipoprotein^[12]. It is widely recognized that apolipoproteins could exert vital role in cardiovascular disease. APOC1 is the smallest apolipoprotein (6.6 kDa) and the component of triglyceride-rich lipoproteins and high-density lipoproteins^[13]. In recent years, apolipoproteins have been gradually discovered to be promising therapeutic targets as well as useful diagnostic and prognostic biomarkers in cancers^[14]. Several studies have been reported that APOC1 was closely associated with different types of cancers. It was shown that APOC1 was upregulated in pancreatic cancer and increased serum level of APOC1 was significantly related to poor prognosis of patients^[15]. Besides, APOC1 can affect colorectal cancer tumor progression by regulating the mitogen-activated protein kinase (MAPK) pathway^[16]. The results of a phase I clinical trial showed that the serum level of APOC1 in patients with advanced lung cancer was significantly higher than that in normal volunteers, which had diagnostic and prognostic value^[17]. It has been reported that APOC1 was upregulated in RCC^[18] and related to the metastasis of the clear cell renal cell carcinoma^[19]. However, the function of APOC1 in the diagnosis and treatment of RCC and the role of it in the RCC immune microenvironment has not been elucidated yet.

In the study, systematic analysis of the RCC data set in The Cancer Genome Atlas (TCGA) was performed to clarify the singularly expressed transcriptomics and immune infiltration phenotype of RCC. Interestingly, we found that APOC1 was dramatically overexpressed and correlated with RCC immunophenotype. In addition, the serum level of APOC1 in the RCC patients is significantly increased, and the expression level of APOC1 was related to the overall survival and disease-free survival of the patients. Knockdown of APOC1 expression inhibits the migration and invasion of RCC cells, which may be mediated by TGF- β , MMP3, and E-cadherin. Overexpression of APOC1 promoted the growth of RCC cells in medium, 3D Matrigel, and nude mice. Besides, overexpression of APOC1 promoted the transition from G1 to S phase, while silencing of APOC1 arrested cycle of RCC cells at G2/M phase via FOXO1/P27/Cyclin D1 and P53/GADD45A/cdc2/ Cyclin B1 pathway. In conclusion, APOC1 may be a promising diagnostic, prognostic marker and therapeutic target correlated with immune infiltration for RCC.

Methods And Materials

Patients and TCGA data retrieval

The RNA-seq and miRNA-seq data of 893 samples with RCC with 128 adjacent control were retrieved from the TCGA data portal. The TCGA dataset (<https://portal.gdc.cancer.gov/>), being comprised of more than two petabytes of genomic data, is publicly available, and this genomic information helps the cancer research community to improve the prevention, diagnosis, and treatment of cancer. A total of 54 paraffin-

embedded tissues of RCC were used in this study, obtained from the patients diagnosed at the Peking Union Medical College Hospital (PUMCH) in 2014. Clinical information related to the samples is summarized in Table 1. The serum of 73 RCC patients and 55 normal controls were collected for the experiment. Informed consents were obtained from all patients included in this study, and the study and all procedures used were approved by the Institutional Research Ethics Committee of PUMCH (S-424).

RNA sequence data processing

The Ensembl database^[20] (<http://www.ensembl.org/index.html>, version 89) was used to identify mRNAs and lncRNAs. In this study, we mainly used the program code written in Perl and R language to analyze and deal with RNA data. By using the edgeR^[21] package to further analyze the data, the differentially expressed mRNAs, lncRNAs and miRNAs were obtained. Some online websites are used as auxiliary analysis and data visualization, such as the Oncomine database (<https://www.oncomine.org/resource/login.html>), GEPIA database^[22] (<http://gepia.cancer-pku.cn/>), UALCAN database^[23] (<http://ualcan.path.uab.edu/>).

Identification of RCC Subtypes Based on Immune Gene Sets

A literature search was performed to determine 29 immune gene sets^[24] to represent tumor immunity. For each RCC dataset, the GSVA package was used for ssGSEA of the 29 immune gene sets^[25]. The ConsensusClusterPlus package was used for consensus clustering and molecular subtype screening of ssGSEA scores^[26]. The best cluster number was determined by the clustering score for the cumulative distribution function (CDF) curve, and the relative changes in the area under the CDF curve were evaluated. TIMER^[27, 28] database was used to visualize the correlation between APOC1 expression and immune infiltration (B cells, CD4 + T cells, CD8 + T cells, neutrophils, macrophages and dendritic cells) in RCC.

Cell lines and cell culture

RCC cell lines Caki-1, ACHN, 769-P and 786-O were purchased from the Cell Bank of the Chinese Academy of Sciences (Beijing, China). All cells lines were authenticated using short tandem repeat (STR) method, performed by this cell bank. Caki-1 and ACHN were grown as a monolayer in DMEM supplemented with 10% fetal bovine serum (FBS). 769-P and 786-O were grown as a monolayer in 1640 supplemented with 10% fetal bovine serum (FBS). Cells were cultured in adhesive sterile culture flasks at 37°C in a 5% CO₂ humidified incubator.

RNA extraction and qRT-PCR

The expression levels of APOC1 mRNA were determined with the use of quantitative real-time polymerase chain reaction (qRT-PCR). The total RNA was extracted from RCC tissues and paracancerous tissues by RNeasy Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions. Then, reverse transcription was conducted with a high-capacity cDNA synthesis kit (Takara, China). After reverse

transcription, qRT-PCR was carried out to evaluate the expression abundance of APOC1 mRNA. The reaction was conducted under optimal conditions: 95°C for 3 min, followed by 40 cycles at 95°C for 6 s, and 60°C for 35 s. The relative mRNA expression value was calculated by 2^{-DDT} method. β -actin was utilized as the internal control. The test was done in triplicate.

Transfection and stable-cell line construction

siRNA duplexes were obtained from Genepharma (Suzhou, China) and transfected into 786-O or 769-P cells using Lipofectamine 3000 (Invitrogen, USA) according to manufacturer's instructions. Human plasmid pCMV-Entry was purchased from OriGene Technologies, Inc. and APOC1 expression construct was generated by the subcloning of PCR-amplified full-length human cDNA into the pCMV-Entry vector. When cell confluence reached about 70–80%, plasmids transfection was performed with Lipofectamine3000 according to manufacturer's instructions.

Western blotting analysis

Equal amounts of whole tissue lysates were resolved by SDS polyacrylamide gel electrophoresis (PAGE) and electro-transferred onto a polyvinylidene difluoride (PVDF) membrane (Pall Corp., Port Washington, NY, USA). Subsequently, the lysates were incubated with a primary anti-APOC1 antibody (ab198288, Abcam, 1:1000 dilution). The immunoreactive signal was detected using the enhanced chemiluminescence kit (Amersham Biosciences, Uppsala, Sweden). The procedures were conducted according to the manufacturer's instructions.

Cell proliferative assay

Cells were seeded in 96-well plates at 2×10^3 /well and cultured for 24, 48, 72 h and 96h. 10 μ L CCK-8 (Dojindo, Japan) was added into the cells, and their viability was measured at 450 nm, using an ELISA plate reader (BioTek, VT), according to the manufacturer's instructions.

Cell migration and invasion assays

Cells were transferred to the top of the transwell chambers in serum-free DMEM medium (1×10^5 cells/well) in migration assay. DMEM medium containing 10 % FBS was added to the lower chambers. Cells were cultured for 24 hours. Cells on the top of membrane were removed by using a cotton ball and cells on the bottom of membrane of the transwell chambers were fixed in 4% formaldehyde and stained with 0.2% crystal violet. Cells were manually counted in 4 randomly chosen fields (one in middle and three in border) under a microscope, and photographs were taken. For invasion assay, the chambers of transwell were covered with 0.5 mg/mL matrigel (BD Biosciences, USA). The following procedure was same as migration assay.

Colony formation assay

Low melting point agarose was bought from Thermo Fisher Scientific (Waltham, MA, USA). The assays were performed in a 6-well plate. Cells were suspended in medium containing 0.4% agar and plated onto

a layer of 0.7% agar (2000 cells/well in 1.5 ml medium, 2 ml bottom agar). After cultured for 2–3 weeks, the colonies were stained with MTT.

Cell cycle assay

Cell cycle assay was performed by flow cytometry. The cells were harvested and fixed with 75% ethanol at 4°C for 24 h, followed by staining in PI (200 µg/mL RNase, 50 µg/mL PI and 0.1% (v/v) Triton X-100 in PBS) for 30 min. Cell cycle was determined by flow cytometry and analyzed by FlowJo software (Version10; FlowJo, LLC, Ashland OR, USA). Finally, the change of cell cycle was checked according to the above procedures.

Xenograft assay in nude mice

Animal study was approved by The Committee of Animal Care & Welfare at the Institute of Materia Medica, Chinese Academy of Medical Science & Peking Union Medical College (No. 0005265). About five weeks old athymic nude mice (16–18 g) were purchased by the Animal House in the Department of Animal Care Center at Institute of Materia Medica, Chinese Academy of Medical Science & Peking Union Medical College. The animals were housed at 24°C with ad libitum access to food and water. All experimental procedures were carried out in accordance with institutional guidelines for the care and use of laboratory animals at the Institute of Materia Medica, Chinese Academy of Medical Science & Peking Union Medical College and the National Institutes of Health Guide for Care and Use of Laboratory Animals (publication No. 85 – 23, revised 1985). Mice were randomly distributed at six per group, an aliquot of 5×10^6 ACHN_NC, ACHN_APOC1 was subcutaneously injected into the right flank of each mouse (n = 6). Tumor volume (mm^3) was measured with a Vernier caliper and calculated using the formula, $(L \times W^2)/2$, where L and W represented length and width of the tumor. After 18 days, all the mice were sacrificed, and the tumor tissues were harvested, weight and photographed. Intraperitoneal injection of 20 mg/kg sodium pentobarbital was applied in the anesthesia process for cell injection and the euthanasia.

Expression level of APOC1 in serum by ELISA

Serum was collected and centrifuged at 12000 rpm for 10 min. After centrifuged, the supernatant was collected and ELISA assay was carried out according to the protocol.

Immunohistochemical (IHC) analysis

IHC assay was done to check protein expression in 54 RCC tissues. In brief, paraffin-embedded specimens were cut into 4µm sections and baked at 65°C for 30 min. The sections were deparaffinized with xylene and rehydrated. Afterward, they were submerged into EDTA antigenic retrieval buffer and microwaved. The sections were treated with 3% hydrogen peroxide in methanol to quench the endogenous peroxidase activity, which was followed by the incubation with 1% bovine serum albumin (BSA) to block nonspecific binding. Rabbit anti-APOC1 antibody (1:500; Abcam, Cambridge, MA, USA) was incubated with the sections at 4°C overnight. As the negative controls, this antibody was replaced with normal goat serum or blocked with a recombinant APOC1 polypeptide, by incubation at 4°C

overnight before the IHC staining. After washed, the tissue sections were treated with biotinylated anti-rabbit secondary antibody (Abcam, Cambridge, MA, USA), followed by the additional incubation with streptavidin horseradish peroxidase complex (Abcam, Cambridge, MA, USA). Tissue sections were immersed in 3-amino-9-ethyl carbazole and counterstained with 10% Mayer's hematoxylin solution, dehydrated, and mounted in crystal mount (Electron Microscope Sciences, Hatfield, PA).

Immunohistochemistry evaluation

The H-score was assigned to paraffin-embedded specimens (possible score 0–9). The H-score was calculated as the product of the intensity of staining (0, negative; 1, weakly positive; 2, moderately positive; and 3, strongly positive) and extent of immune-expression. For immunoreactivity the extent of staining was scored 0–3 (0, no cells staining; 1, < 25% cells staining; 2, 26–69% cells staining; 3, > 70% cells staining). The raw data were then converted to IRS by multiplying the quantity and staining intensity scores. The final scores were: 0, 1, 2, 3, 4, 6, and 9, with 0 to 3 considered weak staining, over 4 strong IRS as described previously[15,16]. The average score over two core punches was used for analysis.

Statistical analysis

Student's t-test was used for comparison of two groups. Correlation between the APOC1 expression and the clinical characteristics in RCC patients were assessed by Spearman's correlation coefficient test. The survival analyses were conducted according to the Kaplan-Meier method. $p < 0.05$ (*), $p < 0.001$ (**) and $p < 0.0001$ (***) were considered statistically significant. All statistical analyses were performed using GraphPad Prism 7.0 and SPSS17.0.

Results

1. Comprehensive analysis of transcriptomics and immune phenotype of RCC

Through integrative analysis of the transcriptomic data of RCC in TCGA, the singularly expressed RNAs were shown in Fig. 1A and 1B. Interestingly, the biological process enrichment analysis showed that RCC differential genes are mainly enriched in immune and inflammation response or regulation pathways (**Supplementary Fig. 1A**). Besides, the transcriptome of RCC patients not only contained large number of changes in the protein-coding genes, but also abnormal expression of many non-coding RNAs (**Supplementary Fig. 1E**). It is worth noting that the expression of APOC1 was significantly increased in the majority of RCC patients. The mRNA expression files were utilized for immunotyping based on 29 immune gene sets via ssGSEA analysis. The RCC patients were divided into three subtypes to represent different level of tumor immunity, Immunity_H (High), Immunity_M (Medium) and Immunity_L (Low) (Fig. 1C). There were significant different infiltration levels of M1 macrophages, naïve B cells, activated dendritic cells, etc. among the three immunotypes (Fig. 1D). The GSEA enrichment analysis showed that the dendritic cell antigen processing, MHC class II processing and natural killer cell related signaling were the dominant different regulatory pathways among the subtypes (Fig. 1E). Besides, the expression levels

of the three immune checkpoints PD-1 (CD247), PD-L1 (PDCD1), CTLA4 (CTLA4), including APOC1, were negatively correlated with high immune levels (Fig. 1F). Altogether, APOC1 was associated with immunity of RCC.

2. APOC1 was highly expressed in RCC

The analysis using TCGA dataset identified that APOC1 was highly expressed and related to immune infiltration level of RCC. To further explore expression of APOC1 in RCC, bioinformatics analyses were performed using the TCGA, GEPIA, and Oncomine databases. Results showed that the expression of APOC1 was higher in major types of tumors than normal tissues, especially in kidney, lung, and breast (Fig. 2A and 2B). Results from eight different RNA-seq data robustly confirmed that the transcription level of APOC1 was significantly upregulated in RCC (Fig. 2C and 2D). Ten pairs of matched tumor and adjacent human tissue samples were used to check the mRNA and protein expression levels of APOC1 in RCC patients. The results also showed that the mRNA and protein expression levels of APOC1 were significantly higher in RCC than that in adjacent tissues (Fig. 2E and 2F). Taken together, all these results suggested that APOC1 was highly expressed in RCC.

3. APOC1 was correlated with RCC immune infiltration level

We further accessed the correlation of APOC1 expression with immune infiltration levels in RCC by TIMER, and the results indicated that APOC1 expression was positively associated with the infiltration levels of B cells, CD8 + T cells, neutrophils, macrophages, and dendritic cells, while negatively associated with CD4 + T cells in kidney renal clear cell carcinoma (KIRC) (Fig. 3A). Interestingly, APOC1 expression was positively associated with the infiltration levels of all above immune cells in kidney renal papillary cell carcinoma (KIRP) with higher relevance of correlation (Fig. 3B). In addition, the expression level of APOC1 was significantly related to a variety of tumor immune biomarkers, such as CD68, CD86 and CTLA4, etc in RCC (Fig. 3C). The copy number variation of APOC1 was correlated with the infiltration level of many immune cells in the tumor microenvironment of RCC (Fig. 3D and 3E). Moreover, the high expression of APOC1 of RCC in decreased natural killer cells, enriched macrophages, enriched mesenchymal stem cell, enriched B cells, enriched CD4 + memory T-cells, enriched CD8 + T-cells, decreased Type 1 T-cells, and enriched Type 2 T-cells subgroups had poorer prognosis (Fig. 3F). These above analyses suggested that high expression of APOC1 might affect prognosis of patients with RCC in part due to immune infiltration.

4. APOC1 might be a diagnostic and prognostic biomarker of RCC

Serum level of APOC1 was detected via ELISA assay from 55 healthy controls and 75 RCC patients. The results showed that the serum level of APOC1 in RCC patients was significantly higher than that of normal controls (Fig. 4A), which indicated that APOC1 might be a diagnostic biomarker of RCC. Besides, survival analysis identified that patients with high APOC1 expression level have a significantly poorer overall survival (Fig. 4B, $P = 0.024$) and relatively lower disease-free survival (Fig. 4C). The mRNA expression level of APOC1 was positively related to the tumor grade of RCC (Fig. 4D) with prognostic

significance using TCGA database (Fig. 4E). The expression rates of APOC1 in RCC with respect to several standard clinicopathological features are presented in Table 1. IHC analyses showed that the expression of APOC1 is significantly associated with age ($P = 0.007$), clinical stage ($P < 0.001$) and Furhman grade ($P < 0.001$). IRS was shown to increase with clinical stage ($P = 0.0246$) (Fig. 4F and 4G). However, it was not shown to be associated with gender ($P = 0.13$) and tumor size ($P = 0.175$). Spearman association coefficients between APOC1 expression and age, clinical stage and Fuhrman grade were 0.2 ($P = 0.005$), 0.309 ($P < 0.001$), and 0.397 ($P < 0.001$), respectively (Table 2). Taken together, these results indicated that expression of APOC1 was associated with many key clinical features of RCC, which might be a diagnostic and prognostic biomarker of RCC.

5. APOC1 promoted the migration and invasion of RCC

The migration and invasion experiments were applied on RCC cell lines to explore the significance of APOC1 in the metastasis process of RCC. Upregulation of APOC1 could promote the migration and invasion of ACHN and Caki-1 cells (Fig. 5A and 5C). On the other hand, downregulation of APOC1 prevented the migration and invasion of 786-O and 769-P cells (Fig. 5B and 5D). The cadherin and matrix metalloproteinase (MMP) family are essential proteins in epithelial–mesenchymal transition (EMT). They could degrade and remodel extracellular matrix and regulate the migration and invasion process. The expression of cadherin family (E-cadherin, N-cadherin, P-cadherin), SPAG5, ITGAV, and MMP family (MMP3, MMP7, MMP9 and MMP10) were checked by Western blot to investigate the role of APOC1 in the migration and invasion of RCC cell lines. It was found that APOC1 could significantly affect the expression of E-cadherin, MMP3, MMP7 and MMP10 (Fig. 5E and 5F). Besides, TGF- β , Slug, Snail and Twist1 are acquainted essential transcription factors that regulated the development of EMT. Studies identified that overexpression of APOC1 induced expression of TGF- β and Slug (Fig. 5G). On the contrary, knockdown of APOC1 expression inhibited expression of TGF- β and Slug. However, overexpression or knockdown of APOC1 had little effect on Snail and TWIST. In summary, APOC1 might promote the migration and invasion of RCC cell lines by mediating expression of TGF- β , MMP3, and E-cadherin.

6. APOC1 promoted the growth of RCC

To explore the effects of APOC1 on the growth of RCC, the experiments of proliferation, growth in 3D Matrigel, colony formation, and nude mice transplantation were carried out. The results showed that overexpression of APOC1 increased the proliferation of ACHN and Caki-1 (Fig. 6A and 6B), while knockdown of APOC1 expression in 786-O and 769-P significantly inhibited the proliferation of RCC cells (Fig. 6C and 6D). Besides, upregulation of APOC1 promoted the growth of ACHN cells in 3D Matrigel and soft agar. In contrast, downregulation of APOC1 inhibited the growth in 3D Matrigel and soft agar (Fig. 6E and 6F). *In vivo* experiments showed that overexpression of APOC1 could significantly promote the growth of RCC cell line ACHN in nude mice (Fig. 6G-6I). In addition, nude mice in the APOC1 overexpression group significantly lost their body weight two weeks after the tumor was implanted (Fig. 6J). Overall, APOC1 plays a vital role in the growth of RCC.

7. APOC1 played vital roles in cell cycle progress and immunomodulatory pathway of RCC

To explore the molecular mechanism of APOC1 in RCC, RNA-seq analysis was performed. Cell lines ACHN_NC and ACHN_APOC1 which have high APOC1 expression were harvested and sequenced. Compared with ACHN_NC group, 100 genes were upregulated and 26 genes were downregulated in ACHN_APOC1 (Fig. 7A and 7B). The top enrichment ontologies and KEGG pathways of these genes using the DAVID website were listed in Fig. 7C and 7D. The results showed that APOC1 may involve in cell cycle regulation, cell adhesion and immunomodulatory pathways. In addition, genes co-expressed with APOC1 were analyzed through the TCGA dataset on the cbiportal website. Using the top 200 APOC1 co-expressed genes for enrichment analysis, most of them are tumor immune response or regulation pathways (Fig. 7E-7H).

8. APOC1 regulated the cell cycle progression of RCC via FOXO1/P27/Cyclin D1 and P53/GADD45A/cdc2/ Cyclin B1 pathway

Flow cytometry analysis showed that overexpression of APOC1 could increase the cell proportion in G1/S phase (Fig. 8A and 8B), while inhibition of APOC1 arrested the cell cycle at G2/M phase (Fig. 8C and 8D). It was shown in Fig. 8E that overexpression of APOC1 inhibited expression of FOXO1, P21 and P27, while knockdown of APOC1 expression induced expression of them. On the contrary, overexpression of APOC1 induced the expression of Cyclin D1 and CDK4 and knockdown of APOC1 expression inhibited their expression. These results suggested that inhibition of APOC1 may activated downstream proteins p21 and p27 by activating FOXO1, thereby inhibiting the activity of the CDK2/cyclin E1 and CDK4/cyclin D complexes, arresting the cell cycle in the G0/G1 phase. In addition, overexpression of APOC1 promoted the expression of SPAG5, PLK1 and Cyclin B1 with the downregulation of P53 and GADD45A (Fig. 8F). The expression of cdc2 was also upregulated with the overexpression of APOC1 and knockdown of APOC1 inhibited it. The master mitotic driver is Cyclin B-CDK1 complex which is completely activated depending on the dephosphorylation of CDK1 at Y15/T14 and phosphorylation at T161. The results shown that overexpression of APOC1 reduced the expression of p-cdc2 (Tyr15) and increased the expression of p-cdc2 (Thr161). In conclusion, APOC1 could regulate the cell cycle progression of RCC via FOXO1/P27/Cyclin D1 and P53/GADD45A/cdc2/ Cyclin B1 pathway.

Table 1
Basic characteristics of patients and association between the
expression of APOC1 and clinicopathologic characteristics in
RCC tissues

	APOC1		P value
	Low (Score ≤ 3) N = 10	High (> 3) N = 36	
Age (years)			0.007
< 60	8	21	
≥ 60	2	15	
Gender			0.13
Male	5	23	
Female	5	13	
Clinical Stage			< 0.001
I	8	14	
II	2	14	
III	0	8	
IV	-	-	
Tumor size			0.175
< 4cm	6	15	
≥ 4 cm	4	21	
Furhman grade			< 0.001
I	5	10	
II	5	23	
III	0	3	

Table 2
Spearman association analysis between APOC1 and clinical pathologic factors

Variables	APOC1 expression level	
	Spearman association	P-value
Age (years)	0.2	0.005
Clinical Stage	0.309	< 0.001
Furhman Grade	0.397	< 0.001

Discussion

RCC refers to cancers that derive from the kidney epithelium, accounting for 90% of kidney cancers. RCC presented a high degree of heterogeneity, showing significant individual differences in gene phenotype, clinical responses, etc^[29]. Sorafenib^[30] and sunitinib^[31] are the major therapeutic drugs of RCCs, which are the vascular endothelial growth factor (VEGF) and its receptor (VEGFR) inhibitors respectively. In recent years, the application of immunotherapy and related immunotherapy drugs in advanced renal cell carcinoma has provided new ideas for the treatment of RCC^[6, 32]. Unfortunately, as these drugs generally slow the progression of disease with only modest objective response rates, it is necessary to identify new molecular targets in RCC for the development of more effective therapeutic strategies^[33].

APOC1 was shown to play important roles in several cancers. APOC1 was upregulated in pancreatic cancer and increased serum level of APOC1 was significantly related to poor prognosis of patients^[15]. The expressions of APOC1 mRNA and protein were increased in prostate cancer tissues and the serum levels of APOC1 were raised in prostate cancer patients^[34]. The mRNA and protein levels of APOC1 were significantly overexpressed in lung cancer at the late stage. However, no prognostic effect of serum levels of APOC1 could be found in lung cancer patients^[17]. However, there is lack of in-depth function and mechanism exploration on the diagnostic and prognostic value of APOC1 in RCC, and its ability to be used as a therapeutic target for the treatment of RCC. Our study systematically explored the value of APOC1 in the diagnosis and prognosis of RCC, as well as its application prospects and mechanism of action as a therapeutic target of RCC, and found that APOC1 is closely related to the RCC immune microenvironment.

In recent years, the public availability of cancer gene expression data, such as Oncomine databases and TCGA databases, has provided a unique opportunity to explore initial hypotheses and concepts that can subsequently be validated by additional experiments^[35]. In this study, the singularly expressed transcriptomics and immune infiltration phenotype of RCC had been clarified through systematic analysis of the RCC data set in TCGA. The identification of RCC immunophenotyping provided important theoretical support for the identification of immune-related biomarkers and targets of RCC. In addition, different infiltration level of immune cells and the analysis of immune pathways related to the occurrence

and development of RCC help to better understand the RCC immune microenvironment. Interestingly, we found that APOC1 was dramatically overexpressed and correlated with RCC immunophenotype. Besides, the copy number variation and expression of APOC1 were significantly associated with the infiltration levels of many immune cells in the tumor microenvironment of RCC. In addition, the expression level of APOC1 was significantly related to a variety of tumor immune biomarkers, such as CD68, CD86 and CTLA4, etc. Moreover, the high expression of APOC1 was related to poorer prognosis in immune cell subgroups with different enrichment degree. APOC1 is the smallest of all apolipoproteins and it is primarily expressed in the liver and activated when monocytes differentiate into macrophages^[36], which might play an important role in the tumor immunity. These above analyses suggested that high APOC1 might affect prognosis in part due to immune infiltration. Besides, the expression level of APOC1 was related to the overall survival and disease-free survival of RCC patients. It is indicated that APOC1 might be a promising immune-related prognostic biomarker. Specific experimental confirmation and mechanism research will be still needed.

The serum level of APOC1 of RCC patients was significantly raised. In addition, through bioinformatics analysis, RT-PCR, Western blot and IHC analysis, it was confirmed that the expression level of APOC1 in RCC tumors was significantly higher than that in adjacent tissues. More interestingly, the expression level of APOC1 is positively correlated with the malignancy of RCC tumors, and has a significant correlation with the prognostic indicators of RCC patients. Based on the above results, APOC1 may also be a promising diagnostic biomarker associated with the progression of RCC.

Knockdown of APOC1 expression inhibited the migration and invasion of RCC cells, while overexpression of APOC1 promoted these processes. Previous study has been identified that APOC1 could promote the metastasis of clear cell renal cell carcinoma via activation of STAT3^[19]. Our study firstly found that the regulation of APOC1 on the migration and invasion of renal cancer cells might be mediated by TGF- β , MMP3, and E-cadherin (Fig. 9A). Furthermore, overexpression of APOC1 promoted the growth of RCC cells in medium, 3D Matrigel, and nude mice. Studies had identified that APOC1 could stimulate the malignant process of RCC via the Wnt3a signaling, however the role and mechanism of APOC1 in the regulation of cell cycle have not been illustrated yet. It was firstly identified that overexpression of APOC1 could promote the transition from G1 to S phase via FOXO1/P27/Cyclin D1 pathway, while silencing of APOC1 arrested cycle of RCC cells at G2/M phase through P53/GADD45A/cdc2/Cyclin B1 pathway. These results reveal the role of APOC1 in the cell cycle of renal cancer cells, and the underlying mechanisms of APOC1 in the regulation of cell cycle (Fig. 9B). Together, these data indicate that APOC1 was critical for mitotic progression, metastasis, and immune infiltration of RCC and may be a promising therapeutic target for RCC. The development of APOC1 neutralizing antibodies may become a potential strategy for the treatment of RCC.

Conclusion

In this article, the immune phenotype and infiltration of RCC were systematically evaluated and analyzed. APOC1 had a strong connection with the tumor immune microenvironment of RCC and could serve as

immune-related biomarker. Besides, our results further confirmed that APOC1 played an important role in development and progression of RCC, and firstly revealed the mechanism of APOC1 in the regulation of renal cancer cell cycle. Not only that, APOC1 may also be a potential serum biomarker to diagnose RCC and a potential prognostic marker for RCC by experimental confirmation. This study provided a novel view of APOC1 in RCC and APOC1 could serve as an immune-related biomarker and therapeutic target of RCC.

List Of Abbreviations

Renal cell carcinoma, (RCC); Apolipoprotein C1, (APOC1); Programmed cell death protein 1, (PD1); Mitogen-activated protein kinase, (MAPK); The Cancer Genome Atlas, (TCGA); Quantitative real-time polymerase chain reaction, (qRT-PCR); Polyvinylidene difluoride, (PVDF); Short tandem repeat, (STR); Immunohistochemical, (IHC); Cytotoxic T-lymphocyte-associated protein 4, (CTLA4); Matrix metalloproteinase, (MMP); Vascular endothelial growth factor (VEGF), Kidney renal clear cell carcinoma, (KIRC); Kidney renal papillary cell carcinoma, (KIRP); Transforming growth factor- β , (TGF- β); Forkhead, (FOX);

Declarations

Consent for publication

Not applicable.

Availability of data and material

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

The experimental protocol was established, according to the ethical guidelines of the Helsinki Declaration and was approved by the Human Ethics Committee of Institutional Research Ethics Committee of PUMCH (S-424). Written informed consent was obtained from individual or guardian participants.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Conceived and designed the research: Jinhua Wang, Dexin Dong, Xifu Wang, Guanhua Du, Liwen Ren. Manuscript writing: Liwen Ren, Jie Yi, Chuncai Wu; Data acquisition and interpretation: Xiangjin Zheng, Jinyi Liu; Manuscript revision/review: Sha Li, Yihui Yang.

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Figures

Figure 1

Comprehensive analysis of transcriptomics and immune phenotype of RCC. (A) Volcano plots showing the differential expression of mRNA, lncRNA, microRNA in RCC; (B) Heatmaps demonstrated differential expression of mRNA, lncRNA, microRNA between RCC and normal samples. (C) The immunophenotype of RCC via ssGSEA analysis. (D) The fraction of various type of immune cells between different

immunophenotypes. (E) The GSEA enrichment of major immune regulatory pathways. (F) The mRNA expression of PD-1, PD-L1, CTLA4 and APOC1 between different immunophenotypes.

Figure 2

APOC1 is highly expressed in renal cell carcinoma. (A) APOC1 expression in tumor and normal tissues was analyzed in website <http://gepia.cancer-pku.cn/>. (B) APOC1 expression in tumor and normal tissues was analyzed in website <http://gepia.cancer-pku.cn/>. (C) APOC1 expression in tumor and normal tissues

was analyzed in website <https://www.oncomine.org/>. (D) APOC1 expression in RCC was analyzed in website <https://www.oncomine.org/>. (E) The mRNA level of APOC1 is up-regulated in human RCC tissue samples via RT-PCR experiment. (F) The protein level of APOC1 is upregulated in human RCC tissue samples by Western blot t.

Figure 3

APOC1 was correlated with RCC immune infiltration level. (A) The expression of APOC1 was significantly correlated with the infiltration level of various types of immune cells of KIRC. (B) The expression of APOC1 was significantly correlated with the infiltration level of various types of immune cells of KIRP. (C) APOC1 was significantly correlated with the expression level of many immune related markers. (D) The copy number of APOC1 was significantly correlated with the infiltration level of various types of immune cells of KIRC. (E) The copy number of APOC1 was significantly correlated with the infiltration level of various types of immune cells of KIRP. (F) Prognostic significance of APOC1 in different infiltrating immune cells subgroup.

Figure 4

APOC1 might be a diagnostic and prognostic biomarker of RCC. (A) The serum level of APOC1 was significantly increased in the RCC patients. (B) High expression of APOC1 was associated with unfavorable overall survival of RCC. (C) High expression of APOC1 was associated with unfavorable disease-free survival of RCC. (D) The mRNA expression of APOC1 in different tissue grades of RCC. (E) The effect of APOC1 expression & tumor grade on RCC patient survival. (F) The protein expression of

APOC1 in different tissue grades of RCC via IHC analysis. (G) The IRS score of APOC1 in different tissue grades of RCC.

Figure 5

APOC1 promoted the migration and invasion of RCC. (A) Caki-1 and ACHN cells with relative lower levels of APOC1 were stably transfected with pCMV6-APOC1-Myc-DDK for increasing APOC1 expression and the results were identified by Western blot. (B) The effects of APOC1 on migration and invasion in ACHN

and Caki-1 cells were measured by Transwell experiments. (C) RCC 786-O and 769-P cells with relative higher levels of APOC1 were transfected with siRNA duplexes of APOC1 for 72 h and detected by RT-PCR. (D) The effects of APOC1 on migration and invasion in 786-O and 769-P cells were measured by Transwell experiments. (E) The effect of APOC1 on the EMT related markers. (F) The effect of APOC1 on the proteins of MMP family. (G) The effect of APOC1 on the EMT related transcription factor.

Figure 6

APOC1 promoted the growth of RCC. (A) The effect of APOC1 on the growth of ACHN cells was detected by CCK8 assay. (B) The effect of APOC1 on the growth of Caki-1 cells was detected by CCK8 assay. (C) The effect of APOC1 on the growth of 786-O cells was detected by CCK8 assay. (D) The effect of APOC1 on the growth of 769-O cells was detected by CCK8 assay. (E) The effect of APOC1 on the colony formation of RCC cells. (F) The effect of APOC1 on the growth of RCC cells in the 3D Matrigel. (G) Overexpression of APOC1 promoted tumor growth in a human RCC xenograft mouse model, Scale Bar: 1cm. (H) Overexpression of APOC1 promoted tumor growth as measured by tumor weight. (I) Overexpression of APOC1 promoted tumor growth as measured by tumor volume; (J) Overexpression of APOC1 caused loss of weight in nude mice;

Figure 7

APOC1 played vital roles in cell cycle progress and immunomodulatory pathway of RCC. (A) Volcano plot of differential expression results; (B) Heatmap of differential expressed RNAs of ACHN cells overexpressed with APOC1; (C) GO enrichment analysis of differentially expressed genes; (D) KEGG pathway analysis of differentially expressed genes. (E-H) Enrichment of genes co-expressed with APOC1 in the TCGA cohort.

Figure 8

The function of APOC1 in regulating renal cell cycle. (A) The effect of overexpression of APOC1 on ACHN cell cycle; (B) The effect of overexpression of APOC1 on Caki-1 cell cycle; (C) Knockdown of APOC1 could arrested the cell cycle of 786-O in the G2/M phase; (D) Knockdown of APOC1 could arrested the cell cycle of 769-P in the G2/M phase; (E) The effect of APOC1 on the expression of G1/S phase related proteins. (F) The effect of APOC1 on the expression of G2/M phase related proteins.

Figure 9

Proposed mechanistic scheme: APOC1 is critical for metastasis and mitotic progression of RCC. (A) APOC1 promoted EMT by regulating expression of TGF- β , MMP3, and E-cadherin. (B) APOC1 promoted cell cycle by regulating FOXO1/P27/Cyclin D1 and P53/GADD45A/cdc2/ Cyclin B1 pathway.

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