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Original Article

Epigenetic differences in NCOA2: a novel biomarker that predicts prognosis in clear cell renal cell carcinoma

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Abstract: Objectives: Kidney cancer is one of the top ten cancers worldwide, and clear cell renal cell carcinoma (ccRCC) is the most common pathohistological type of kidney cancer. This study aimed to decipher the diagnostic and prognostic value of NCOA2 for ccRCC survival based on its expression and methylation. Methods: We explored the mRNA and protein expression, DNA methylation, prognosis, cell function, and relevant immune infiltration of NCOA2 in ccRCC using data from public databases. Furthermore, GSEA (Gene Set Enrichment Analysis) was used to dissect the cell functions and signal pathways associated with NCOA2 involved in ccRCC and evaluated the close correlation between NCOA2 expression and immune cells. Finally, RT-qPCR (quantitative reverse transcription PCR) and IHC (immunohistochemistry) were utilized to verify the expression of NCOA2 in ccRCC among the tumor and adjacent normal tissues collected from patients. Results: NCOA2 was lowly expressed in ccRCC tissue, which resulted from its methylation. High NCOA2 expression and low beta value of one of the CpG sites predicted better prognosis in patients with ccRCC. GSEA results and analysis of immune infiltration revealed that NCOA2 was associated with PD-1/PD-L1 expression and infiltration of other immune cells in ccRCC. Conclusions: NCOA2 has great potential to serve as a novel biomarker that can predict prognosis in ccRCC and may become a new therapeutic target in patients with late-stage ccRCC.

Keywords: NCOA2, biomarker, prognosis, renal cell carcinoma, DNA methylation, immune infiltration

Introduction

As of 2020, more than 430,000 new cases of renal cancer were diagnosed worldwide, including 180,000 new disease-related deaths, making it a severe threat to public health [1]. Accounting for approximately 75% of all renal cell carcinoma, ccRCC is the most common pathological subtype and has become a serious concern to clinicians and scientists for its difficulty in early diagnosis and treatment in terminally ill patients [2]. Although imaging technologies, including enhanced computed tomography (CT) and magnetic resonance imaging (MRI) and even more advanced imaging systems such as targeted positron emission tomography-computed tomography (PET-CT) imaging with radiolabeled antibodies have emerged, many patients already have developed metastatic lesions at the time of diagnosis and lost the chance to undergo radical surgeries [3]. While tyrosine kinase inhibitors (TKI) like sunitinib are highly recommended and widely employed in patients with metastatic ccRCC, therapeutic resistance appears inevitable in the final stages [4-6]. Immunotherapies have recently made remarkable progress in the field of cancer. As an advanced treatment, TKI combined with immune checkpoint inhibitors (ICI), which have been proposed for patients with late-stage ccRCC in the latest guidelines, has significantly improved the prognosis of many target patients [7]. However, not all terminal patients are compatible with this new therapy. Hence, novel biomarkers, by which ccRCC can be detected at an early stage or even preclinically with more specificity, and indicators predicting the usage suitability of immunotherapy in patients in late stages are urgently needed to improve the prognosis of ccRCC.

Nuclear receptor coactivators include seven subtypes that interact with nuclear hormone receptors, including steroid, thyroid, retinoid, estrogen, and androgen receptors, to stimulate their transcriptional activities in a ligand-dependent way [8]. Nuclear receptor coactivators play important roles in reproduction, stress, and behavior because hormones function almost everywhere in the body and are implicated in various processes [9]. As key regulators of the nucleus signal pathway, the prognostic and therapeutic value of these members in cancer is still barely understood [10, 11], and relevant biological processes and functions involved in ccRCC have never been reported.

Epigenetic regulation often participates in tumorigenesis [12]. DNA methylation, a common type of epigenetic alteration, is mostly connected with transcriptional silencing, which can result in the loss or downregulation of tumor suppressor genes, facilitating tumor formation and progression [13]. In addition, defective epigenetic regulation can also affect tumor immunogenicity and immune responses [14]. This reveals the therapeutic potential of targeting epigenetic alterations combined with immunotherapies.

In this study, we first analyzed the expression profile of subtypes of nuclear receptor coactivators in the KIRC (kidney renal cell carcinoma) dataset of TCGA and chose NCOA2 through a screening procedure to evaluate its various roles in ccRCC. In addition to the ability of NCOA2 itself to predict survival, we investigated the prognostic value of methylation of NCOA2 and relevant immune infiltration in ccRCC based on the analysis of gene function. We confirmed the low expression of NCOA2 in ccRCC at the mRNA and protein levels with the specimens collected from patients.

Materials and methods

Data downloaded from public databases

First, we downloaded expression data and clinical data of all of the subtypes of nuclear receptor coactivator in ccRCC and another two common pathohistological types of kidney cancer from TCGA database through UCSC Xena (https://xenabrowser.net/) to analyze their expression along with prognostic value in ccRCC and demonstrate their expression profiles in kidney caner. Two datasets from ONCOMINE (https://www.oncomine.org/resource/main.html), Jones Renal and Lenburg Renal were used to validate NCOA2's expression in ccRCC. Then we referred to cBioPortal (https://www.cbioportal.org/) and UALCAN (http://ualcan.path.uab.edu/) for the methylation data of ccRCC and normal kidney tissue. Beta values of each CpG site of NCOA2 in cancer and normal tissues were also downloaded from UCSC Xena.

Gene set enrichment analysis

Genes co-expressed with NCOA2 in ccRCC were accessed through cBioPortal and those which satisfied the inclusion criteria ($|r|>0.7$, $P<0.05$) were selected to perform the Gene Ontology analysis. We also utilized these 'correlated' genes along with DAVID Tool: Functional Annotation, to show the outcome of KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway which imply the probable functions related to NCOA2 in ccRCC. Other results came from the GSEA software with the expression matrix of ccRCC downloaded from TCGA and rearranged according to the expression of NCOA2 in each tumor specimen. The protein-protein interaction (PPI) was conducted in STRING (string-db.org).

TIMER algorithm analysis

Tumor IMmune Estimation Resource (TIMER, https://cistrome.shinyapps.io/timer/) was employed to analyze the relationship between NCOA2 expression and immune cell infiltration of CD4+ T cells, CD8+ T cells, B cells, neutrophils, dendritic cells and macrophages, respectively. Immune checkpoint molecules, PD-1 and PD-L1 were also included.

Validation of expression of NCOA2 in ccRCC by clinical specimen

The specimens used in this study to confirm the expression pattern of NCOA2 in ccRCC came from patients who received nephrectomy or partial nephrectomy in the Urology Surgery Department of Wuhan Union Hospital (Wuhan, China) and were diagnosed as ccRCC pathologically afterwards. The patients or their family members, from whom the collection of specimens came, gave consent before the surgery. The tissues were ground by a high speed grind-

Table 1. Primer sequences

		Sequences
GAPDH	Forward	5'-GAGTCAACGGATTTGGTCGT-3'
	Reverse	5'-GACAAGCTTCCCGTTCTCAG-3'
NCOA2	Forward	5'-AACAAATGACCCCAACCTGC-3'
	Reverse	5'-TAGACCCAGAACCAGGCAAG-3'

ing mill (KZ-II, Sevicebio, Wuhan, China). The TRizol reagent (Thermo, Massachusetts, USA) was utilized to extract RNA from the tissues. The NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, USA) was used to examine the purity and concentration of the RNA solution. 1 μg of tissue RNA was applied for reverse transcription with RT SuperMix (Vazyme, Nanjing, China). RT-qPCR was conducted (CFX96 Touch; BIO-RAD, America) with the SYBR Green mix (Vazyme, Nanjing, China) by ΔΔCq method. Samples were normalized by GAPDH. The primers, which were designed at Primer3web (primer3.ut.ee), were produced by TSINGKE (www.tsingke.net/shop/). Detailed primer sequences were listed in **Table 1**. RCC tissues and corresponding normal tissues were fixed in 10% formalin, dehydrated, and embedded in paraffin. The antibody used to perform IHC came from ABclonal (A10280, https://abclonal.com.cn/, dilution: 1:200).

Statistical methods

The data were analyzed with GraphPad (Version 8.4.3) or SPSS (Version 22.0). High and low expression of NCOA2 were determined based on the median mRNA value in each dataset. In the same way, hyper- and hypo-methylation groups were established. Of note, as for the survival analysis in the subgroup of ccRCC patients, the high and low expression groups were determined based on the whole cohort initially and every patient was fixed to a certain, high or low, group whichever subgroup he or she belonged to. The difference in continuous indexes with normal distribution between two groups was determined by Student's t test. Pearson correlation coefficient was utilized to examine the correlation of mRNA expression of NCOA2 with its DNA methylation. Prognostic value of NCOA2 and its methylation in ccRCC in predicting OS or DFS was illustrated in the form of Kaplan-Meier curve, in which time dependent-receiver operating characteristic (td-ROC) analyses and log-rank (Mantel-Cox) test were used and $P<0.05$ was considered significant.

Results

NCOA2 is specifically lowly expressed in ccRCC and of diagnostic and prognostic significance

The RNA-sequencing data of ccRCC and normal kidney tissue from 535 and 72 patients, respectively, from TCGA were analyzed. All the NCOA family members, except NCOA3 and NCOA5, were lowly expressed in ccRCC compared to normal tissue (**Figure 1A**). The survival information of these patients, from which the specimens were collected, was applied to determine the prognostic value of each gene combined with their expression profiles in ccRCC. The results revealed that NCOA1, NCOA2, NCOA4, and NCOA7 had prognostic value for overall survival (OS) and disease-free survival (DFS) (**Figure 1B**). Since we aimed to find a biomarker specific to ccRCC, we compared the expression of NCOA1, NCOA2, NCOA4, and NCOA7 in ccRCC and another two common pathohistological types of kidney cancer, including papillary renal cell carcinoma (KIRP) and chromophobe cell carcinoma (KICH), using the data in TCGA (**Figure 1C-F**). All these genes except NCOA2 are generally downregulated in all 3 subtypes of kidney cancer so they are considered as lacking in specificity in diagnosis of ccRCC. Conversely, it's safe to conclude that NCOA2 is the most favorable biomarker for ccRCC among these candidates although it's also downregulated in KIRP. In addition, the Jones Renal ($P<0.001$, **Figure 1G**) and Lenburg Renal ($P=0.013$, **Figure 1H**) datasets from ONCOMINE were employed to validate the expression pattern of NCOA2 in ccRCC. The ROC curve of NCOA2 in ccRCC is illustrated in **Figure 1I** (AUC=0.8521, $P<0.0001$), where the high expression of NCOA2 in ccRCC is associated with more favorable OS ($P<0.001$, **Figure 1J**) and DFS ($P=0.01$, **Figure 1K**).

Low NCOA2 expression in ccRCC is correlated with DNA methylation that predicts prognosis

As we preliminarily demonstrated the role of NCOA2, we then aimed to investigate the mechanism that led to the low expression of NCOA2 in ccRCC. Higher methylation levels in ccRCC compared to normal kidney tissues were recorded according to the data from UALCAN

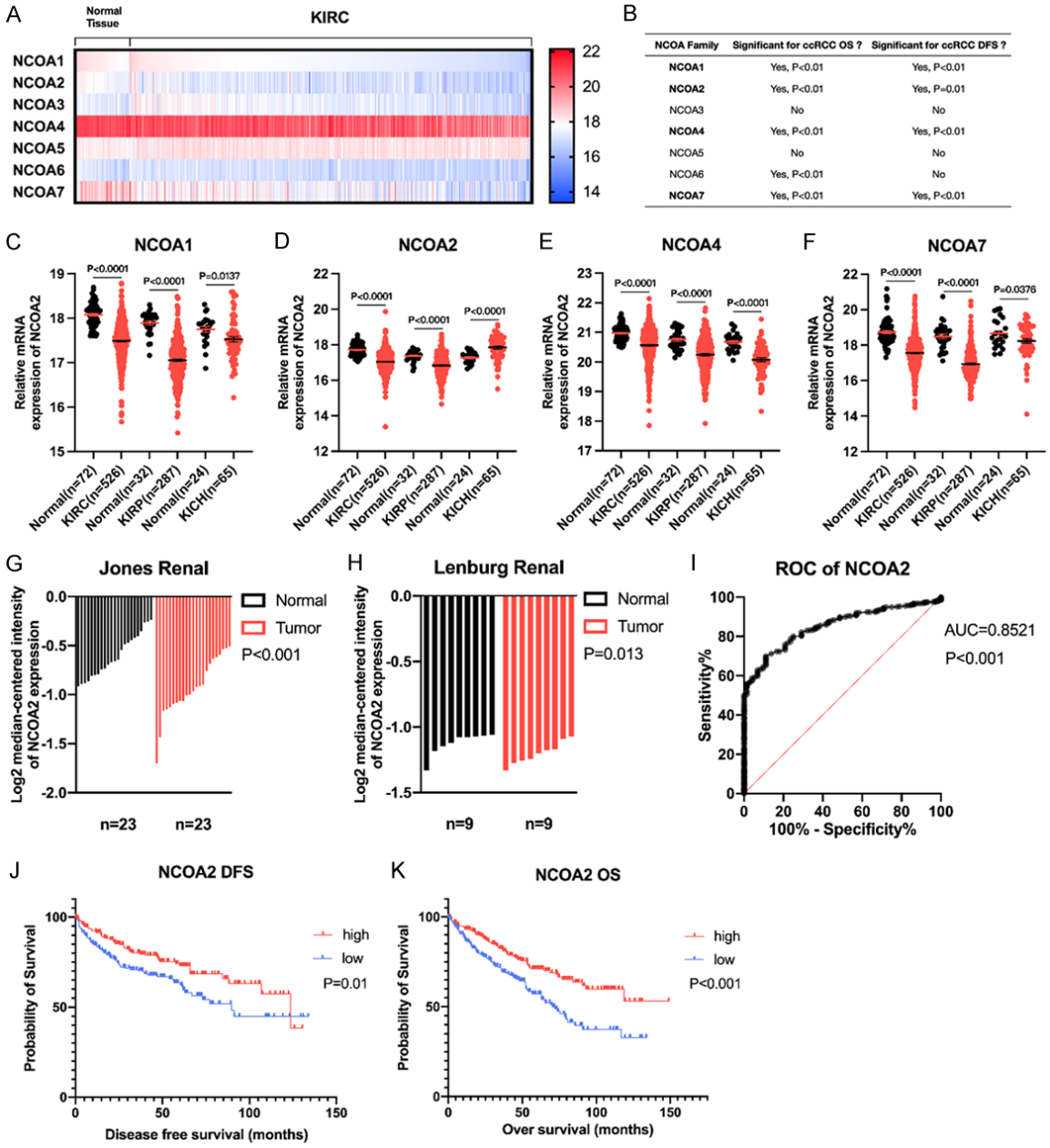


Figure 1. The expression pattern of NCOA1-7 and diagnostic and prognostic value of NCOA2 in ccRCC. (A) Heatmap of expression pattern of NCOA1-7 in ccRCC. (B) Prognostic value of NCOA1-7 in ccRCC. Expression of NCOA1 (C), NCOA2 (D), NCOA4 (E), NCOA7 (F) in KIRC, KIRP and KICH. Expression of NCOA2 in normal tissue and ccRCC in datasets (G, H) from ONCOMINE. (I) ROC curve of NCOA2 in ccRCC. The prognostic value of NCOA2 in OS (J) and DFS (K) of ccRCC. Clear cell renal cell carcinoma (ccRCC).

(*P*<0.001, **Figure 2A**). A correlation analysis between mRNA expression and methylation level of NCOA2 in ccRCC tissues was conducted, and it displayed that the mRNA expression of NCOA2 in ccRCC correlated negatively with its DNA methylation (r=-0.14, *P*=0.0112, **Figure 2B**). The beta value of each CpG site of NCOA2 in each specimen, tumor, or normal tissues are demonstrated as a heatmap in **Figure 2C**. Of these 41 CpG sites, cg24857609, cg27287166, cg07020398, cg26060583, cg22304838, cg01538165, and cg01012082 were filtered out for their beta values, and all exhibited a significant negative correlation (r<0, *P*<0.05) with the mRNA expression of NCOA2 in ccRCC. The methylation level of each of these sites is

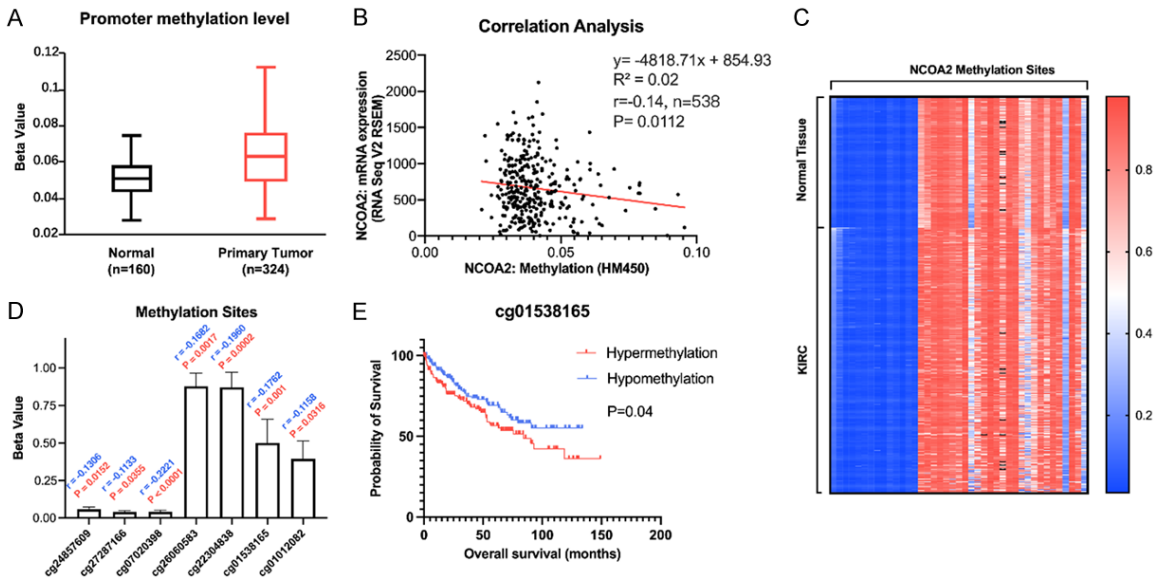


Figure 2. NCOA2 expression is associated with its DNA methylation in ccRCC. A. Promoter methylation level of normal tissue and primary tumor tissue. B. The correlation of NCOA2 expression with methylation in ccRCC tissue. C. Heatmap of beta value of all CpG sites of NCOA2 in each normal tissue and ccRCC tissue. D. Beta value of those CpG sites, methylation level of which is negatively correlated with NCOA2 expression in ccRCC. Pearson correlation coefficient and *P* value of methylation level of each site with the mRNA expression of NCOA2 are indicated. E. Prognostic value of methylation of cg01538165. Clear cell renal cell carcinoma (ccRCC).

displayed in **Figure 2D**. The low expression observed of NCOA2 in ccRCC was probably caused by its methylation at the above sites of this gene. To determine whether the methylation level of NCOA2 had any prognostic value, a survival analysis was carried out within these sites. The hypermethylation of cg01538165 (50% high beta value) predicted a poorer OS in ccRCC patients (*P*=0.04, **Figure 2E**).

Expression of NCOA2 associates with clinical parameters and predicts survival in some subgroups of ccRCC patients

To comprehensively explore the clinical value of NCOA2 in patients with ccRCC, we analyzed the correlation between its expression and clinical parameters. As illustrated in **Table 2**, the expression of NCOA2 had a close correlation with the M stage (*P*=0.005), longest dimension of tumor (*P*=0.005), person neoplasm status (*P*=0.014), disease-free status (*P*=0.006), and vital status (*P*<0.001) of the patients. Further, a survival analysis was performed in the different subgroups of patients with ccRCC and found that higher expression of NCOA2 predicted a better OS in males (*P*=0.003, **Figure 3A**) and females (*P*=0.014, **Figure 3B**), patients over 60 years old (*P*<0.01, **Figure 3C**), patients diagnosed at disease stage I or II (*P*=0.003, **Figure 3D**), patients with G3/4 or G4/4 tumors (*P*=0.001, **Figure 3E**), patients with T1 or T2 tumors (*P*<0.001, **Figure 3F**), patients without metastasis of the lymph node (*P*<0.001, **Figure 3G**), and patients without cancer metastasis (*P*=0.001, **Figure 3H**). As for DFS, higher expression of NCOA2 was associated with better survival in male patients (*P*=0.006, **Figure 4A**), patients over 60 years old (*P*=0.004, **Figure 4B**), patients with G3/4 or G4/4 tumors (*P*= 0.01, **Figure 4C**), patients with or without metastasis of the lymph node (*P*=0.01, **Figure 4D**; *P*=0.008, **Figure 4E**), and patients without cancer metastasis (*P*=0.014, **Figure 4F**).

GSEA and PPI reveal probable functions of NCOA2 in ccRCC

The GO (Gene Ontology) and KEGG Pathway results are displayed in **Figure 5A** and **5B**, respectively. NCOA2 was implicated in pathways including ubiquitin-mediated proteolysis, renal cell carcinoma, EGFR tyrosine kinase inhibitor resistance, mTOR signaling pathway, PD-L1 expression, and PD-1 checkpoint pathway in cancer. The expression matrix of ccRCC downloaded from TCGA, which was rearranged according to the expression of NCOA2 in each

Table 2. The association of NCOA2 expression with clinical parameters in ccRCC patients

Clinical parameters		NCOA2		*P* value
		Low expression	High expression	
Age	≤60	130	132	0.965
	>60	132	133	
Sex	Male	171	171	0.812
	Female	90	94	
Prior Cancer Diagnosis Occurrence	Yes	35	35	0.903
	No	221	228	
Primary Tumor Laterality	Left	128	121	0.413
	Right	132	144	
Neoplasm Disease Stage	Stage I	125	138	0.217
	Stage II	23	33	
	Stage III	66	56	
	Stage IV	46	36	
Neoplasm Histologic Grade	G1	6	8	0.165
	G2	111	112	
	G3	96	110	
	G4	46	29	
T stage	T1	127	142	0.373
	T2	33	35	
	T3	97	81	
	T4	4	7	
N stage	N0	106	132	0.075
	N1	7	9	
	NX	148	124	
M stage	M0	192	223	0.005
	M1	44	34	
	MX	23	8	
Longest Dimension	≤2	176	219	0.005
	>2	58	38	
Shortest Dimension	≤0.5	197	213	0.696
	>0.5	37	44	
Person Neoplasm Status	Tumor free	164	190	0.014
	With tumor	81	57	
Disease Free Status	Disease free	133	171	0.006
	Recurred/Progress	71	54	
Patient's Vital Status	Alive	156	197	<0.001
	Dead	103	67	

ccRCC, Clear cell renal cell carcinoma.

specimen, was applied to perform the GSEA. The GSEA results (**Figure 5C-H**) implied NCOA2's role in tumor progression and immunity of PI3K events in ERBB2 signaling, PID/IL1 pathway, FCGR3A mediated IL10 synthesis and B cell receptor signaling pathway. The PPI is presented in **Figure 5I**, where most of the proteins interacting with NCOA2, such as AR, CARM1, NR3C1, ESR1, NR5A2, RXRA, and PPARG, validated its role in intracellular receptor and steroid hormone-mediated signaling pathways, which suggested that biological processes associated with tumorigenesis or disease progression were probably bridgeable with endocrine signaling pathways by NCOA2.

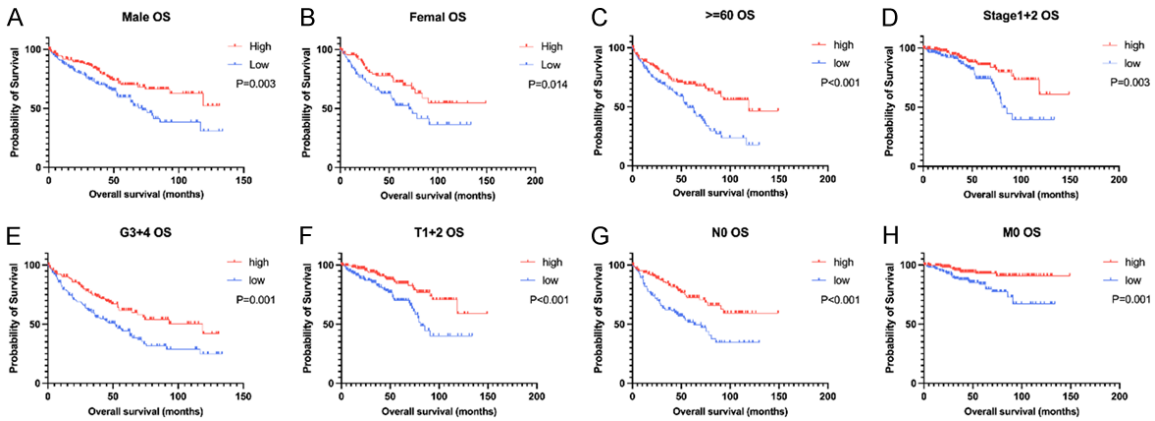


Figure 3. Prognostic value of NCOA2 as for OS in subgroup patients of ccRCC. A. Male patients. B. Female patients. C. Patients over 60 years old. D. Patients diagnosed at disease stage I or II. E. Patients with G3/4 or G4/4 tumor. F. Patients with T1 or T2 tumor. G. Patients without metastasis of lymph node. H. Patients without cancer metastasis. Clear cell renal cell carcinoma (ccRCC).

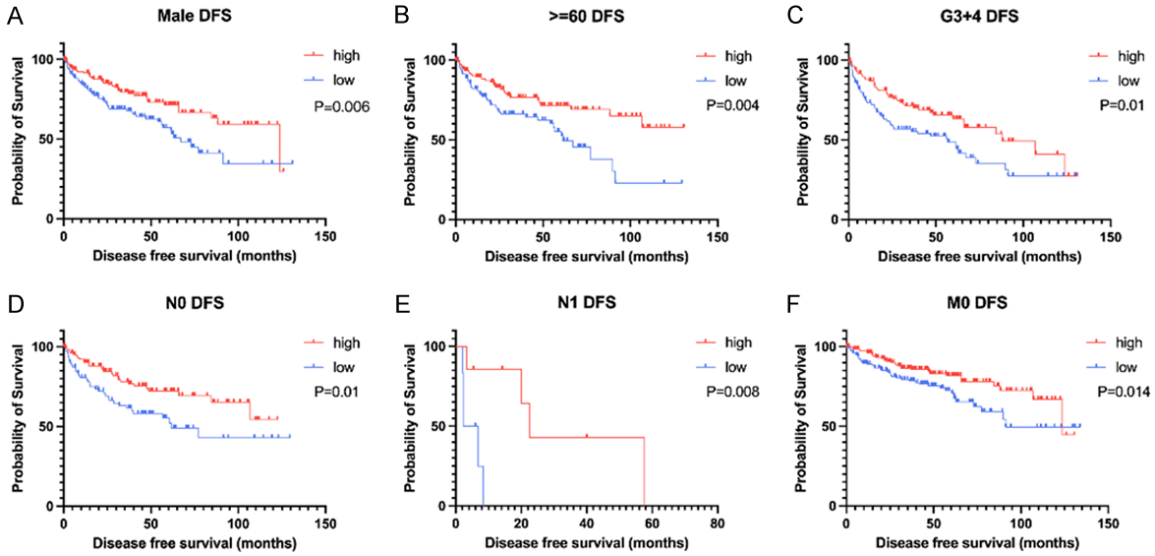


Figure 4. Prognostic value of NCOA2 as for DFS in subgroup patients of ccRCC. A. Male patients. B. Patients over 60 years old. C. Patients with G3/4 or G4/4 tumor. D. Patients with metastasis of lymph node. E. Patients without metastasis of lymph node. F. Patients without cancer metastasis. Clear cell renal cell carcinoma (ccRCC).

NCOA2 is correlated with immune cell infiltration and PD-L1 (CD274) in ccRCC

ccRCC has long been regarded as one of the most immune-infiltrated tumors and immunotherapy effective [15]. Since NCOA2 was implicated in some immune pathways in ccRCC through gene enrichment analysis, we turned to the TIMER to get an insight into the correlation of NCOA2 mRNA expression with immune cell infiltration in ccRCC. As exhibited in **Figure 6A** and **6B**, after adjusting for tumor purity, NCOA2 expression depicted a positive correlation with the infiltration level of B cells (r= 0.328, *P*=6.03e-13), CD8+ T cells (r=0.137, *P*=4.10e-03), CD4+ T cells (r=0.32, *P*=2.19e-12), macrophages (r=0.477, *P*=8.24e-27), neutrophils (r=0.46, *P*=2.22e-25), and dendritic cells (r=0.378, *P*=7.25e-17). We estimated the correlation of NCOA2 with PD-1 and PD-L1 in ccRCC and found that NCOA2 correlated positively with PD-L1 (r=0.622, *P*=9.25e-51, **Figure 6C**). Finally, we analyzed the correlation between NCOA2 expression and immune infiltration in ccRCC. NCOA2 expression demonstrated a significant positive correlation with

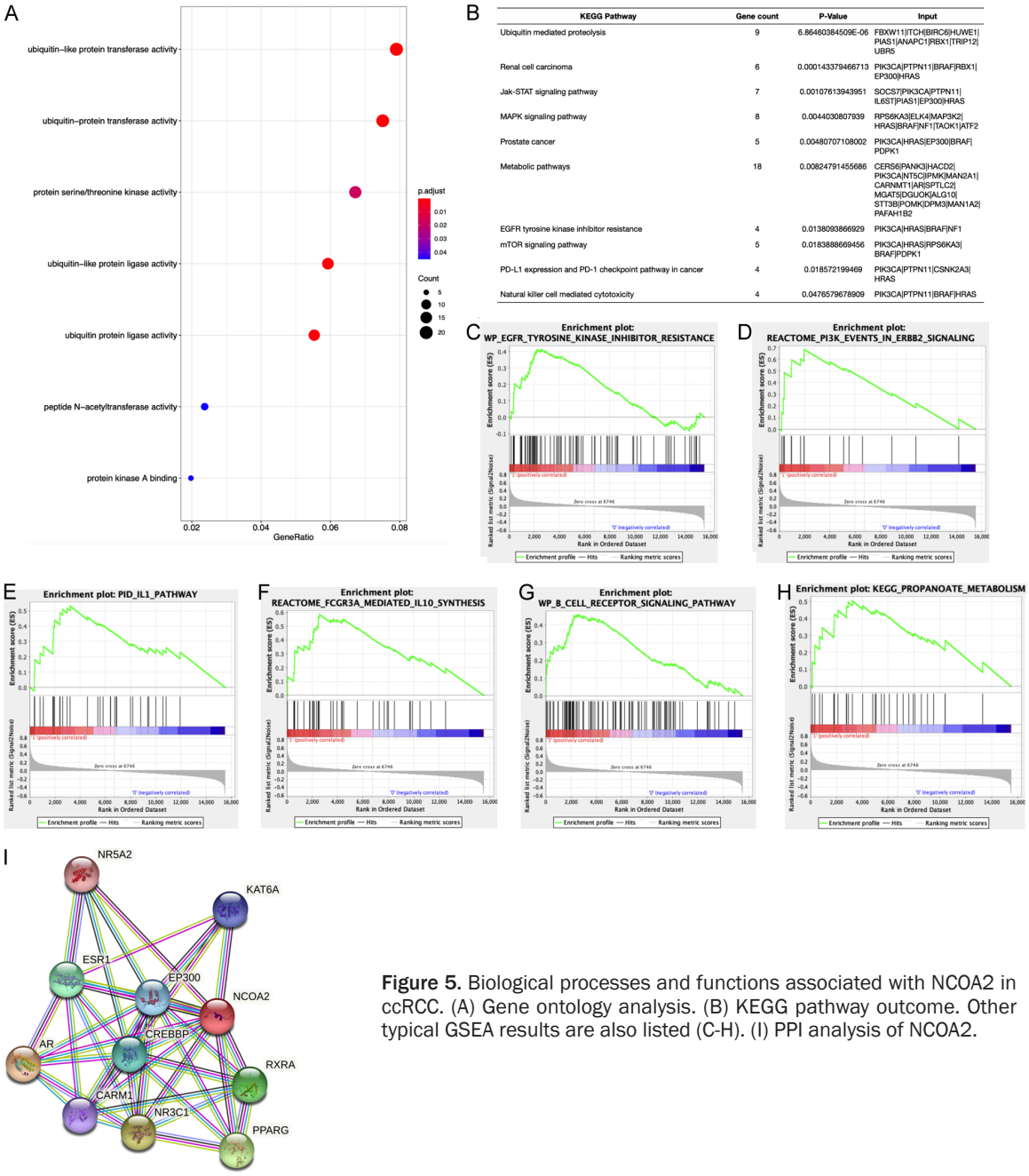
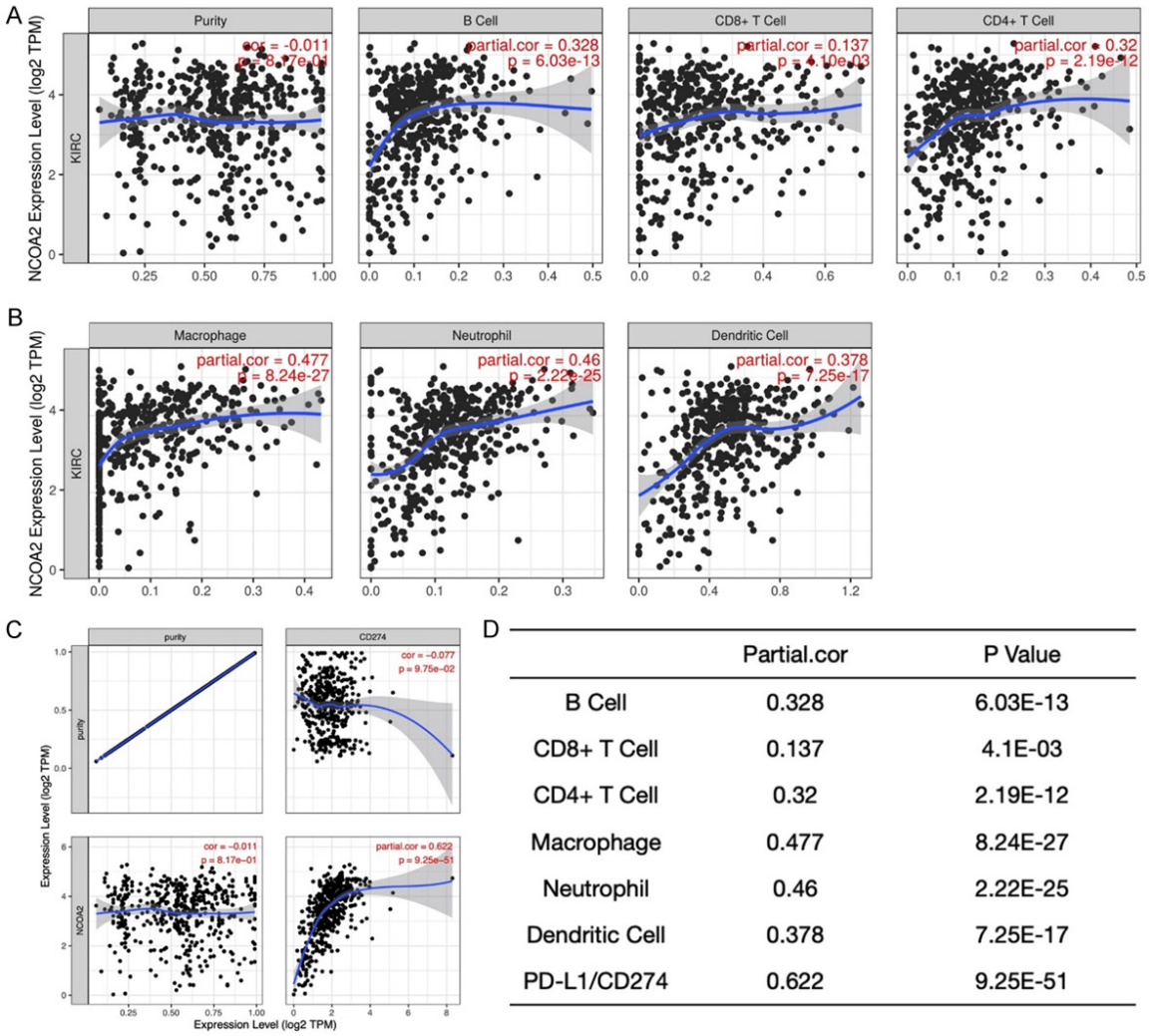


Figure 5. Biological processes and functions associated with NCOA2 in ccRCC. (A) Gene ontology analysis. (B) KEGG pathway outcome. Other typical GSEA results are also listed (C-H). (I) PPI analysis of NCOA2.

the infiltration level of all of the main specific or nonspecific immune cells in ccRCC, as displayed in **Figure 6D**. Overall, the correlation of NCOA2 with immune cell infiltration indicated that the influence of NCOA2 expression in ccRCC prognosis resulted, to some degree, from the immune infiltration, which may provide a new therapeutic target in addition to its co-expression with PD-L1 in ccRCC considering the recent popularity of immune therapy in cancer.

Expression pattern of NCOA2 in ccRCC is validated through RT-qPCR and immunohistochemistry

Thirty-eight pairs of tumor and adjacent normal tissues were collected from patients who received nephrectomy or partial nephrectomy and were diagnosed as ccRCC pathologically. We analyzed the expression pattern of NCOA2 among these specimens and found that NCOA2 was lowly expressed in ccRCC compared



	Partial.cor	P Value
B Cell	0.328	6.03E-13
CD8+ T Cell	0.137	4.1E-03
CD4+ T Cell	0.32	2.19E-12
Macrophage	0.477	8.24E-27
Neutrophil	0.46	2.22E-25
Dendritic Cell	0.378	7.25E-17
PD-L1/CD274	0.622	9.25E-51

Figure 6. Immune infiltration associated with NCOA2 in ccRCC. (A) Infiltration level of specific immune cells including B cell, CD8+ T cell and CD4+ T cell. (B) Infiltration level of nonspecific immune cells including macrophage, neutrophil and dendritic cell. (C) Correlation of PD-L1 with NCOA2 expression in ccRCC. After adjusted for tumor purity, Pearson correlation coefficient and *p* value are shown in the table (D). Clear cell renal cell carcinoma (ccRCC).

to the corresponding adjacent normal tissue in most patients (29 out of 38, $P<0.0001$, **Figure 7A**), which was consistent with the data from the public database. To validate the expression of NCOA2 in ccRCC at the protein level, we performed IHC, and the result depicted that NCOA2 protein was lowly expressed in ccRCC compared to corresponding adjacent normal tissue in all three pairs of tissues used ($P<0.0001$, **Figure 7B-D**).

Discussion

NCOA2, a member of the nuclear receptor coactivators, functions as a transcriptional coactivator for nuclear hormone receptors, including steroid, thyroid, retinoid, and vitamin D receptors. It acts as an intermediary factor for the ligand-dependent activity of these nuclear receptors, which regulate their target genes upon binding of cognate response elements and is involved in various cancers [16, 17]. NCOA2 is frequently up-regulated in patients diagnosed with metastatic prostate cancer, mediating the progression of metastatic castration-resistant prostate cancer [18, 19]. In gastric cancer, NCOA2 is an oncogene; thus, the knockdown of NCOA2 inhibits tumor growth by interfering with the Wnt signaling pathway [20]. Moreover, NCOA2 is also involved in disease development through fusions with other genes. The ETV6-NCOA2 fusion can induce arrest in T-cells at its early developmental stage

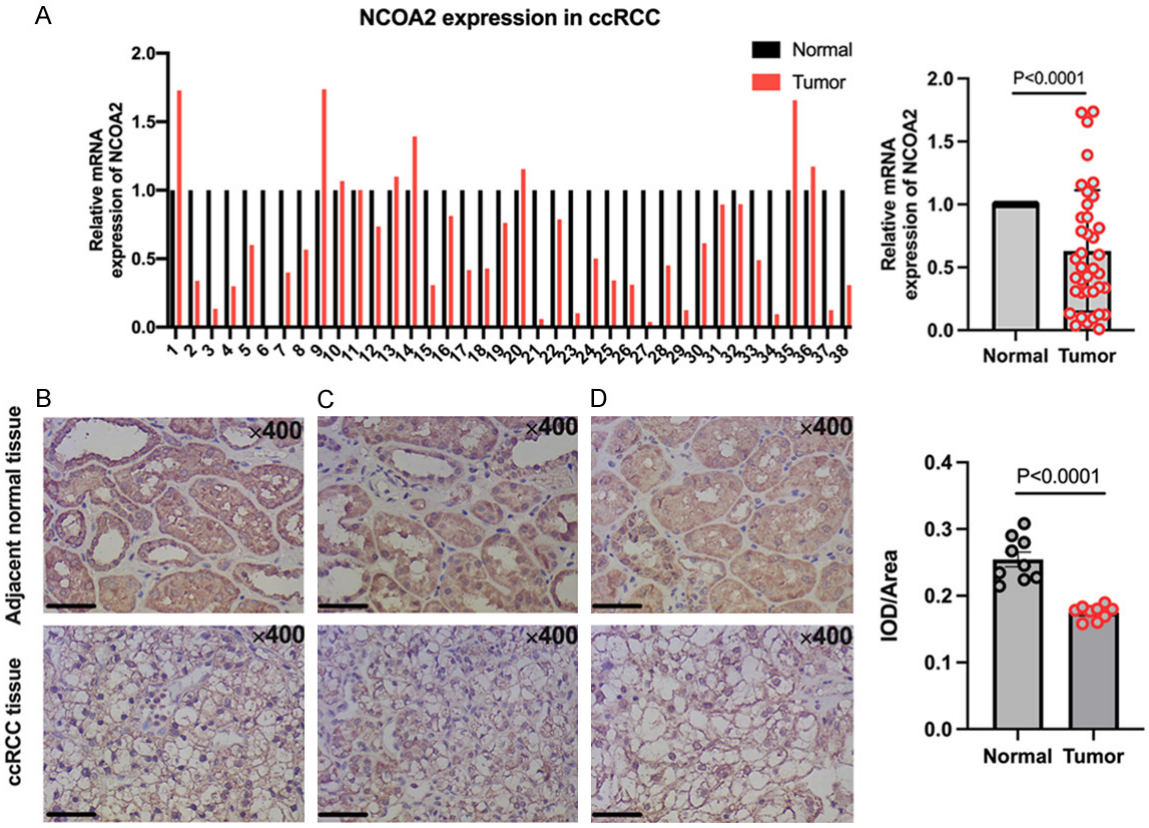


Figure 7. Confirmation of NCOA2 expression in ccRCC with patient-derived specimens. Relative mRNA expression of NCOA2 in ccRCC and corresponding normal tissues were shown in (A). IHC results of ccRCC and corresponding normal tissues were shown at lower and upper side of (B-D), respectively. Scale bars: 50 μm. Clear cell renal cell carcinoma (ccRCC).

and promote leukemia [21]. Recurrent MEIS1-NCOA2 fusions have been reported in several low-grade spindle cell sarcomas cases [22]. However, the role of NCOA2 in ccRCC remains poorly understood, and in our study, we first recognized NCOA2 as a tumor suppressor gene in ccRCC and revealed its diagnostic and prognostic value, especially its ability to predict survival based on the methylation level of one of its methylation sites.

Epigenetics, although the definition remains unclear, mostly refers to biological events regulating DNA-templated processes based on chromatin [13]. Recently, research has revealed that epigenetic alterations play important roles in tumorigenesis, like breast and prostate cancer [23]. Further, epigenetic aberrations frequently happen in ccRCC and are considered significant events in ccRCC progression [24]. As a vital epigenetic regulator, aberrant DNA methylation is a well-defined marker closely related to tumorigenesis [25]. The loss of the von Hippel-Lindau (VHL), which is widely known in renal cell cancers and leads to the accumulation of hypoxia-inducible factors in ccRCC [26], is associated with epigenetic silence for hypermethylation at a CpG island in the promotor region [27]. As for the prognostic value of epigenetic activities, some subpopulations of these epigenetic alterations may have the potential to be developed as clinical biomarkers in colorectal cancer (CRC) [28]. Chromatin alterations are also important epigenetic events; distinct chromatin alterations can be found in all stages of lung cancer, from tumorigenesis to tumor growth and metastasis. Therefore, these stage-specific epigenetic events can be used as tools for early lung cancer diagnosis with considerable sensitivity and specificity [29]. Apart from the fields of cancer, epigenetic mechanisms can affect Covid-19 (the coronavirus disease caused by the SARS-CoV-2 virus) outcomes by regulating IFN signaling,

angiotensin-converting enzyme-2 (ACE2), and other genes associated with immunity [30]. Here, we compared the promoter methylation level of normal kidney tissue with that of the primary tumor and compared different methylation sites of NCOA2 in ccRCC. Finally, we recognized the prognostic value of cg01538165 for its hypermethylation correlation with a poorer OS, which is consistent with previous reports about the prognostic role of epigenetic events.

During the last 15 years, medical interventions for ccRCC have rapidly developed from cytokine methods to targeted therapy against VEGF [31]. Nowadays, as immune therapy for cancer, especially those against PD-1/PD-L1, gains popularity and is validated to be incredibly effective [32, 33], novel medical approaches for ccRCC based on specific immunity agents have been brought into clinical trials and applied to patients [34]. Our study gave insight into the biological functions of NCOA2 involved in ccRCC through GSEA and PPI. Of these results, immune pathways where NCOA2 was implicated in the PID/IL1 pathway, FCGR3A mediated IL10 synthesis and PD-L1 expression, and PD-1 checkpoint pathway is worth highlighting. In turn, we referred to TIMER and analyzed the correlation between NCOA2 expression and immune infiltration in ccRCC. Further, PD-L1 was found to be positively correlated with NCOA2 expression in ccRCC (r=0.622, *P*=9.25e-51). Altogether, NCOA2 may become a novel therapeutic target for patients with ccRCC.

In addition to analyzing the data from the public database, we confirmed the expression pattern of NCOA2 in ccRCC through RT-qPCR and IHC within the specimens collected from patients receiving nephrectomy or partial nephrectomy. The results suggest NCOA2 as a powerful prognostic biomarker for ccRCC. However, it is worth noting the limitations of this study. Restricted by sample numbers, we did not include more clinical samples for IHC for a more accurate result. Besides, nearly all methylation and prognostic analyses were based on information from a public database; thus, more experimental research is needed to validate the role of NCOA2 in ccRCC and the underlying mechanisms.

In conclusion, NCOA2 expression is low in ccRCC and negatively correlated with DNA methylation. Both high NCOA2 expression and hypomethylation of cg01538165, one of the CpG sites of NCOA2, mean better OS survival in ccRCC. Furthermore, NCOA2 expression conceivably regulates the infiltration level of specific and nonspecific immune cells. Hence, NCOA2 probably contributes to immune infiltration and could serve as a powerful prognostic biomarker in patients with ccRCC. On the other hand, considering the significance of the methylation of NCOA2 and its close correlation with immune infiltration in ccRCC, medical treatment targeting epigenetic regulation of NCOA2 or combined with immune therapy may become a novel approach to attenuating tumor progression in patients with ccRCC.

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Disclosure of conflict of interest

None.

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