METTL13 Inhibits Progression of Clear Cell Renal Cell Carcinoma with Repression on PI3K/AKT/mTOR/HIF-1 α Pathway and c-Myc Expression

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Research

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Abstract

Background: Clear cell renal cell carcinoma (ccRCC) is the most common and aggressive type of renal malignancy. Methyltransferase like 13 (METTL13) functions as an oncogene in most of human cancers, but its function and mechanism in ccRCC remain unreported.

Methods: qRT-PCR, western blot and immunohistochemistry were used to detect METTL13's expressions in tissues. The effects of METTL13 on ccRCC cells' growth and metastasis were determined by both functional experiments and animal experiments. Weighted gene co-expression network analysis (WGCNA) was performed to annotate METTL13's functions and co-immunoprecipitation (co-IP) was used to determine the interaction between two proteins.

Results: METTL13 was lowly expressed in ccRCC tissues compared to normal kidney tissues and its low expression predicted poor prognosis for ccRCC patients. In vitro study indicated METTL13's inhibition on ccRCC cells' proliferation, viability, migratory ability and invasiveness as well as epithelial-mesenchymal transition (EMT). Bioinformatic analyses showed various biological functions and pathways METTL13 was involved in. In ccRCC cells, we observed that METTL13 could negatively regulate PI3K/AKT/mTOR/HIF-1 α pathway and that it combined to c-Myc and inhibited c-Myc expression. In vivo experiment confirmed that METTL13 inhibited ccRCC cell growth and metastasis.

Conclusions: In general, our finding suggests that associated with favorable prognosis of ccRCC patients, METTL13 can inhibit growth and metastasis of ccRCC cells with multiple potential molecular mechanisms. Therefore, it's likely for METTL13 to serve as a new diagnostic and therapeutic target for ccRCC in the future.

Background

As the most frequently diagnosed cancer of kidney and the ninth most common of all types of cancer, renal cell carcinoma (RCC) was estimated to cause 14,770 deaths in United States with 73,820 new cases reported in 2019 [1] and the situation in China was similar [2]. Resection is still the major and the most effective therapeutic method for localized RCC, while metastasis occurs in approximate 25% cases, which makes it difficult for patients to undergo surgery [3]. Among all the histological and molecular subtypes of RCC, clear cell RCC (ccRCC) is the most common subtype, accounting for about 75% [1, 4]. Besides, ccRCC is resistant to radiotherapy and chemotherapy, which makes patients' prognosis far from satisfying [5, 6]. Thus, it's urgent and important to identify crucial biomarkers and to have comprehensive insights into deep molecular mechanisms playing in ccRCC with the aim to provide molecular bases and inspirations for ccRCC diagnosis, monitoring and treatment.

Protein methyltransferase like 13 (METTL13), also called FEAT, is coded by gene METTL13, which is located at chromosome 1q24.3. Purified from rat livers in 2011 a Japanese researcher, METTL13 was found to be abnormally overexpressed in several human cancers including lung cancer and to drive tumorigenesis in transgenic mice [7]. After that, a study indicated that post-transcriptionally regulated by

miR-16, it inhibits apoptosis of lung, breast and liver cancer cells [8]. In 2016, researchers detected high expression levels of METTL13 in the blood plasma of patients with breast, ovarian and lung cancer [9], following which it was identified as a recurrence predictor for breast cancer [10]. As a methyltransferase protein, it can specifically di-methylate the Lys55 of eEF1A, resulting in the increase of its translational output and tumorigenesis of lung and pancreatic cancer [11, 12]. In 2019, scholars demonstrated that METTL13 is positively transcriptionally regulated by HN1L and it can enhance hepatocellular carcinoma development by up-regulating TCF3 and ZEB1 [13]. Despite the studies above proving the oncogenicity role of METTL13, an article elucidated its downregulated expression in bladder cancer and its tumor-suppressing functions [14]. However, studies targeted at METTL13 are still very deficiency and unintegrated with uncertainty of its roles in various cancers; meanwhile, its expressions and biological functions in RCC remain unknown, which are worth further exploration.

In this study, it was revealed that METTL13 was down-expressed in ccRCC tissues compared to normal kidney and its low expression was associated with unfavorable prognosis. It repressed proliferation, migratory ability and invasiveness of ccRCC cells with inhibition on PI3K/AKT/mTOR/HIF-1 α signaling pathway and c-Myc expression and with potential participation in other mechanisms. These findings may provide insights into understanding of METTL13's molecular functions in ccRCC as well as inspiration for ccRCC diagnosis and therapy.

Materials And Methods

Bioinformatic analyses

Website UALCAN (<http://ualcan.path.uab.edu/>) was used to visually obtain gene expressions in different sample types (ccRCC tissues and normal kidney tissues), different pathological grades and clinical stages of ccRCC with expression data provided by The Cancer Genome Atlas (TCGA) database. METTL13 expressions in ccRCC tissues and adjacent normal tissues were also acquired from a dataset (GSE53757) of the Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo/>) [15]. Website GEPIA (<http://gepia.cancer-pku.cn/>) directly produced survival curves of ccRCC patients with high and low METTL13 expression levels based on an appropriate expression threshold. Transcriptome data of kidney renal clear cell carcinoma (KIRC) cohort was downloaded from TCGA database and differentially expressed genes (DEGs) was filtered out with $|\log FC| > 1$ and false discovery rate (FDR) < 0.05 as the criteria. We performed weighted gene co-expression network analysis (WGCNA) with the WGCNA package in R (The WGCNA package in R). ClusterProfiler R package was used to determine gene modules' gene ontology and KEGG pathway enrichments regarding FDR < 0.05 as threshold.

Patient samples

All the ccRCC tissues and their corresponding adjacent normal tissues were obtained from the urology surgery department of the first hospital of China Medical University (Shenyang, China). This study was approved by Research Ethics Committee of China Medical University and all the patients had supplied the written informed consent.

Cell lines and cell culture

Human cell lines, 786-O, 769-P, OS-RC-2, Caki-1 and ACHN were purchased from Chinese Academy of Sciences Type Culture Collection Cell Bank (Shanghai, China). 786-O, 769-P and OS-RC-2 cells were cultured in RPMI medium (Hyclone; GE Healthcare), while Caki-1 cells and ACHN cells were treated with McCoy's 5A medium Hyclone; GE Healthcare) and MEM medium (Hyclone; GE Healthcare), respectively. All the mediums contained 10% fetal bovine serum (FBS; Biological Industries, Beit-HaEmek, Israel) and cells were cultured in an atmosphere of 37°C along with 5% CO₂.

Cell transfection

Two strands of siRNAs targeting at METTL13 were designed and purchased from JTSBIO Co. (China), sequences of which were as following: si-METTL13#1 (sense: GCGGGGUGCUACAUAUAAUATT; anti-sense: UAUUUAUGUAGCACCCCGCTT), si-METTL13#2 (sense: GCUCUGCCCUUCAGAUCUUTT; anti-sense: AAGAUCUGAAGGGCAGAGCTT). Usage of LipofectamineTM3000 (Invitrogen, USA) was involved in siRNA transfection with the protocol provided by manufacturer's guide- lines. METTL13 overexpression plasmid was purchased from GeneChem (Shanghai, China) and transfection was performed according to the manufacture's guidance.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from tissue samples and cells with the use of RNAiso Plus (Takara Biotechnology, Dalian, China) under the guidance of manufacture's recommendations, following which Prime Script RT Master Mix (Takara Biotechnology, Dalian, China) was utilized to conduct reverse transcription to synthesize cDNA. With the use of a SYBER Premix Ex TaqTMKit (Takara Biotechnology, Dalian, China), qRT-PCR was conducted by LightCyclerTM 480 II system (Roche, Basel, Switzerland), after which the 2^{-ΔΔCt} method was used to calculate the relative expression level of each sample referring to internal β-actin or GAPDH expression. Information of primer sequences is listed in Supplementary table 1.

Western blotting assay

Total protein from patient samples or cells was obtained by RIPA lysis buffer with 1% Phenylmethylsulfonyl fluoride (PMSF) contained and protein concentrations were detected by bicinchoninic acid (BCA) assay kit (Beyotime Institute of Biotechnology). Equal mass of proteins (30 μg/lane) were used for electrophoresis in 10% SDS- polyacrylamide, followed by PVDF membrane (0.2 μm) transfer, membrane block by 5% non-fat milk, primary and secondary antibody incubation as well as image capture by an EasySee Western Blot kit (Beijing Transgen Biotech, Beijing, China) and a chemiluminescence system (Bio-Rad, CA, USA). Information of primary antibodies is listed in Supplementary table 2.

5-Ethynyl-2'-deoxyuridine (EdU) assay

EdU assay was performed with the usage of an EdU kit (BeyoClick™, EDU-488, China). Cells were co-cultured with EdU working solution (1:1,000) in the atmosphere of 37°C and 5% CO₂ for 2~4 hours, followed by fixation with 4% paraformaldehyde for 30 min and treatment with 0.3% Triton X-100 for 30 min at room temperature. Then, according to the manufacture's protocol, cells were co-incubated with click reaction solution for 30 min at room temperature in a dark environment, after which cells were treated with Hoechst solution for 10 min. We used a fluorescence microscope (Olympus Corporation, Japan) to capture images with a magnification of 400 × and cell counting was conducted by ImageJ software.

Cell viability assay (CCK-8 assay)

Counting Kit-8 (CCK-8) solution (Bimake, USA) was added to each well of 96-well plates to the concentration of 0.5 mg/ml, where 2.0×10^3 cells had been initially seeded. After Incubation for one hour at 37°C with 5% CO₂, absorbance at 450 nm was measured by plate reader (Model 680; Bio-Rad Laboratories).

Wound-healing assay

When the density of cells in 6-well plates reached above 90%, we used a 1-mL pipette tip to vertically scratch an artificial wound in the middle of the wells. Then cells were washed with PBS and new FBS-free medium was added. Images were obtained with the help of an inverted microscope (EVOS XL system, AMEX1200; Life Technologies Corp, Bothell, WA, USA) under a magnification of 100 ×. After cultured for 48 h, cell images were re-obtained.

Cell migration and invasion assay

8-μm-pore transwell chambers in 24-well plates (Corning Costar, Corning, NY, USA) were used. Chambers coated with Matrigel (BD, San Diego, CA, USA) were for cell invasion detecting, while those without Matrigel coating were used to determine cell migration. 600μl 10%-FBS containing medium was placed into each bottom chamber, while equal number of suspended cells ($1.0 \sim 1.5 \times 10^4$ cells for migration assay, $3.0 \sim 4.0 \times 10^4$ cells for invasion assay) in 200μl medium without FBS were imbedded onto each upper chamber. After incubated at temperature of 37°C along with 5% CO₂ for certain time (24h for 786-O, Caki-1, OS-RC-2 and 48h for ACHN) suspended cells in the upper chamber were washed out, while cells adhering to the bottom membrane were stained by crystal violet. Images were gained by using the inverted microscope described previously under a magnification of 200 × and cell counting was dependent on software *ImageJ*.

Co-immunoprecipitation (Co-IP) assay

Cells were lysed by RIPA lysis buffer containing 1% PMSF and 1% protease inhibitor. A certain amount of cell lysate was isolated as input, while 5μg primary antibody (METTL13, abcam, ab186002; c-Myc, Santa Cruz, sc-40) or homologous IgG (Santa Cruz Biotechnology) was co-incubated with remaining lysate at

4°C overnight. Then, 30-μl protein A/G-beads was co-incubated with the lysis solution at 4°C for an hour, after which beads was extracted and washed by washing buffer three times. Next, protein was isolated from beads into 2 × protein loading buffer after co-incubation at 100°C for 15min. Western blot was finally conducted.

Immunohistochemistry assay

Tissues previously formalin-fixed and paraffin-embedded were sliced into 4-μm slides. Following procedures were as previously described [16], which involved use of rabbit anti-METTL13 antibody (GTX120626, GeneTex, USA) and an UltraSensitive™ SP (Mouse/Rabbit) IHC kit (Maxin-Bio, Fuzhou, Fujian, China) according to the manufacture's guidance. Images were captured by the inverted microscope with magnifications of 200 × and 400 ×.

Animal experiments

Experiments with animals involved were approved by China Medical University Ethics Committee of Medical Experimental Animal Welfare and were conducted following the institute's guidelines. Female BALB/c-nude mice of 4-6 weeks old, purchased from Beijing Vital River Experimental Animal Technology Co. Ltd., were housed in a pathogen-free environment at Experimental Animal Department of China Medical University. As for the tumorigenicity study, 1×10^6 OS-RC-2 cells (empty vector or METTL13 overexpression) in 150μl serum-free 1640 medium containing 40% Matrigel were injected subcutaneously into flank of each mouse, 30 days after which mice were euthanized and tumors were excised. Primary tumors were measured for their size and weight. and as for the metastasis study, 1×10^5 OS-RC-2 cells (empty vector or METTL13 overexpression) in 150μl pathogen-free PBS were injected into per mouse via its lateral tail vein. After 45 days, lungs were separated and metastatic tumors were counted. HE staining was involved in observing serial histological sections of the lungs.

Statistical analysis

Each experiment was performed independently for at least 3 times and data were expressed as the mean ± standard deviation (SD). Software GraphPad Prism of version 8.0 (La Jolla, CA, USA) was used perform all the statistical analysis, which involved Mann-Whitney U-test and Student's t test. As for all the data, $P < 0.05$ was regarded as statistically significant. * indicates $P < 0.05$; ** indicates $P < 0.01$, *** indicates $P < 0.001$.

Results

Low expression of METTL13 is associated with poor outcome of ccRCC

Despite the oncogenic roles of METTL13 in most of human cancers, not only TCGA database but also GEO dataset showed that METTL13 was transcriptionally downregulated in ccRCC tissues (Fig. 1a, b). Meanwhile, by using the website UALCAN, we detected significant negative correlations between

METTL13 expression levels and tumor grades as well as cancer stages of ccRCC (Fig. 1c, d). According to Kaplan-Meier survival curves provided by GEPIA on the basis of TCGA database, it was found that patients with higher METTL13 expression levels were more likely to have better prognosis (Fig. 1e). By analyzing 50 pairs of our own patient samples via qRT-PCR assay, we observed significant decrease of METTL13 mRNA expression levels in ccRCC tissues compared to adjacent normal tissues (Fig. 1f, g), which was followed by a similar result of western blot confirming its down-expression in ccRCC at protein level (Fig. 1d). Immunohistochemistry results suggested that METTL13 protein expressions declined with increase of tumor grades (Fig. 1i). Therefore, we concluded that down expression of METTL13 was associated with ccRCC occurrence and unfavorable prognosis.

Knockdown of METTL13 inhibits ccRCC cells' proliferation, migration and invasion

We first detected METTL13 protein expression conditions in 5 ccRCC cell lines (OS-RC-2, 760-P, ACHN, Caki-1 and 786-O) and a normal renal proximal tubule epithelial cell line, HK-2. Results showed that METTL13 was significantly down-expressed in most of the cancer cell lines (Fig. 2a). According to the results, two cell lines, 786-O and Caki-1, in which METTL13 was relatively high-expressed, were chosen to transfect with siRNAs to silence METTL13 expression. Two strands of METTL13 siRNA were designed and their knockdown efficiencies were validated by immunoblotting assay (Fig. 2b). After transfecting the siRNAs, we found that proliferation and viability of the two cell lines were significantly enhanced (Fig. 2c, d). Furthermore, with knockdown of METTL13, wound healing rates of 786-O cells and Caki-1 cells were respectively upregulated (Fig. 2e), which demonstrated promotion of cell migratory ability, further validated by migration assay (Fig. 2f). Meanwhile, silencing METTL13 expression improved cells' invasiveness (Fig. 2f). Western blotting results revealed that N-cadherin expression was upregulated in METTL13-silenced ccRCC cells, whereas E-cadherin was distinctly down-expressed (Fig. 2g). In general, inhibition of METTL13 expression facilitated proliferation, viability, migration and invasion of ccRCC cells.

METTL13 promotes ccRCC cells' proliferation, migration and invasion

Then, with lentiviral of vector expressing METTL13, we respectively constructed METTL13 stable-overexpressed OS-RC-2 and ACHN cell lines (Fig. 3a). Results of EdU assay suggested significant decrease in proliferating cells' portion after repressing METTL13 expression (Fig. 3b). In accordance with our expectation, METTL13 upregulation gave rise to not only cell viability inhibition (Fig. 3c) but also restraint on cell migration and cell invasiveness (Fig. 3d, e). The alterations in expressions of EMT-related proteins, N-cadherin and E-cadherin, were shown in (Fig. 3f), which were opposite to what resulted from siRNA disposals. These data suggested upregulation of METTL13 promotes growth and metastasis of ccRCC.

Functional annotation of METTL13

Then we excavated potential molecular mechanism of METTL13 in ccRCC with bioinformatic analyses used. After scale-standardization, we brought all the DEGs in ccRCC including METTL13 into WGCNA.

The merged dynamic result showed that genes were divided into eight modules, the black, blue, green, magenta, pink, purple, red and turquoise module (Fig. 4a). METTL13 and genes with similar expression mode were located in the turquoise module (Fig. 4b). Functional enrichment analysis of the turquoise module was shown in Fig. 4c, which suggested that METTL13 had tremendous potential to participate in metabolism regulations, including metabolism-related pathways like the HIF-1 signaling pathway. Furthermore, by extracting nodes in METTL13's secondary connection and drawing network, we noticed that despite location in turquoise module, METTL13 directly connected to many genes situated in the blue module, which share high correlation with METTL13 (Fig. 5a). Meanwhile, the interactions between METTL13 and other modules were mainly dependent on the blue module. As for genes directly linked to METTL13, results of functional enrichment analyses were shown in Fig. 5b, based on which we predict that METTL13 affects tumor metastasis not only via EMT regulation but also by modulating cell adhesion. Taken together, METTL13 might regulate various biological functions as well as signaling pathways in ccRCC.

METTL13 inhibits PI3K/AKT/mTOR/HIF-1 α signaling pathway in ccRCC

With the guidance of bioinformatic analyses, we noticed that METTL13 participated in regulating HIF-1 signaling pathway. As a core factor participating in HIF-1 signaling pathway, hypoxia-inducible factor-1 α (HIF-1 α) has been reported to affect multiple biological behaviors of renal cell carcinoma cells [17,18]. Then, we tried to figure out what impact METTL13 had on HIF-1 α . Results showed that silencing METTL13 resulted in significantly increase in HIF-1 α protein levels in Caki-1 cells, whereas in OS-RC-2 cells, overexpression of METTL13 led to an opposite effect (Fig.6a). However, HIF-1 α mRNA expressions were not influenced by METTL13 expression alterations (Fig. 6b), which suggested METTL13 might regulate HIF-1 α in a post-transcriptional manner. It's been widely acknowledged that PI3K/AKT/mTOR signaling pathway importantly participates in HIF-1 α protein translation [17]. Then, via our validation by respectively silencing and overexpressing METTL13, we detected that METTL13 negatively regulated the phosphorylation levels of PI3K, AKT and mTOR without obvious impact on their total expressions (Fig. 6c, d). Taken together, we elucidated that METTL13 could inactivate the PI3K/AKT/mTOR/HIF-1 α pathway in ccRCC cells.

METTL13 binds to c-Myc and inhibits c-Myc expression

Meanwhile, METTL13 has been confirmed to own properties of protein binding and protein modification [11,12]. Out of great interest, we surveyed IntAct [19], a protein-protein interaction database. The result displayed a number of protein interactors of METTL13, which was visualized by software Cytoscape [20] and was shown in Fig. 7a. According to this result, we selected c-Myc, the most classic member of Myc family, as a target interactor of METTL13 to conduct further validations because of its critical role in tumorigenesis and metabolism. By performing co-immunoprecipitation assay, we were convinced that METTL13 could physically bind to c-Myc (Fig. 7b), following which we detected distinct repression that METTL13 had on c-Myc expression (Fig. 7c). Similarly, after alterations of METTL13 expressions, no significant impact on c-Myc mRNA expression levels was observed (Fig. 7d), indicating another post-

transcriptional regulation modification. In conclusion, METTL13 negatively and post-transcriptionally regulates c-Myc expressions in ccRCC, while numbers of other potential interactors of METTL13 are worth further validations and explorations.

METTL13 inhibits tumor growth and metastasis in vivo

To further investigate the biological functions of METTL13 in vivo, OS-RC-2 cells stably overexpressing METTL13 were subcutaneously injected into BALB/c nude mice while OS-RC-2 cells transfected with empty vector was processed in the same way as negative control. After 4 weeks, tumors in the METTL13 overexpression group showed significantly larger sizes and higher weights by comparison to tumors in the negative control group (Fig. 8a-c). After extracting proteins from the tumors and performing western blot assay, we detected significantly lower protein expressions of HIF-1 α and c-Myc in METTL13 group (Fig. 8d), respectively, which was accordant to what we previously observed by experiments in vitro. Next, tail vein injection was performed. METTL13-overexpressed and empty vector-expressed OS-RC-2 cells were respectively injected into BALB/c nude mice, 45 days after which lung colonization was analyzed. Results indicated lungs excised from mice of METTL13 overexpression group were observed with presence of fewer metastatic tumors (Fig. 8e, f), suggesting METTL13's inhibition on metastatic ability of ccRCC cells.

Discussion

Despite the oncogenic role it plays with its enhanced expression in multi-types of tumors reported before, METTL13 is lowly expressed in ccRCC and associated with better prognosis according to public datasets, which aroused our interest in further research. With qRT-PCR and western blot used, we detected significant decrease in mRNA and protein levels of METTL13 in ccRCC tissues compared to normal adjacent tissues and a negative relevance was observed between expressions of METTL13 and pathological grades of ccRCC via immunohistochemistry. Next, four ccRCC cell lines were selected for cell functional experiments, by which we confirmed significant repression that METTL13 had on cells' proliferation, migration and invasion. Similarly, METTL13 inhibited tumor growth as well as metastasis in vivo. Furthermore, alterations of ccRCC cells' metastatic ability by METTL13 might result from regulations of epithelial-mesenchymal transition (EMT). Based on these, we identify METTL13 as an anti-tumor gene in clear cell renal cell carcinoma that affects a variety of biological behaviors of cancer cells, which is opposite to its role in most of the other malignancies. Therefore, METTL13 not only can be considered as a potential biomarker for ccRCC but also should be paid attention to when it comes to therapies for other tumors because of its inverse role in ccRCC.

To investigate potential molecular mechanisms, we performed bioinformatic analyses with transcriptome data provided by TCGA datasheet, via which we identified a number of biological functions and signaling pathways METTL13 was enriched in along with other differentially expressed genes of similar expression mode. According to the bioinformatic analysis result, METTL13 had great potential to participate in metabolism regulation, including glycolysis, gluconeogenesis, TCA cycle, fructose and mannose

metabolism, which had been proved to be closely associated with occurrence and development of ccRCC [21–24]. What appealed to us was that HIF-1 signaling pathway was involved and it's also been reported to play an important role in cancer metabolism [25, 26]. HIF-1 α , an essential transcription factor that the HIF-1 signaling pathway is constructed around, has brought about scholars' interest with its valuable and crucial roles in various diseases, including malignancies [27–30]. Previous studies have stated abnormally high expression of HIF-1 α in ccRCC tissues with its multiple tumorigenic functions, regulating and regulated by various genes, under normoxia or hypoxia [31–34]. Selected as a potential downstream target gene, HIF-1 α was observed to be negatively and post-transcriptionally modulated by METTL13 in our experiments, which partially validates our bioinformatic analyses. With sufficient oxygen supplied, HIF-1 α protein can be ubiquitinated and subsequently degraded [36], while more types of posttranslational regulation have been discovered, which can affect not only stability but also transcriptional activity of HIF-1 α [37]. Additionally, the PI3K/AKT/mTOR signaling pathway that has promotion on HIF-1 α protein translation was observed to be inhibited by METTL13 by us. However, it still requires further efforts to determine whether METTL13 regulates HIF-1 α expression from other aspects or not and how it specifically participates in the HIF-1 signaling pathway. Besides, the roles that it plays in other biological behaviors and processes of ccRCC, especially metabolisms, are left to discover. Besides, extremely high relevance between METTL13 and many other genes was detected by WGCNA and further analyses revealed participation of these genes in several extra biological functions or pathways, referring to which we suppose METTL13 is able to regulate cell adhesion in ccRCC by possibly connecting to other molecules. Thus, METTL13's inhibition on ccRCC metastasis can result from more than EMT regulation, which is worth following research.

Meanwhile, as a methyltransferase protein, METTL13 increases translational activity to accelerate tumorigenesis in lung cancer and pancreatic ductal adenocarcinoma by di-methylating eEF1A [12], which reminded us of its protein-binding function. The database IntAct, which aims at collecting data of protein interaction, provided us with more than 20 genes potentially combining with METTL13. Among these interactors, TRAF2 influences mitochondrial apoptosis of ccRCC [38]; KLK6's expressions are negatively associated with renal carcinoma grades [39]; higher HLA-E mRNA level predicts better prognosis of ccRCC patients [40]; FAS can be regarded as a biomarker for predicting survival of renal cancer patients who have received nephrectomy [41]. The result also involved Myc, a family of transcriptional factors encoded by the proto-oncogene family, which extensively functions in cancer formation and development [42]. c-Myc is the most classic and important member of the Myc family as an oncogenic transcriptional factor, which has been reported in succession to control various biological behaviors of ccRCC cells with abundant mechanisms [43–45]. Meanwhile, c-Myc's significant role in metabolism regulation is increasingly fascinating worldwide interest [45, 46]. Therefore, we chose c-Myc as a target interactor for accuracy validation of the information from the database and for extension of our research. Via a series of experiments, we were convinced of the interaction between METTL13 and c-Myc but also METTL13's restraining effect on c-Myc expression. However, it remains a subsequent task to figure out whether METTL13's inhibition on c-Myc expression is due to the physical connection between them or not and the deeper mechanisms hidden behind as well. On the other hand, the potential combinations between

METTL13 and the other interactors are expected to be further validated, which will provide us with more comprehension of unknown regulating mechanisms in ccRCC.

Generally speaking, our research not only states METTL13's role in repressing progression of clear cell renal cell carcinoma but also provides a variety of its potential molecular mechanisms, many of which are tremendously worth validating and exploring in the future.

Conclusion

Collectively, our research demonstrated METTL13's significant role in clear cell renal cell carcinoma, as featured by inhibiting growth and metastasis of cancer cells. Moreover, we summarized a variety of biological processes and molecular mechanisms that METTL13 might be involved in, according to which we validated its inhibition on PI3K/AKT/mTOR/HIF-1 α pathway. Moreover, we confirmed that METTL13 could bind to c-Myc and restrain its expression. On the basis of these, we identify METTL13 as an anti-tumor factor in ccRCC for the first time, which has a great potential to act as a new diagnostic biomarker and therapeutic target in the future.

Abbreviations

ccRCC: clear cell renal cell carcinoma; METTL13: methyltransferase like 13; HIF-1 α : hypoxia-inducible factor-1 α ; c-Myc: myelocytomatosis virus oncogene cellular homolog; 5-EdU: Ethynyl-2'-deoxyuridine; qRT-PCR: Quantitative real-time PCR; IHC: immunohistochemistry; WGCNA: weighted gene co-expression network analysis; Co-IP: co-immunoprecipitation; PI3K: phosphatidylinositol-4,5-bisphosphate-3-kinase; mTOR: mammalian target of rapamycin; AKT: protein kinase B;

Declarations

Ethics approval and consent to participate

The use of human tissue samples involved in the present study was approved by The Ethics Committee of the First Hospital of China Medical University (Shenyang, China) and all the participants had written informed consent before enrollment. The Animal Ethics and Welfare Committee of China Medical University had provided approval before all the animal experiments performed.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author's contributions

ZL designed the experiment, analyzed the data and wrote the manuscript; ZL and CP performed all the experiments; ZL and TS conducted bioinformatic analyses; ZZ and CK provided experimental reagents and materials and supervised the study. All authors have read and approved the final version of the manuscript.

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Figures

Figure 1

Low expression level of METTL13 is associated with poor prognosis of ccRCCs. a, b METTL13 expression levels in ccRCC tissues and normal kidney tissues according to TCGA database (a) and GSE53757 dataset (b). c, d Expression levels of METTL13 in ccRCC tissues of different tumor grades (c) and of different cancer stages (d) in TCGA dataset. e Kaplan–Meier analysis of ccRCC patients' overall survival based on METTL13 expression in TCGA database. f, g Relative mRNA levels of METTL13 in 50 ccRCC tissues and their corresponding adjacent normal kidney tissues detected by qRT-PCR assay. h METTL13 protein levels in 13 pairs of ccRCC tumors (T) and their corresponding adjacent normal kidney tissues (N) tested by western blotting. i IHC staining of METTL13 in ccRCC tissues of different

pathological grades. Mann-Whitney U-test was performed for statistical analysis for qRT-PCR and western blotting results. $**P < 0.01$.

Figure 2

Knockdown of METTL13 promotes ccRCC cell proliferation, viability, migration and invasion. **a** METTL13 protein expressions in five ccRCC cell lines and one immortalized ccRCC cell line by western blotting. **b** Knockdown efficiencies of two siRNAs targeting METTL13 in two cell lines detected by western blotting. **c-e** Cell proliferation, viability and migration were measured by EdU assay (**c**), CCK-8 assay (**d**) and wound-healing assay (**e**), respectively. **f** Cell migration assay and invasion assay were respectively used to detect cells' migratory ability and invasiveness. **g** Expression levels of EMT-related proteins (N-cadherin, E-cadherin) from cells were detected via western blotting. Each experiment was conducted independently at least three times. Student's t-test (two-tailed) was used for statistical analysis, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Bar graphs: mean $\pm$ S.D., $n = 3$.

Figure 3

Overexpression of METTL13 promotes proliferation, viability, migration and invasion of ccRCC cells in vitro. **a** METTL13 protein levels in ccRCC cells (OS-RC-2 and ACHN cells) respectively transfected with METTL13 expressing plasmid (METTL13) and control vector (vector) confirmed by western blotting. **b** Cell proliferation was detected by EdU assay. **c** CCK-8 assay was performed to measure cell viability. **d** Cell migration rate was observed via wound-healing assay. **e** Migratory ability and invasiveness of ccRCC cells were detected by migration assay and invasion assay, respectively. **f** EMT-related protein levels were detected by performing western blotting. Each experiment was conducted independently triplicate. Student's t-test (two-tailed) was used for statistical analysis, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Bar graphs: mean $\pm$ S.D., $n = 3$.

Figure 4

Functional annotation of METTL13 in ccRCC. a DEGs were divided into modules by weighted gene co-expression network analysis (WGCNA). b Correlation analysis of genes and identification of METTL13 location in modules. c GO and KEGG functional enrichment analyses of the turquoise module. GO, gene ontology; KEGG, Kyoto Encyclopaedia of Genes and Genomes; BP: biology process; CC: cellular component; MF: molecular function.

Figure 5

Functional annotation of genes directly linked to METTL13 in WGCNA. a Network of nodes in METTL13's secondary connection. b GO and KEGG functional enrichment analyses of genes directly linked to METTL13 in WGCNA. GO, gene ontology; KEGG, Kyoto Encyclopaedia of Genes and Genomes; BP: biology process; CC: cellular component; MF: molecular function.

Figure 6

METTL13 negatively regulates PI3K/AKT/mTOR/HIF-1α signaling pathway. a, b Protein levels (a) and mRNA levels (b) of HIF-1α were respectively detected by western blot and qRT-PCR after silencing or overexpressing METTL13. b HIF-1α mRNA levels were measured by qRT-PCR. c, d Protein levels of p-PI3K, PI3K, p-AKT, AKT, p-mTOR and mTOR were determined by western blot after knocking down METTL13 (c) or overexpressing METTL13 (d). Each experiment was carried out at least for three times independently. Student's t-test (two-tailed) was used for statistical analysis, *P < 0.05, **P < 0.01, ***P < 0.001. Bar graphs: mean ± S.D., n = 3.

Figure 7

METTL13 binds with c-Myc and inhibits c-Myc expression. **a** Genes interacting with METTL13 were provided by database IntAct (<https://www.ebi.ac.uk/intact/>) and visualized by software Cytoscape. Each string represents an interaction accession. **b** The interaction between METTL13 and c-Myc was confirmed by co-immunoprecipitation (Co-IP). **c, d** Protein levels (**c**) and mRNA levels (**d**) of c-Myc were respectively detected by western blot and qRT-PCR after silencing or overexpressing METTL13. Each experiment was performed independently triplicate. Student's t-test (two-tailed) was used for statistical analysis, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Bar graphs: mean $\pm$ S.D., $n = 3$.

Figure 8

METTL13 inhibits ccRCC cells' growth and metastasis in vivo. a Image of BALB/c nude mice executed 30 days after injection of cells transfected with empty vector or with METTL13 overexpression plasmid. b Image of excised tumors from the mice. c The weights of the isolated tumors. d western blot was used to measure protein expressions of METTL13, HIF-1α and c-Myc in the nude mice tumors. e Cells overexpressing empty vector or METTL13 were injected into nude mice through tail vein. Representative

images of lungs with metastatic tumors excised from the mice and H&E staining of the lung tissues were shown. f The numbers of metastatic tumors in lungs per field were measured under a magnification of 100 x. Each experiment was conducted at least three times. Student's t-test (two-tailed) was used for statistical analysis, *P < 0.05. Bar graphs: mean $\pm$ S.D., n = 3.

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