

Loss of Setd2 Induces the Upregulation of Genes Related to Akt/Mtor Signaling Pathway

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Abstract: Patients with polycystic kidney disease (PKD) have a high risk of developing renal cell carcinoma (RCC). SET domain-containing 2 (SETD2) is the only molecule known to regulate lysine trimethylation (H3K3me3) of histone H3 in human tissue, and SETD2 is identified as a tumor suppressor in ccRCC. Although there are some studies revealing some mechanism about PKD developing ccRCC, the underlying mechanism remains largely reported. We collected the Kidney samples from SETD2 conditional knockout mice described before (Rao, 2021) and detected the expression levels of some important genes related to Akt/mTOR signaling pathway. Besides, we found that SETD2 is closely related to Akt/mTOR signaling pathway and can be regulated by Western blot analysis, qRT-PCR and immunofluorescence. For clinical translation, the cross-talks between SETD2 and Akt/mTOR signaling may provide a potential strategy to prevent tumorigenesis in patients with ccRCC therapy.

1 INTRODUCTION

Renal cell carcinoma (RCC) is the most common type of malignant tumor of kidney, which is originated from the renal tubular and happened for 90% of cancer in kidney (Rao, 2020). However, little is known about the relationship between PI3K/AKT pathway and the tumorigenesis.

Clear cell renal carcinoma (ccRCC) is the most common tumor among all kinds of renal tumors, accounting up to 75% of RCCs (Miller, 2010; Moch, 2016). On the level of chromosome, loss of the short arm of chromosome 3, which contains VHL, PBRM-1, SETD2, BAP-1, KDM5C, and MTOR, is detected in approximately 95% of sporadic ccRCC (D'Avella, 2020). Furthermore, about 70% of ccRCC tumors have VHL mutation and 15% of cases show a suppression of VHL by hypermethylation (Sato, 2013). With the help of whole exome sequencing, there are another three major gene alterations on chromosome 3p found in ccRCC, PBRM1 (~40%), SETD2 (~12%), and BAP1 (~10%) (Petitprez, 2021). On the level of epigenetics, Setd2 as a histone methyltransferase catalyzing tri-methylation of H3K36 plays a key role in ccRCC tumorigenesis, while KDM5C, KMT2D and KDM6A were that encoded histone modifying enzymes and that identified in ccRCC (de Cubas, 2018). Recently, a seminal study shed new light on the SETD2 function that delineated Setd2 not only as a histone methyltransferase, but also as a competitive DNA binding protein, shows a multilevel regulation during the transition from PKD to ccRCC (Rao, 2021; Liu, 2019).

The SETD2 protein is the only molecule known to

regulate lysine trimethylation (H3K3me3) of histone H3 in human tissue. And it participates in transcriptional regulation, DNA damage repair, chromosome segregation and alternative splicing and so on (Li, 2013; Pfister, 2015; Zhang, 2014; Kanu, 2015). According to the research, SETD2 gene is widely mutated in a variety of human tumors, and the increase of SETD2 has been an identity of cancer types, including intestinal cancer (Yuan, 2017), gastrointestinal tumors (Yuan, 2017; Huang, 2016), lung cancer (Walter, 2017; Lee, 2019), osteosarcoma (Sakthikumar, 2018). And mutations or loss of SETD2 can cause tumors. In the ccRCC cells, the researchers found that the mutations of SETD2, which has not only contributed to Epigenetic dysfunction, but also has an effect on the regulation of cell metabolism. In the epigenetic dysfunction, the loss of SETD2 can make the levels of protein of H3K3me3 decrease (Li, 2015), and in the regulation of cell metabolism, the loss of SETD2 causes that the metabolic processes of creatine, glycosaminoglycan, and carbohydrates are down-regulated, and oxidative phosphorylation and lipid production are enhanced through a metabolic network guided by PGClα (Liu, 2019; Li, 2015). And SETD2 has shown that it regulates β-catenin activity on transcriptional and post-transcriptional levels, and regulates Wnt/β-catenin signaling (Rao, 2021). However, some signaling pathways which are regulated by SETD2 have not been revealed.

Here, we used a mouse model of overexpression of c-MYC, deficiency of KSP and Setd2 to identify the potential role of Setd2 gene in regulation of PI3K/AKT pathway. We reported that loss of Setd2 induced upregulation of PI3K/AKT pathway and renal tubule

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morphological alteration.

2 MATERIALS AND METHODS

2.1 Mouse Strains

Setd2^{fl/fl} mice were generated by Shanghai Biomodel Organism Co., which uses conventional homologous recombination in embryonic stem cells (Yuan, 2017). The *Ksp^{Cre}* mice (B6. CgTg (Cdh16-cre) 91Igr/J) and *MYC^{R26StopFL/+}* (C57BL/6N-Gt (ROSA) 26Sor^{tm13(CAG-MYC,-CD)Rsky}) were purchased from The Jackson Laboratory. Setd2^{fl/fl} mice were mated with *Ksp^{Cre}* mice to generate *Ksp^{Cre}*; Setd2^{fl/fl} (Setd2^{KO}) mice in C57BL/6 background. *MYC^{R26StopFL/+}* mice were mated with *Ksp^{Cre}* mice to generate *Ksp^{Cre}*; *MYC^{R26StopFL/+}* (*MYC^{-OE}*) mice in C57BL/6 background. Setd2^{KO} mice were mated with *MYC^{-OE}* mice to generate *Ksp^{Cre}*; *MYC^{R26StopFL/+}* Setd2^{fl/fl} (*MYC^{-OE}*; Setd2^{KO}) mice housing under same condition.

2.2 Quantitative qRT-PCR

cDNA was synthesized by reverse transcription using TaKaRa (Primer Script RT reagent kit) and subjected to quantitative RT-PCR with Kit, *Col4a4*, *Sgk2*, *Col27a* and GAPDH primers in the presence of the SYBR Green Real-time PCR Master Mix (Thermo). Relative abundance of mRNA was calculated by normalization to Akt or GAPDH mRNA.

2.3 Western Blot Analysis and Antibodies

From each sample, 30µl of total protein was separated by 8%-12% SDS-PAGE gels and transferred onto nitrocellulose membranes. Membranes was blocked by 5% skim milk for one hour at room temperature, and then incubated with primary antibodies at 4°C overnight, washed in TBST (TBS containing 1% Tween20), incubated with a horseradish peroxidase (HRP)-

conjugated secondary antibody for 1.5 hours at room temperature, and developed by ECL reagent (Thermo Fisher Scientific). The immunoblots were quantified by Bio-Rad Quantity One version 4.1 software. Primary antibodies against Phospho-mTOR (D9C2, #5536), mTOR (7C10, #2983) were purchased from Cell Signaling Technology Inc. Antibody GADPH (D110016) and Anti-Rabbit IgG mouse monoclonal antibody was purchased from BBI Life Sciences Corporation.

2.4 Hematoxylin-Eosin Staining (HE Staining)

First of all, we remove the paraffin from the section by using the xylene I and II, anhydrous ethanol I and II, 95% ethanol 85% ethanol, 75% ethanol and water. And then the sections without wax were soaked in the hematoxylin about 7-8 minutes to stain the nucleus, washed water about 10 minutes. Having stained the nucleus, the sections were soaked in the eosin about 10 seconds to stain the cytoplasm. Finally, the sections were soaked in 75%,80%,95% ethanol, anhydrous ethanolIandII and xylene IandIIin turn to remove water, and then block sections by neutral gum.

2.5 Immunofluorescence technique

First of all, we remove the paraffin from the section by using the xylene I and II, anhydrous ethanolIandII95%, 90%, 80% and 70% ethanol and water in turn. And then we put the sections into solution mixed by citric acid A and B and water at high temperature, and antigen repair at low temperature about 15 minutes. Having cooled for 1 hours and washed by PBS, we break membranes by 0.5% Triton, clock samples by 10% BSA for 1 hour and then the sections were soaked with primary antibodies at 4°C overnight. The next day, the sections were incubated by secondary fluorescence antibody without light about 1 hour after the temperature of sections return to room temperature and washing them by PBS for three times. And then, we use DAPI to seal sections.

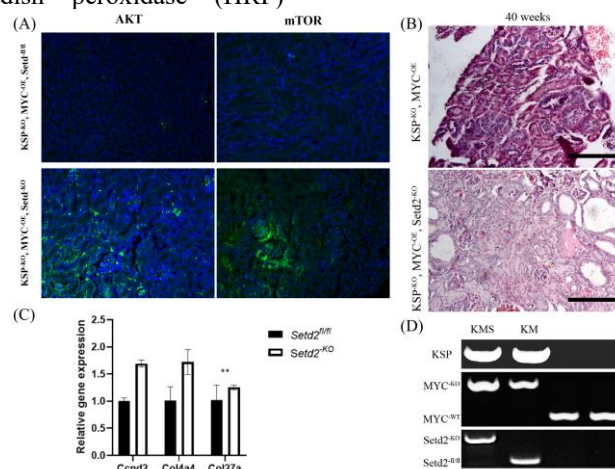


Figure 1: Loss of Setd2 induces upregulation of genes involved in Akt/mTOR signaling pathway and aberrant renal tubule morphology in mice. (a) Representative immunofluorescence images of kidney sections from 40-week-old Setd2^{fl/fl} and Setd2^{KO} showing protein expression upon SETD2 deficiency. Scale bars, 80 µm. (b) Representative hematoxylin and eosin images of kidney sections from Setd2^{fl/fl} and Setd2^{KO} mice. Scale bars, 80 µm. (c) RT-qPCR analysis of Akt/mTOR target genes. (d) PCR image shows the genotyping of mice of interest.

3 RESULTS

3.1 KSP-KO, MYC-OE and Sted2-KO mice genotyping and morphology identification

Sted2 as a major mutation gene in ccRCC, has been shown to participate in several biological process including histone methylation, transcriptional regulation, chromosome segregation, DNA damage repair, and alternative splicing, but single Setd2 knockout is not sufficient to induce kidney tumor¹. Here, we generated, KSP-cre, MYC and Setd2, three genes knockout mice to explore pathological changes and tumorigenesis in kidney. Genotyping of mice is performed by PCR, which shows that Setd2 gene has been successfully knockout relative to the KM mice at the age of 40 weeks.

The results of hematoxylin and eosin images show that KSP^{cre}, MYC^{OE}, and Setd2^{fllox/fllox} mice exhibited normal renal tubule structure and no obvious aberrant morphology at the age of 40 weeks, while the mice of Setd2^{-KO} show a different pattern, which has multiple cysts and aberrant renal tubules and several abnormal renal tubule cells with clear cytoplasm and stained lightly in comparison with their control littermates. Moreover, deficiency of Setd2 shows glomeruli structural abnormality and fibrosis compared to control littermates. These results demonstrate that KSP-cre, MYC, and Setd2^{-KO} results in aberrant renal tubule and glomeruli morphology and renal tubule cell alteration and multiple cysts formation with tissue fibrosis. qRT-PCR analysis of genes related to PI3K/AKT signaling pathway (Cnd2, Col4a4, Col27a) in *Setd2^{fl/fl}* and *Setd2^{-KO}*, we can find that the deficiency of *Setd2* can upregulate PI3K/Akt signaling pathway.

3.2 Deficiency of Sted2 facilitates mTOR and AKT expression

AKT/mTOR pathway plays a pivotal role in tumorigenesis and the expression changes among this pathway are evidence to generate tumor. In this study, IF image shows that it is obvious that there are overexpression in KSM mice renal tissue as opposite to the control group which shows no significant green fluorescence in the same photographic condition.

4 DISCUSSION

Setd2 is inactivated in a variety of human tumors and is involved in tumor genesis and clinical progression. Epigenetic studies based on SETD2 are currently receiving attention from researchers. Besides, our study is based on the recent clinical reports indicating that mutations of the SETD2 gene happen in up to 12% of early-stage ccRCC, and in primary ccRCC the numbers of H3K36me3- positive nuclei are reduced an average of approximately 20% (Hacker, 2016). According to the research, SETD2 is widely mutated in a variety of human tumors, and the increasement of SETD2 has been an identity of cancer types, for example its activation can promote intestinal tumorigenesis of colorectal cancer

(Yuan, 2017) and the fusion-oncogene-driven lung adenocarcinomas (LADs) had frequent SETD2 mutations (Huang, 2016).

The precious study showed that the mice with ectopic expression of c-MYC in renal epithelial cells, under the control of Ksp1.3/Cre only appear PKD, but ccRCC is generated when the Setd2 is knockout (Rao, 2021). In this article, we showed that the mice with Setd2 knockout and the ectopic expression of c-MYC under the control of KSP generate the ccRCC.

In addition, the SETD2 can interact with a variety of proteins to influence transcription. And there are some studies revealing that SETD2 can regulate cellular signaling and responses through modification of nonhistone substrates, such as STAT1 (Chen, 2017) and α -tubulin in some biological processes (Park, 2016). In this study, we found that SETD2 can regulate the Akt/mTOR signaling pathway, which provides a potential strategy about ccRCC therapy.

5 CONCLUSION

In conclusion, we compared the mouse model of PKD driven by c-MYC with that of ccRCC with the deficiency of *Setd2* and c-MYC, which will have effect on preclinical research. Besides, we found that the deficiency of *Setd2* can upregulate mTOR signaling pathway. For clinical translation, the cross-talks between SETD2 and mTOR signaling may provide a potential strategy to prevent tumorigenesis in patients with ccRCC therapy.

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