

Original Article

KDM6B induces epithelial-mesenchymal transition and enhances clear cell renal cell carcinoma metastasis through the activation of SLUG

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Abstract: Clear cell renal cell carcinoma (ccRCC) is one of the most common kidney cancers; epithelial-mesenchymal transition (EMT) is associated with carcinoma invasion and metastasis. There have been several studies about the molecular regulation of EMT, but the relationship between histone demethylase and EMT is little known. Here, we reported KDM6B has high expression level in ccRCC and is positively correlated with poor ccRCC prognosis. KDM6B, also known as JMJD3, is a histone demethylase, can remove repressive histone H3K27me3 marks from chromatin, thereby activating gene expression. We found that the knockdown of KDM6B could inhibit ccRCC tumorigenesis in vitro; furthermore, KDM6B could induce EMT in ccRCC cells by activating the expression of master transcription factor SLUG. ChIP assays revealed that KDM6B stimulated SLUG expression by demethylate histone H3K27me3. The knockdown of KDM6B strongly inhibited ccRCC cell invasion in vitro, while the overexpression of KDM6B shown the opposite trend. Meanwhile, our analysis of the ccRCC tissue found that KDM6B expression was significantly corresponded with lymph node metastasis. Together, our data provide a novel epigenetic mechanism regulating tumor cell invasion and EMT, and provide a biomolecule for ccRCC diagnosis and prognosis.

Keywords: ccRCC, KDM6B, EMT, SLUG

Introduction

Every year, there are 120,000 persons dying from kidney cancer, and this number is still in increase, which makes kidney cancer become the thirdly most common urological malignancy tumor in world [1]. Renal-cell carcinoma (RCC) has high incidence of all adult malignancies tumor [2]. Clear cell renal cell carcinoma (ccRCC) comprising 83% of RCC [3]. Moreover, ccRCC is also a therapy-resistant carcinomas, so it ordinarily responding very poorly to chemotherapy or radiotherapy. All these facts point that searching early diagnostic markers for ccRCC is very important.

Epigenetic modification plays an important role in cancer development have been confirmed in many studies. Histone methylation is a kind of important epigenetic modification, which

enables the gene promoter to be accessible or inaccessible to transcription factors, thereby regulating target gene expression [4, 5]. H3K27me3 is a gene transcription silence marker, it cooperates with polycomb proteins silence gene promoters [6-9]. H3K27 methylation and demethylation is a dynamic process that can be catalyzed by EZH2, KDM6A and KDM6B, also known as JMJD [10]. KDM6B has been reported to activate gene expression by removing H3K27me3 marks on gene promoters dependent on Fe²⁺ and α -ketoglutarate [10-16]. It executes key functions in differentiation, development and oncogenic stress-induced cell senescence in cancer [10, 17, 18]. Epithelial-mesenchymal transition (EMT) has been identified as potentially playing a significant role in RCC like other cancers [19-21]. EMT is a process determines several critical stages involved in cancer progression and invasion,

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embryonic development through multiple mechanisms [22]. Various molecules and signaling pathways can regulate EMT process have been found [23]. But how epigenetic mechanisms cooperate with signaling networks to influence the expression of EMT-related transcription factors, such as SLUG, has not been well investigated.

In this study, we provided that the expression of KDM6B is significant high in ccRCC tissue compared with non-tumorous tissues, and the increased KDM6B expression is positively correlated with cancer stage, tumor size and lymph node metastasis. Furthermore, we also found the high level of KDM6B expression has a positively correlates with poor prognosis. We unraveled a novel epigenetic mechanism about the role of KDM6B EMT and cancer cell invasion in ccRCC. In addition, the present work provided a new clinical biomarker to predict the prognostic and developing a novel therapeutic target.

Materials and methods

Cell culture

The human ccRCC cell lines Caki-2 and ACHN were used. Cells were cultured in RPMI-1640 medium supplemented with 2 mmol/l glutamine, amphotericin B (2.5 µg/ml) and 10% fetal bovine serum (FBS), 100 U/ml penicillin, 0.1 mg/ml streptomycin. Cells were incubated at 37°C at 5% CO₂. The non-cancerous Human Embryonic Kidney cell line (HEK-293) was used as control.

Antibodies and reagents

ChIP grade anti-trimethyl-histone H3 (Lys-27) monoclonal antibodies were purchased from Abcam (ab6002); rabbit KDM6B/JMJD3 antibody was from Abcam (ab169197); β-actin was purchased from Abgent (AM1021B); EMT Antibody Kit was purchased from Cell Signaling Technology (9782S).

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from cells using TRI Reagent (Sigma- Aldrich) according to the manufacturer's protocol. First-strand cDNA was synthesized from 2 µg of total RNA using TransGen first-strand cDNA synthesis system. Subse-

quently, the qRT-PCR was used to analyze resulting cDNA. The qRT-PCR conditions were as follows: 5 min at 95°C, denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 60 s, carry out 35 cycles. As follows are the primers of KDM6B: 5'-CCTC-GAAATCCCATCACAGT-3' and 5'-GTGCCTGTCAG-ATCCCAGTT-3'; β-actin: 5'-GGCATCCTGGGCTAC-ACTGA-3' and 5'-GTGGTCGTTGAGGGCAATG-3'; E-cadherin: 5'-GAAATCACATCCTACTGCCC-3' and 5'-GTAGCAACTGGAGAACCATTGTC-3'; γ-catenin: 5'-GGACAAGAACCCAGACTACC-3' and 5'-GTGGCATCCATGTCATCTCC-3'; Fibronectin: 5'-GATAAATCAACAGTGGGAGCGG-3' and 5'-GTC-TCTTCAGCTTCAGGTTTACTC-3'; N-cadherin: 5'-CTCCATGTGCCG GATAGC-3' and 5'-CGATTCA-CCAGAAGCCTCTAC-3'; Vimentin: 5'-ATTGAGATT-GCCACCTACAG-3' and 5'-ATCCAGATTAGTTTCC-CTCAG-3'; SNAI1: 5'-TCTAATCCAGAGTTTACCTT-CC-3' and 5'-GAAGAGACTGAAGTAGAGGAG-3'. All qRT-PCR results were performed at least three times.

Western blot analysis

The total extract protein was resolved on SDS-PAGE gel, and then was transferred onto the PVDF membrane. The blots were blocked with 5% dried skimmed milk at 37°C for 1 hr, followed by incubation with primary antibody at 4°C overnight, β-actin antibodies was used as control. Subsequently, the blots were washed thrice for 10 minutes each with 1x TBST and were then incubated with secondary antibody for 1 hr at 37°C. The blots were then washed three times for 10 min each with 1x TBST. The blots were developed on X-ray films (Amersham or Kodak) after incubation with ECL reagent (Immunocruz, Santa Cruz biotechnology).

ChIP assay

Cells were grown to 95% confluence for each location analysis reaction. Next washed thrice with PBS and chemically cross-linked with 1% formaldehyde for 30 min at 37°C. Cells were lysed and sonicated to solubilize and shear crosslinked DNA. Sonication was performed for 10-15 cycles (1 s-ON, 1 s-OFF). The chromatin extract was taken to incubate at 4°C overnight with 3 µg of antibody. Qiagen PCR purification kit was used to immunoprecipitated DNA. qChIP was analyzed by quantitative PCR using

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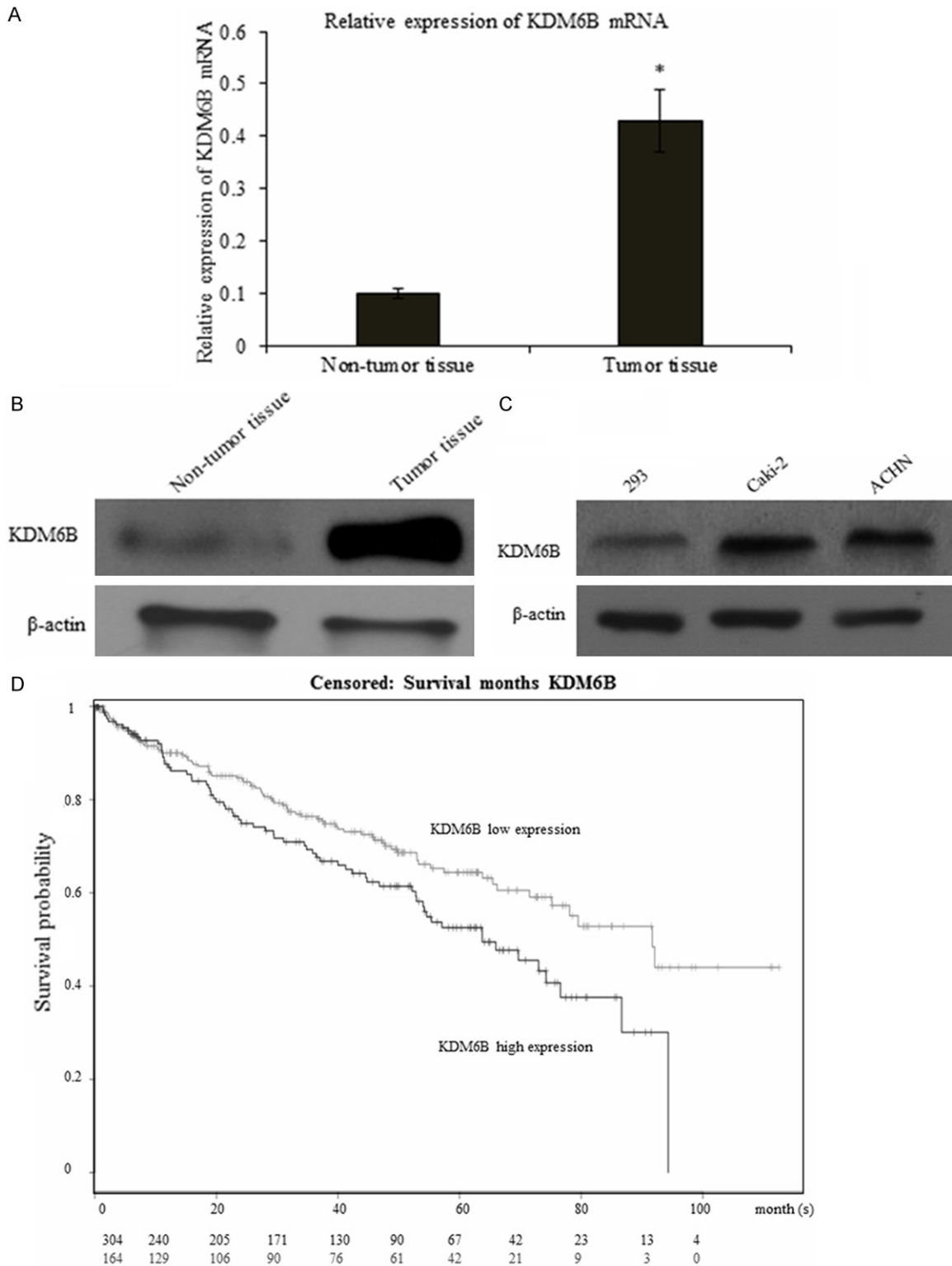


Figure 1. KDM6B is frequently up-regulated in ccRCC and positively correlated with poor ccRCC prognosis. A. qRT-PCR was used to detect KDM6B mRNA in non-tumor tissue and tumor tissue from clear cell renal cell carcinoma (ccRCC) patients. B. Western blot was used to measure the protein expression of KDM6B in non-tumor tissue and tumor tissue from ccRCC. C. KDM6B protein levels between Human kidney epithelial cell line 293 and ccRCC cell lines Caki-2 or ACHN were detected by Western blot. D. The online tool was used to plot the Kaplan-Meier survival curves.

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Table 1. Clinicopathologic variables in 145 ccRCC patients

Variables	No. (n = 145)	KDM6B protein expression		P value
		Low (n = 70)	High (n = 75)	
Gender				
Male	75	35	40	0.688
Female	70	35	35	
Age				
< 60	66	37	29	0.085
≥ 60	79	33	46	
Tumor diameter				
Small (≤ 3 cm)	72	43	29	0.006
Large (≥ 3 cm)	73	27	46	
Pathological grade				
I-II	61	22	39	0.012
III-IV	84	48	36	
pT status				
pT1	76	47	29	0.001
pT2-4	69	23	46	
pN status				
pN0	60	39	21	0.001
pN1-2	85	31	54	
Lymph node metastasis				
Yes	71	27	44	0.016
No	74	43	31	
Smoker status				
Never smoke	43	17	26	0.171
Smoker	102	53	49	
Differentiation				
Well/moderate	77	37	40	0.954
Poor	68	33	35	

1640 medium for 24 hr, and then applied cells ($1-2 \times 10^5$) suspended on the upper chamber in 0.5 ml of serum-free medium. In the lower chamber, RPMI 1640 containing 10% FBS was used as chemoattractant. The chambers were incubated for 24 hr in the CO₂ incubator. Matrigel was removed from the membrane, using cotton swab scraped non-migrated cells off the top of the membrane, and cells on the lower surface of the filter were fixed with 4% formaldehyde and stained with 0.5% crystal violet. Cells were counted under a microscope in five different fields in duplicate wells, in at least three independent experiments.

Immunohistochemistry (IHC)

145 Samples were selected from ccRCC patients with complete clinicopathologic information. All patients were informed to understand their tissue would be used for re-

specific primers as follows. The final target DNA sequence for common ChIP assays was amplified and resolved on 1% agarose DNA gels.

Monolayer colony formation assay

The proliferation of control cells and Caki-2/shKDM6B was used CCK8 (Beyotime, China) according to the manufacturer's instructions. For the colony formation assay, 6-cm cell culture dishes planted about 1,000 cells per dish and incubated for 10 days in 37°C. PBS was used to wash plates then stained with Giemsa.

Matrigel invasion assays

Cell invasion assays was performed by BioCoat Matrigel invasion chambers (BD Biosciences). First, cells were starved with serum-free RPMI

search purposes. The study was approved by the ethics committee of the hospital.

The tissue first incubated at 60°C for 1 hr, and then incubated in xylene by 10 minute twice and was rehydrated in graded alcohol to water. 0.01 M sodium citrate buffer was used for antigen retrieval. The tissue samples were blocked in 10% normal goat serum in PBS and incubated at 4°C overnight in primary antibody solution of anti-KDM6B at a 1:500 dilution. The rest of the staining was done following the manufacturer's protocol. For detection of bound antibody, DAB was used.

Statistical analysis

All data were presented as the mean ± standard deviation (SD) from at least three separate experiments. The χ^2 test was used to analyze

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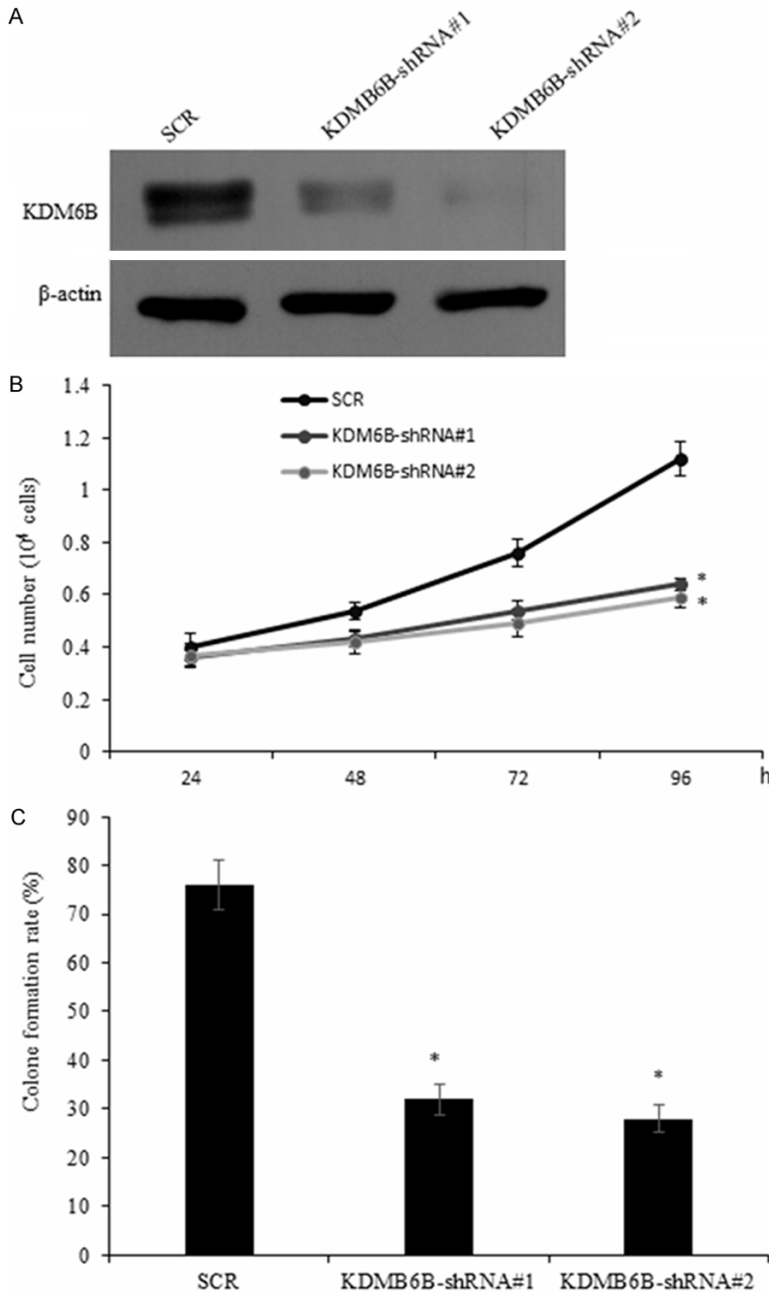


Figure 2. ccRCC tumorigenesis is inhibited by KDM6B knockdown in vitro. A. Western blot analysis verified shRNA-mediated interference of KDM6B expression in Caki-2 cells. B. The CCK-8 assay was used to draw the growth curves of Caki-2 cells, cells were transfected with shKDM6B or SCR. C. Graph representative inhibition of foci formation in monolayer culture due to the interference of KDM6B expression.

the relationship between KDM6B expression and clinicopathological characteristics. A *P value of 0.05 was considered statistically significant. Numerical data were calculated using Microsoft Excel and analyzed using SPSS 17.0.

Results

KDM6B is frequently up-regulated in ccRCC and positively correlated with poor ccRCC prognosis

To investigate the expression of the H3K27 demethylase genes, KDM6B, we collected tumor tissues and adjacent non-tumor tissues from 145 ccRCC patients. The transcription levels of KDM6B were determined by qRT-PCR. We found the mRNA levels of KDM6B were significantly increased in tumor tissues compared to the adjacent non-tumor tissues (**Figure 1A**). To support the changes in mRNA levels of KDM6B, the protein levels were measured by western blotting. Consistent with mRNA levels, the protein levels of KDM6B were also obviously increased in tumor tissues compared to adjacent normal tissues (**Figure 1B**). While we used Human kidney epithelial cell line 293 and ccRCC cell lines Caki-2 or ACHN, the similarly results were found, the expression of KDM6B was higher in the Caki-2 or ACHN cells compared with the normal 293 (**Figure 1C**). In brief, KDM6B is high expressed in ccRCC.

To further explore the relationship between KDM6B and ccRCC, we analyzed KDM6B expression in the cohort of 145 ccRCC patients. IHC staining showed that KDM6B protein was significantly up-regulated in ccRCC compared to normal tissues. Interestingly, KDM6B expression positively correlated with advanced tumor node metastasis (TNM) stage ($P < 0.01$), lymph node metastasis ($P = 0.016$), and tumor size ($P =$

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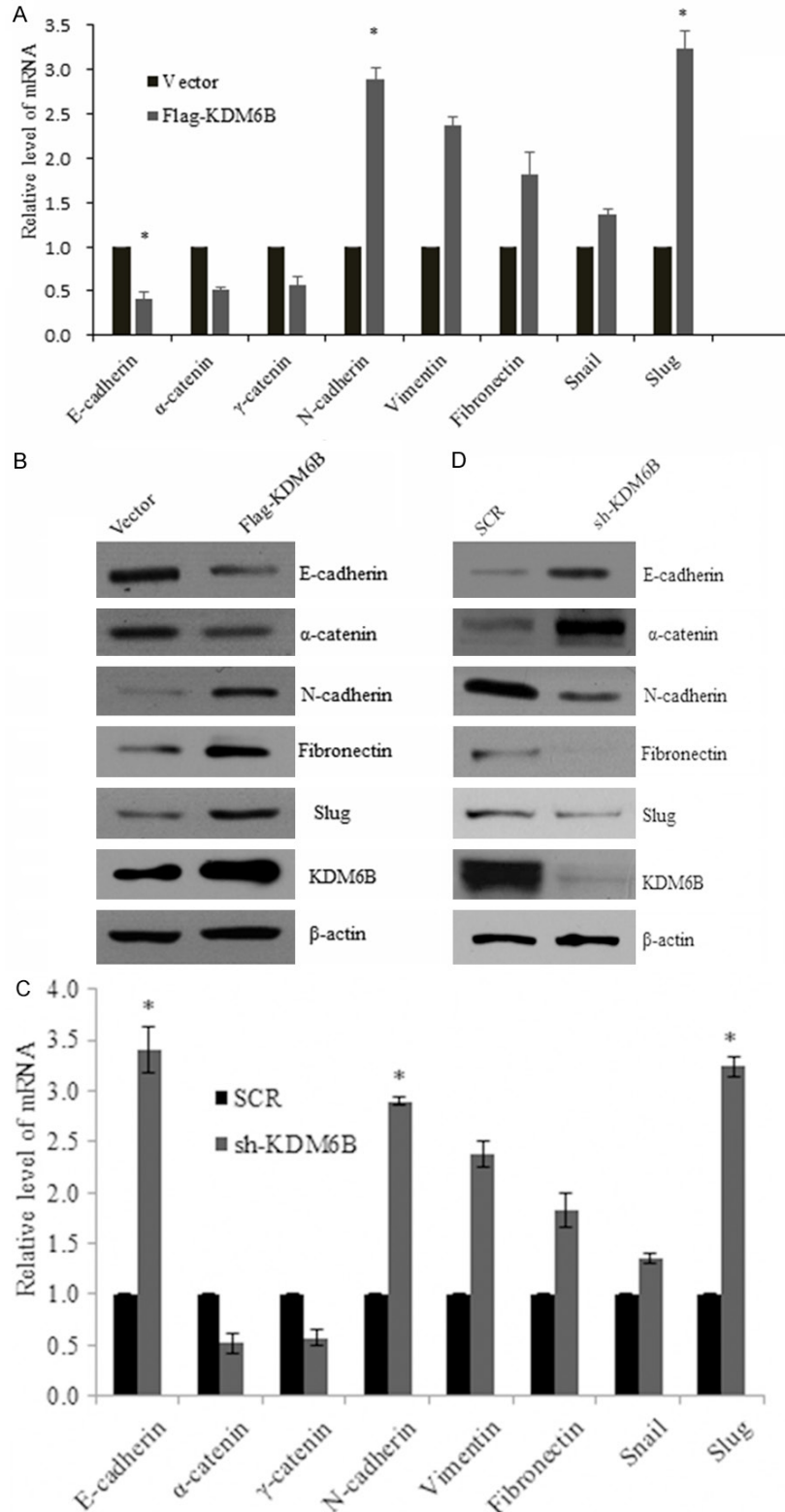


Figure 3. KDM6B promotes EMT by inducing SLUG. A. KDM6B was overexpressed in Caki-2 cells, qRT-PCR was used to measure mRNA levels of epithelial and mesenchymal markers. B. Western blot analysis revealed a significant decreased epithelial markers and increased mesenchymal marker in Caki-2/KDM6B cells. C. qRT-PCR was used to measure mRNA levels of epithelial and mesenchymal

markers change in Caki-2/shKDM6B cells. D. In Caki-2/shKDM6B cells, western blot showed an opposite result compare with Caki-2/KDM6B cells, epithelial markers was increased and mesenchymal marker was decreased.

0.006). However, there were no significant relationships between KDM6B and other factors, such as age, gender, smoking status, and differentiation (**Table 1**).

We next investigated the prognosis of KDM6B overexpression in Renal-cell carcinoma, the database of Cbioportal website was used for analysis. Remarkably, follow-up data showed that the survival rate of patients with high expression of KDM6B was significantly lower than that with low expression of KDM6B (Hazard Ratio = 1.49, $P = 0.01375$, **Figure 1D**).

ccRCC tumorigenesis is inhibited by KDM6B knockdown in vitro

To investigate the mechanism of KDM6B in ccRCC, KDM6B was knocked down in Caki-2 cells, a clear cell renal cell carcinoma cell lines, using two different shRNAs. qRT-PCR revealed that mRNA levels of KDM6B were down-regulated more than 80% in Caki-2 cells when transfected KDM6B shRNA (Caki-2/shKDM6B), compared with Caki-2 cells transfected scramble shRNA (SCR), which was as a control. The

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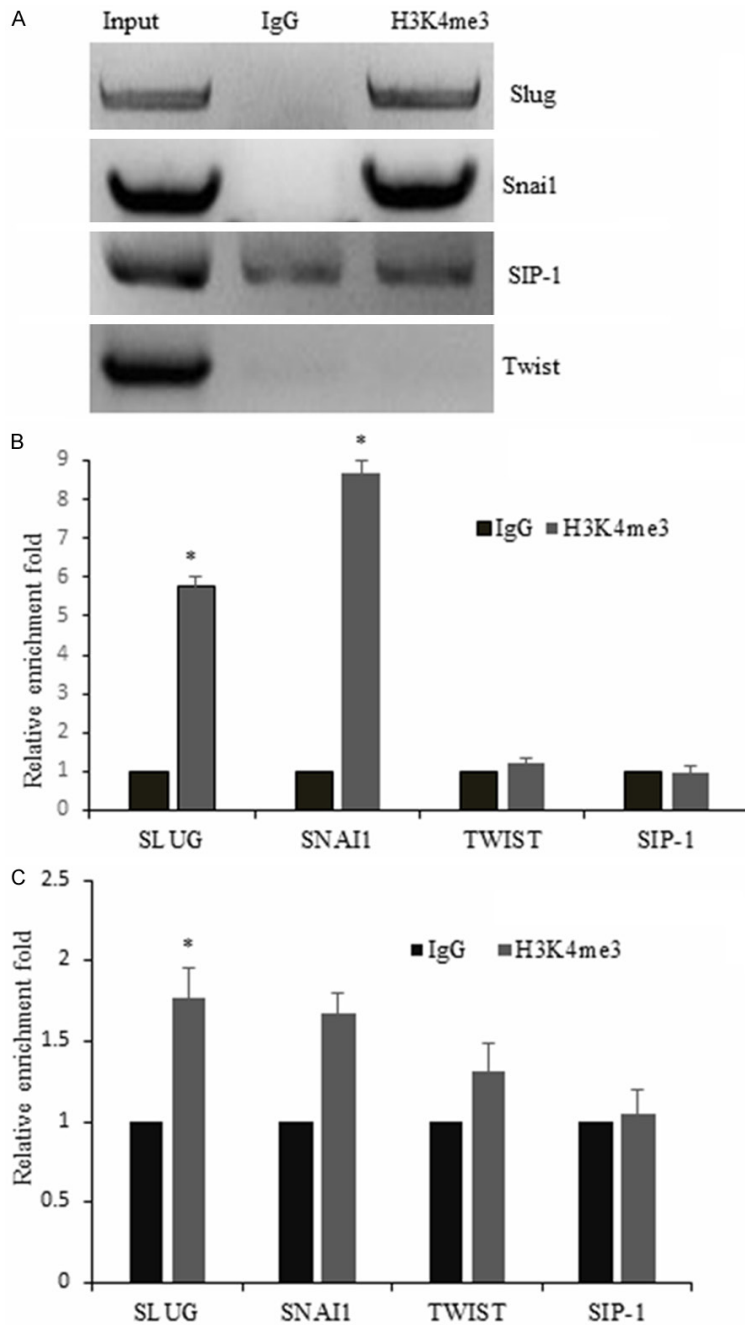


Figure 4. KDM6B promotes SLUG transcription via modulating H3K27 methylation. A. Caki-2 cells were transfected with KDM6B shRNA#2. ChIP analyses were performed with an H3K27me3 antibody. B. qRT-PCR was performed with specific primers for *SLUG*, *SNAI1*, *TWIST* or *SIP-1* mRNA. Data represent the mean \pm SD of three independent experiments. C. Reconstituted expression of RNAi-resistant KDM6B in Caki-2/shKDM6B cell, ChIP analyses were performed with an H3K27me3 antibody, subsequent qRT-PCR was performed with specific primers for *SLUG*, *SNAI1*, *TWIST* or *SIP-1* mRNA. Data represent the mean \pm SD of three independent experiments.

KDM6B shRNA 2# was the more effective knockdown sequence than shRNA 1#, and was used for further experiments (Figure 2A).

Compared with control cells, cells with an interference of KDM6B effectively inhibited cell tumorigenesis, including cell growth rate and foci formation frequency in vitro assays (Figure 2B and 2C). In a word, the abnormal expression of KDM6B strongly influenced ccRCC tumorigenesis.

KDM6B promotes EMT by inducing SLUG

EMT is a complex process, which involve cytokines, oncogenes, and growth factors, they employ different mechanisms to maintain or to induce EMT [23, 24]. EMT is common associated with a decrease or loss of epithelial markers, such as E-cadherin, and a gain of mesenchymal markers, such as N-cadherin. To determine whether KDM6B was required for EMT, we first overexpressed KDM6B in Caki-2 cells (Caki-2/KDM6B), next we detected the transcripts of EMT-associated genes. The result showed that up-regulation of KDM6B was associated with the increased mesenchymal markers, whereas epithelial markers were decreased (Figure 3A). Consistently, western blot showed that the induction of mesenchymal markers was significantly promoted, and epithelial markers were decreased (Figure 3B). And then we utilized KDM6B-shRNA to specifically knock-down KDM6B in Caki-2 cells. As shown in Figure 3C and 3D, an opposite result can be seen, the epithelial markers were up-regulated, and the mesenchymal markers were down-regulated. These results emphasized that KDM6B is required for EMT.

To gain further insight into the molecular mechanisms by which KDM6B regulates EMT and malignant conversion, we aimed to identify transcription factors whose expression was regulated by

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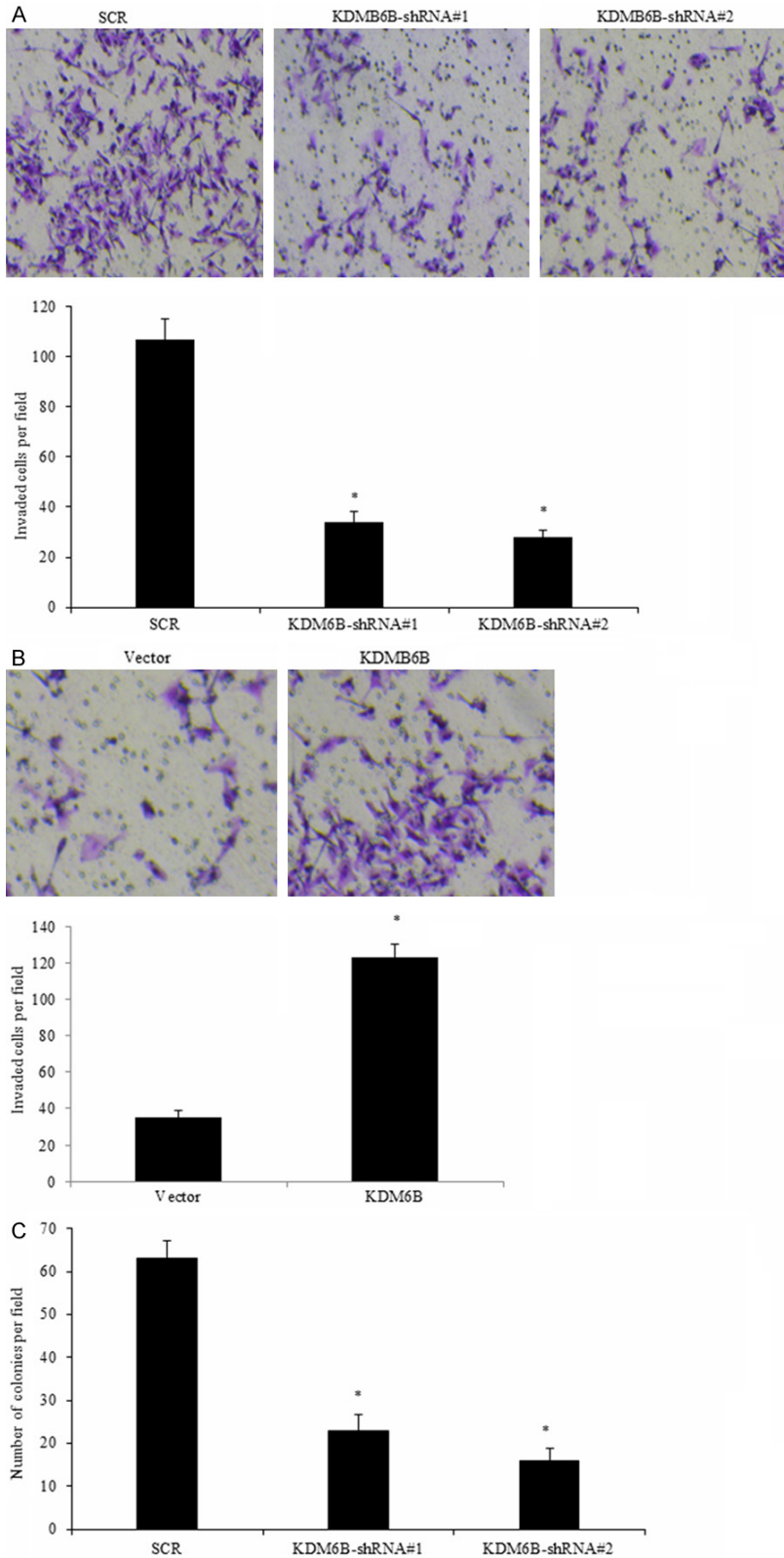


Figure 5. KDM6B promotes clear cell renal cell carcinoma cell invasion. A. 786-O cells were transfected with KDM6B shRNA and then subjected to the in vitro invasion assay 72 hr later. The graph indicates the average number of invaded cells

per field. Three independent experiments were carried out, and the data are shown as the mean with SD (* $P < 0.05$). B. 786-O cells were transfected with KDM6B and then subjected to the in vitro invasion assay 72 hr later. Representative photos were shown. Three independent experiments were carried out, and the data are shown as the mean with SD (* $P < 0.05$). C. Caki-2 cells were transfected with shRNA and then subjected to the soft agar colony formation assay. The graph indicates the average number of colonies per field. Three independent experiments were carried out, and data are shown as the mean with SD (* $P < 0.05$).

KDM6B. Because the transcription factors, including SNAI1, SLUG, SIP-1, and TWIST1 are implicated as master transcription factors that mediated EMT [23], we were interested in testify whether KDM6B regulated their expression in Caki-2 cells. qRT-PCR profiling revealed that SLUG was significantly activated in Caki-2/KDM6B cells and suppressed in Caki-2/shKDM6B cells, suggesting that SLUG expression is dependent on KDM6B (Figure 3A and 3C). The expression of TWIST1 and SIP-1 are slightly affected while knockdown of KDM6B in Caki-2 cells, data not shown. Western blot also confirmed that the overexpression of KDM6B could increase the expression of SLUG while KDM6B knockdown reduced the expression of SLUG in Caki-2 cells (Figure 3B and 3D).

KDM6B promotes SLUG transcription via modulating H3K27 methylation

We found KDM6B not only influenced SLUG in the protein levels, but also in the mRNA levels, so *may* be KDM6B regulated SLUG expression via modulating H3K27 demethylation at the promoter regions. To verify this hypothesis, we performed ChIP assay with anti-H3K27me3 in Caki-2/shKDM6B cells. As shown in **Figure 4A** and **4B**, H3K27me3 at the SLUG promoter obviously increased when KDM6B was knockdown. When expression of RNAi-resistant KDM6B in Caki-2 cells is reconstituted, it was successful to reduce H3K27me3 at the SLUG promoter (**Figure 4C**). These results indicated that the kinase activity of KDM6B regulates SLUG expression via modulating H3K27 demethylation.

KDM6B promotes clear cell renal cell carcinoma cell invasion

To further invest the function of KDM6B for EMT process, we next sought to determine whether KDM6B influence cancer cell grow or invasion, because EMT is associated with malignant properties, such as cancer cell invasion, metastasis and anchorage-independent growth [22, 23, 25, 26]. Besides, our previous data have been discovered that KDM6B was correlated with lymph node metastasis. So we supposed KDM6B maybe have function in carcinoma cell invasion. In support of our hypothesis, KDM6B significantly high expressed in invasive carcinoma, such breast cancer and kidney cancer [27, 28].

To directly examined whether KDM6B was required for tumor cell invasion, we knockdown KDM6B in the highly invasive ccRCC cell line 786-O. Matrigel invasion assays revealed that the invasiveness of 786-O cells through the Matrigel-coated membrane was significantly inhibited by KDM6B depletion (**Figure 5A**). As expect, overexpression of KDM6B promoted the invasion in 786-O cells through the Matrigel-coated membrane (**Figure 5B**). We next assessed anchorage-independent cell growth in the Caki-2 cells. As shown in **Figure 5C**, the silencing of KDM6B expression suppressed the colony formation in soft agar.

Discussion

Histone methylation plays a key role in the regulation of gene transcription, cell cycle and DNA repair, and its abnormality is also related with tumor formation [29]. There are several site-specific histone methyltransferases and demethylases can be dynamically regulated histone methylation, which is a reversible process [30]. Abnormal expression or mutation of histone methylation frequently occur in cancers [31]. KDM6B contains the JmjC domain at its C-terminus, catalyzes histone H3K27me3 demethylation, thereby promoting gene transcription. Several cancers are link to H3K27me3 alternation, including melanoma, colon, gastric, stomach, ovarian, breast, ovarian and kidney cancers [32, 33].

In this study, we first examined KDM6B expression in the ccRCC patient's tissue samples and ccRCC cell lines. To our surprised, both mRNA and protein levels of KDM6B were significantly higher in ccRCC tissues than in the adjacent non-tumor tissues, and ccRCC cell lines compared with human kidney epithelial cell line 293. Subsequently, we examined KDM6B expression profiles and its correlations with clinicopathologic parameters and prognosis in 145 ccRCC specimens by IHC. As expected, the data revealed KDM6B overexpression was significantly correlated with tumor size, pathological grade, pT status and pN status. Unfortunately, patients with high KDM6B expression had significantly worse overall survival than patients with low expression of KDM6B. However, the reason why the overexpression of KDM6B caused poor prognosis has not been well understood. Based on these studies, we found KDM6B could activate the expression of SLUG, make a hypothesis that KDM6B maybe function as an oncogene and also suggest that KDM6B may play an important role in the tumorigenesis of Clear cell renal cell carcinoma.

The major fractions of cancer-related death is result from cancer metastasis, it is a multistep process associated with EMT. One important feature of EMT is a shift in the expression levels of epithelial markers and mesenchymal markers, such as cadherins, a decrease in E-cadherin and an increase in N-cadherin. Then results in

transitioning of epithelial cells to mesenchymal-like cells, which promotes the tumor cells invade ability, primary tumor cells can metastasizing through adjacent tissues and blood vessels and form secondary tumors at distant sites. This program driven by activity of mesenchymal transcription factors such as Twist, Snail, Slug, which is regulate by some signaling pathways such as TGF β , Notch and Wnt. In our study, we found that by inducing SLUG, KDM6B could promote EMT and cancer cell invasion.

Although several studies argued KDM6B acts as a tumor suppressor, and it can control the expression of INK4A/ARF to play function in oncogenic signaling-induced cell senescence [17, 18]. However, there also have some studies supported that KDM6B is highly expressed in several kind of cancers and associated with tumor progression. In conclusion, our study provides a direct evidence for KDM6B works as a promoter of EMT and involves in ccRCC cell invasion. We demonstrate that KDM6B plays a key role in ccRCC and therefore provide a novel therapeutic target and a prognostic marker in ccRCC.

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Disclosure of conflict of interest

None.

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