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The Expression of Histone Demethylase JMJD1A in Renal Cell Carcinoma

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Background: Hypoxia-inducible factor 1α has been shown to play a central role in RCC tumorigenesis by acting as a transcription factor. Histone demethylase JMJD1A is an iron- and 2-oxoglutarate-dependent dioxygenase which catalyze the demethylation of mono- and dimethylated H3K9. JMJD1A can be upregulated by hypoxia via HIF-1 and associated with cancer.

The expression of JMJD1A was determined in 10 kidney cancer tissue and adjacent tissue by quantitative polymerase chain reaction, western blotting and immunohistochemistry. Furthermore, the expression of JMJD1A was investigated in cell line 786-0 through adding nickle or cobalt ion to mimic hypoxic environment.

The expression of JMJD1A was higher in cancer tissue than adjacent tissue, and in hypoxic environment than normal environment. In cancer tissue, the JMJD1A mainly located around blood vessels which indicated that JMJD1A is involved tumor angiogenesis. Conclusion: the increased expression of JMJD1A might be associated with the progression of kidney cancer.

Key words: renal cell carcinoma, histone demethylase, JMJD1A, hypoxia-inducible factor, iron

Renal cell carcinoma (RCC) is one kind of serious cancer, which accounts for 3% of adult malignancies and about 209,000 new cases are reported per year worldwide [1]. Mutations of the *VHL*(von Hippel-Lindau) tumor suppressor gene are very common in RCC [2], but largely absent in other cancers. Inactivation of VHL cause the increase of hypoxia-inducible factor 1α (HIF-1α) protein stabilities because of deficiency in ubiquitination mediated by VHL and proteasomal degradation [3]. Many researches indicate that HIF-1a plays a central role in the tumorigenesis of RCC by acting as a transcription factor for several proteins that are important in tumoral adaptation to a tissue microenvironment that is low in oxygen [4]. These genes regulated by HIF-1 include vascular endothelial growth factor(*VEGF*), glucose transporter-1 (GLUT-1), and so on [5]. HIF-1 also affects the expression of several jumonji-domain histone demethylase genes, including *JMJD1A*, *JMJD2B*, *JMJD2C* and *JARID1B* [6-9].

JMJD1A (Jumonji domain-containing protein 1A),also called JHDM2A(JmjC domain-containing histone demethylation protein 2A) or KDM3A(Lysine demethylase 3A), belongs to iron- and 2-oxoglutarate-dependent dioxygenases. JMJD1A specifically demethylates mono- and dimethylated H3 'Lys-9' residue(H3K9me1/2), which is an important epigenetic mark associated with transcription repression [10]. In hypoxic environment, the expression of JMJD1A is upregulated, which enhances hypoxic gene expression [11-12]. These researches also indicates that the upregulation of JMJD1A expression is associated with cancer development [6,12]. The relationship between JMJD1A and the development of renal cell carcinoma need further investigation.

In this study, we showed that increased expression of JMJD1A in cancer tissue and mimicking hypoxic environment with quantitative real-time reverse transcription PCR(qRT-PCR), western blotting and immunohistochemistry. We provide evidence that histone demethylase JMJD1A is regulated by hypoxia and associated with the progression of kidney cancer.

Materials and methods

Clinical sample collection. The cancer tissues and matched adjacent tissues in 10 RCC patients were obtained through radical nephrectomy from Peking University Shenzhen Hospital (Guangdong, China).Written informed consent was obtained from all patients and the study was approved by the Institutional Review Board of Peking University Shenzhen Hospital.

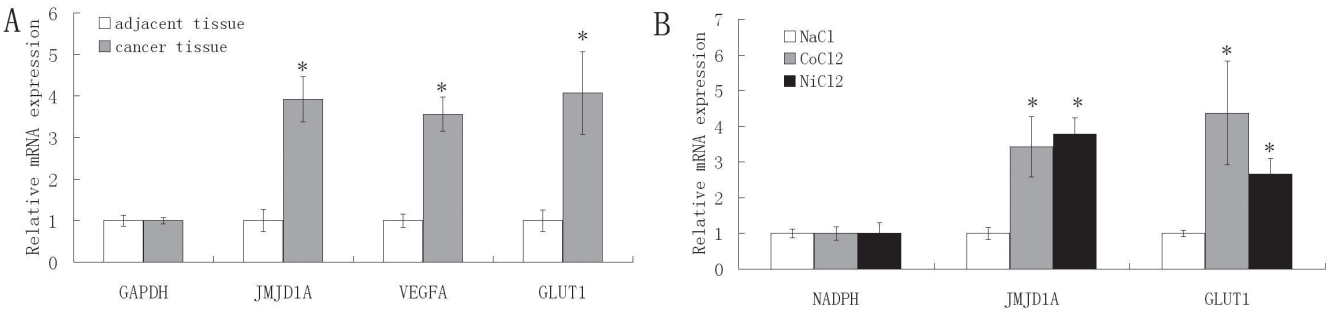

Figure 1. Expression study by qRT-PCR of JMJD1A and related genes in cancer tissue and hypoxic environment. qRT-PCR was conducted for three genes encoding GLUT1, VEGF and JMJD1A to see their expression, and GAPDH gene served as the internal control. A. In cancer tissues and matched adjacent tissues of RCC (10 patients), relative mRNA expressions including VEGFA, JMJD1A and GLUT1 were indicated. B. relative mRNA expressions including JMJD1A and GLUT1 in 786-0 cell (three parallel experiments) treated with $CoCl_2$ or $NiCl_2$ (* p<0.05) .

Cell culture and treatment. RCC cell line 786-0 was cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO,USA)supplemented with 10% fetal bovine serum (FBS) (Hyclone,USA) and maintained at 37 °C in a humidified atmosphere of 5% CO2 and 95% air (20% O2). For mimicking hypoxic environment, 786-0 cells were treated with 1mM $NiCl_2$ or 0.25mM $CoCl_2$ for 24 hour, and physiological saline treatment as control(three parallel experiments).

qRT-PCR. Total RNA was isolated from patients' tissue and cultured cells by Trizol reagent (Invitrogen, US) according to the manufacture's protocol. 2 μg of total RNA was subjected to cDNA synthesis using a reverse transcription system according to the manufacturer's protocol (Fermentas, USA). The synthesized cDNA was directly used for qPCR amplification with SYBR Green I PCR kit (Invitrogen, USA) as recommended by the manufacturer. All used primers are listed in table 1. The relative fold change in expression of each mRNA was calculated using the ΔΔCt method relative to GAPDH mRNA.

Western blotting. The patients' tissues and treated cells were homogenized using supersonic method. The lysis buffer (50 mMTris-HCl,pH 8.0, 150 mM NaCl, 5 mM EDTA, pH 8, 1% NP-40) included 1 mM PMSF and inhibitor cocktail (Calbiochem). Proteins were quantified using Bicinchoninic Acid (BCA) Protein Assay kit (Pierce). 40 μg of protein from each sample was resolved by reducing loading buffer and then separated with 10% SDS-PAGE. After protein was transferred onto Polyvinylidene fluoride(PVDF) membranes, the membranes were saturated with 5% skim milk in TBST (50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20) for 2 hours and then incubated with 1:5000 primary antibody(polyclonal antibody JMJD1A from Sigma, monoclonal antibody to HIF-1α from Invitrogen, monoclonal antibody to GLUT1 from Abcam and monoclonal antibody to GAPDH from Epitomics) overnight. NC membrane was incubated with 1:5,000-diluted peroxidase-coupled goat anti–rabbit/mouse IgG (secondary antibody, MERCK) for 1 h. After being wash with TBST four time(5 mins/time), the NC membranes were exposed to chemical luminescence substrate(Pierce) and then to the X-ray film.

Immunohistochemical analysis. After fixation in 10% neutral buffered formalin, RCC cancer tissues and adjacent tissues were dehydrated through an ascending series of graded ethanol, embedded in paraffin wax, and sectioned into 5 μm sections. Briefly, tissue sections were deparaffinized, rehydrated, and treated with 3% hydrogen peroxide in methanol for 15 minutes to quench endogenous peroxidase activity. After washing in 10mM PBS (pH 7.4), antigen retrieval was performed by heating the slides for 10 minutes in a boiled 0.01M citrate buffer (pH 6.0) in a staining jar. Afterward, sections were incubated with 8% normal BSA to block nonspecific binding. Sections were then incubated overnight at 4°C with 1:100 dilution of anti-JMJD1A (sigma, USA) primary polyclonal rabbit antibodies. After being washed in PBS, sections were treated with MaxVision HRP-Polymer anti-rabbit IHC Kit (Maixin Bio, Fujian, China) at room temperature for 15 minutes and subsequently DAB kit (Maixin Bio, Fujian, China) was used as a chromogen to visualize the peroxidase activity. The negative immunohistochemical control procedure included replacement of the primary antibodies by antibody diluent.

Table 1. Primers used in the experiment

gene	Forward	Reverse	product length
JMJD1A	GTCAACTGTGAGGAGATTCCAGC	AACTTCAACATGAATCAGTGACGG	63
GLUT1	CGGGCCAAGAGTGTGCTAAA	TGACGATACCGGAGCCAATG	283
VEGFA	CTTGCCTTGCTGCTCTAC	TGGCTTGAAGATGTACTCG	192
GAPDH	GCTCTCTGCTCCTCCTGTTC	GACTCCGACCTTCACCTTCC	100

Statistics. A two-tailed Student's t-test was applied to perform statistical analysis and P values of 0.05 or less were considered significant.

Results

The expressed upregulation of JMJD1A in kidney cancer tissue and hypoxic environment at mRNA level. In order to identify the association of JMJD1A and renal cell carcinoma, we first measured the VEGFA and GLUT1 expression in cancer tissue with qRT-PCR. It was found that both genes expression was higher in cancer tissue than in adjacent tissue (Fig 1A), which is consistent with previous report by Liang et al and Lidgren et al [13,14].Then we evaluated the expression of JMJD1A in cancer tissue in 10 RCC patients and found that JMJD1A also was upregulated (Fig 1A).

To prove that the expression of JMJD1A was regulated by hypoxia, we mimicked hypoxic environment by adding Co^{2+} or Ni^{2+}. There were researches indicated that Co^{2+} or Ni^{2+} addition can increase HIF-1α protein stabilities and expression of hypoxia related genes [15-17]. After adding $CoCl_2$ or $NiCl_2$, the expressions of GLUT1 and JMJD1A were upregulated which indicated that JMJD1A is one of hypoxia regulated gene.

The increased stability of HIF-1α protein effects JMJD1A gene expression. To investigate if the upregulation of JMJD1A was induced by increased HIF-1α protein, we performed western blotting analysis. In RCC cancer tissue, the stability of HIF-1α is obviously higher than in adjacent tissue, and in the same time JMJD1A and GLUT1 expression increase at protein levels (Fig 2A). Previous research has indicated that HIF-1 alpha expression was greater in RCC than in normal tissue [18], so induced expression of JMJD1A and GLUT1 may be important for RCC development. In mimicking hypoxic environment, the increased stability of HIF-1α protein also upregulated expression of JMJD1A and GLUT1.

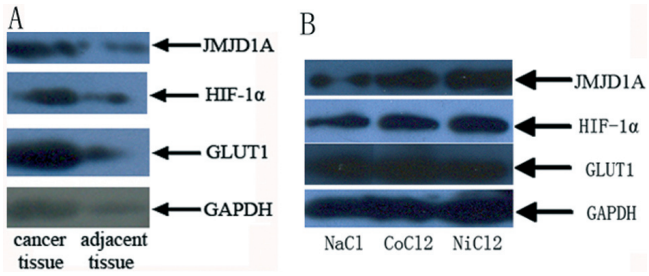

Figure 2. HIF-1α induce the expression of JMJD1A in RCC cancer tissue and hypoxic environment. A, western blotting analysis of JMJD1A, HIF-1α and GLUT1 in cancer tissue and adjacent tissue. B. western blotting analysis of JMJD1A, HIF-1α and GLUT1 in 786-0 cell treated with $CoCl_2$ or $NiCl_2$.

JMJD1A is mainly located around blood vessesl and micro vessels. To determine the effect of JMJD1A overexpression on cancer development, we performed immuno- histochemical analysis for JMJD1A tissue location. The results indicated that JMJD1A expression is stronger in cancer tissue than in adjacent normal tissue. JMJD1A is especially located around vessel of cancer tissue, which suggest that JMJD1A may be involved in VEGFA expression. The overexpression of VEGFA is beneficial for tumor angiogenesis and RCC is a highly vascularized cancer [19], so regulation of JMJD1A on VEGFA may be important for RCC development.

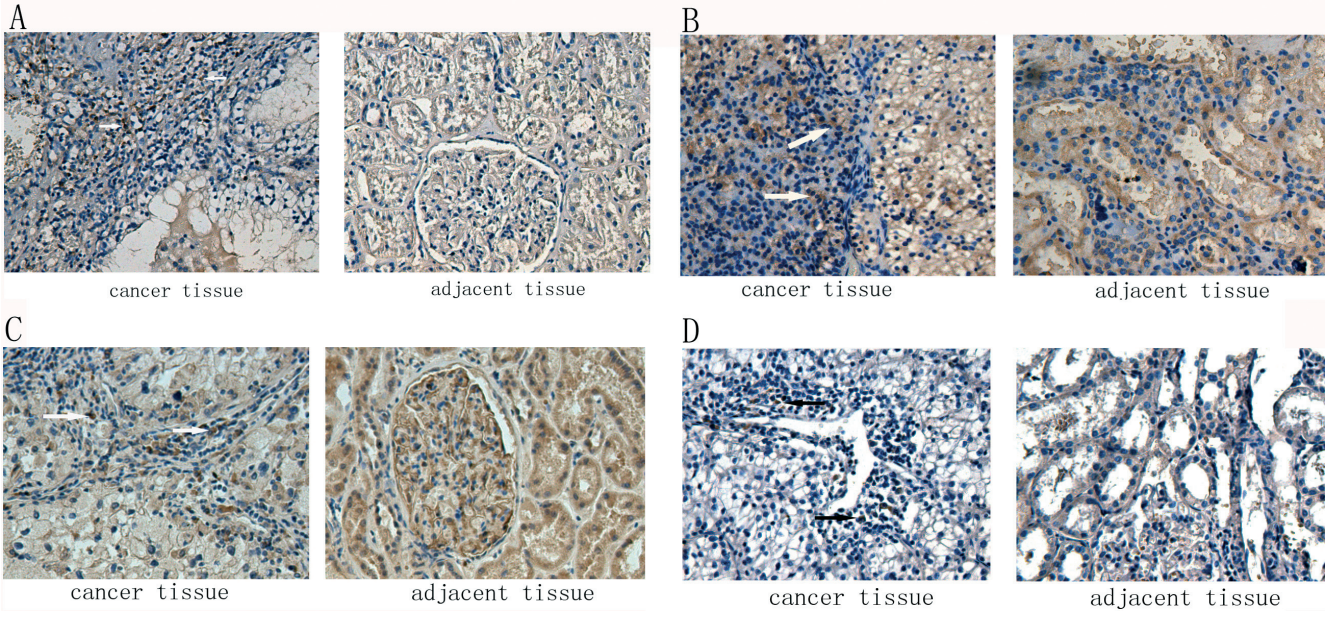

Figure 3. The JMJD1A mainly located around vessel of cancer tissue. JMJD1A expression is strong in cancer tissue, especially around vessel, and weak in adjacent tissue. A,B,C and D represent different RCC patients' tissue.

Discussion

Renal cell carcinoma (RCC) is one heterogeneous malignancy whose incidence rate has notably increased in recent years without any evident reason [20]. But, most of RCC patients have been resistant to classic treatments such as chemotherapy, radiotherapy and hormonal therapy, so it is important to understand the RCC development and search for new treatment modalities [21]. Histone modifications such as acetylation and methylation have important regulatory roles for processes such as gene transcription and DNA damage repair, and misregulation of histone modification actively contributes to human cancer [22].Many histone demethylases such as KDM5B(JARID1B/PLU-1), and KDM8 which belong to iron- and 2-oxoglutarate-dependent dioxygenases are involved in cancer development including prostate cancer and breast cancer [23-25]. Loss of HIF-1alpha expression has been described in RCC cell lines and primary tumors(26),which means that hypoxia signaling pathway is important for RCC development. JMJD1A can be upregulated by hypoxia and HIF-1 due to hypoxia responsive element (HRE) in the promoter of the human JMJD1A gene [7]. All these imply JMJD1A has important function on tumor progression, at least in RCC development.

Cobalt compounds or nickel compounds can alter the histone modification [27].They can affect the activity of histone demethylase such as inhibiting function of nickel ions on JMJD1A by replacing the ferrous iron in the catalytic centers [28]. Chronic exposure to nickel compounds has been connected to the increased risks of cancer, and alteration of histone modification may be one important factor [29].

In this research, the upregulation of JMJD1A in RCC cancer tissue and hypoxic environment indicated that JMJD1A may participate in the progression of RCC through hypoxia signal pathway. H3K9me1/2 is an important transcriptionally repressive histone marks, so demethylase activity of JMJD1A on H3K9me1/2 is essential for the expression of many genes including cancer related genes. It is useful for RCC diagnosis and treatment to understand the JMJD1A mechanism on RCC development.

In conclusion, our data supported that JMJD1A is an important factor in RCC progression and its upregulated expression is common in RCC cancer tissue. To investigate the target genes regulated by JMJD1A deserves further research of RCC.

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