The expression and significance of histone H3K27 demethylases in renal cell carcinoma

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Abstract

**Background:** Histone H3K27 demethylases UTX and JMJD3 are important regulatory factors that modulate gene expression via altering the physical state of chromatin. Previous researches have indicated abnormal status of H3K27 methylation caused in carcinogenesis, so we investigated the expression pattern of UTX and JMJD3 in renal cell carcinoma (RCC) and their role in cancer development.

**Methods:** The mRNA expressions of *UTX* and *JMJD3* were determined in cancer tissues and adjacent tissues of 36 primary RCC patients by quantitative real time polymerase chain reaction (qRT-PCR). The protein contents of UTX and JMJD3 were measured with western blotting and immunohistochemical analysis.

**Results:** The transcripts of UTX and JMJD3 were significantly increased in cancer tissues than adjacent tissues (p < 0.05). And the mRNA levels of cyclin dependent kinases 4 and 6 (CDK4/CDK6) inhibitor p16INK4a was also higher in cancer tissue (p < 0.001). The western blotting result indicated that two of the demethylases were increased in cancer tissue. The level of H3K27me3 was lower in cancer tissue compared to adjacent tissue, although the expression of H3K27 methyltransferase EZH2 was also increased (p < 0.05). The data indicate that two H3K27 demethylases may play critical roles in regulation of H3K27 methylation state in RCC development. Further the immunohistochemical analysis proved that expressions of UTX and JMJD3 were upregulated in cancer tissues compared to adjacent tissues.

**Conclusions:** Our results suggest that UTX and JMJD3 shown higher expression level in cancer cells compared to adjacent normal tissue, which may be important for primary RCC development. Whether the H3K27 demethylases be used as biomarkers in early diagnosis of RCC or not, need to be explored.

**Keywords:** Renal cell carcinoma, Histone H3K27 demethylase, UTX, JMJD3, Epigenetics

**Background**

Kidney cancer is the third most common urological malignancy, which caused estimated 120,000 deaths per year worldwide [1]. The incidence of kidney cancer has significantly increased over the past 20 years [2]. In Europe, kidney cancer accounts for nearly 3% of all cancer cases [3]. Kidney cancer includes two most common types, renal cell carcinoma (RCC) and urothelial cell carcinoma (UCC). RCC is the most common kidney cancer in adults, and generally resistant to chemotherapy and radiation therapy [4, 5]. A radical or partial nephrectomy of the tumor at a localized stage remains the mainstay for curative
therapy [6]. Unfortunately, distant metastases are present at the time of initial diagnosis in approximately one third of patients, and the tumor will recur in another third even after nephrectomy with a curative intent [7, 8]. In addition, there is lack of specific diagnostic markers for RCC, which is an important reason for poor prognosis [9]. A better understanding of the molecular basis of RCC has facilitated the development of novel and more selective diagnostic and therapeutic approaches.

A lot of studies shown that epigenetic modification played important role in cancer development [10]. Histone methylation was essentially posttranslational and epigenetic modification, which was miswritten, misinterpreted and miserased in many human cancers including RCC [11]. Histone methylation can be achieved at certain amino acids such as lysine (K) or arginine(R) in the N-terminus of histone H3 and H4 by the addition of one, two, or three methyl groups. H3K27 methylation is reversible process which can be catalyzed by histone lysine methyltransferase 6 (KMT6, also known as EZH2), and demethylases 6A (KDM6A, also known as UTX) and 6B (KDM6B, also known as JMJD3) [12]. H3K27me3 was suppressor marker which inhibits the expressions of specific target genes via altering the physical state of chromatin. EZH2 (enhancer of zeste homolog 2) can be regarded as oncogene and is frequently overexpressed in a wide variety of cancers [13], like RCC [14, 15]. Several studies identified the UTX (ubiquitously transcribed TPR gene on the X chromosome) and JMJD3 (jumonji domain-containing protein 3) as H3K27-specific demethylases which contribute to the activation of genes [16-20]. Both UTX and JMJD3 were considered as tumor suppressors, especially inactivating somatic mutations of UTX frequently occurred in multiple tumor types including RCC [21]. But the relationship between expressions of two enzymes with cancer development was largely unknown. In this study, the expressions of UTX and JMJD3 and their clinical significance were investigated.

**Methods**

**Patients and tissue specimens**

The 63 clear cell RCC (ccRCC) patients were diagnosed firstly with computed tomography (CT) combined with clinical symptoms, and then confirmed with clinicopathological examination in the Fourth Clinical Medical College of Hebei Medical University (Shijiazhuang, China). All the cancer tissues and paired adjacent tissues were obtained through radical nephrectomy. Written informed consent was signed from all patients before the operation and the study was approved by Human Research Ethics Committee of The Fourth Clinical Medical College of Hebei Medical University. Histological
classification was performed according to the standard provided by Fuhrman et al [22] and post-operative pathological staging was performed according to guide request (Table 1).

**Quantitative real time polymerase chain reaction (qRT-PCR)**

Total RNA was extracted from cancer tissues and adjacent tissues with Trizol reagent kit (Invitrogen, Carlsbad, USA) according to the manufacturer’s protocol. The total RNA concentration was determined with NanoDrop ND-1000 (Thermo Scientific, Wilmington, USA). 2μg of total RNA was used to cDNA synthesis using a reverse transcription system according to the manufacturer's instructions (Invitrogen, Carlsbad, USA). The mRNA expressions of UTX, JMJD3, EZH2 and p16INK4a were analyzed using SYBR green PCR Mix (Tiangen, Beijing, China), and 18S rRNA as internal reference. qRT-PCR was performed using 7500 RealTime PCR System (Applied Biosystems, Foster City, USA). Primer sequences were synthezed by Sangon (Shanghai, China) and included UTX forward 5′- TTTGTCAATTAGTCCTGAACCTC -3′ and UTX reverse 5′- AAAAAGGCAGCATTCTCCAGTGC -3′, JMJD3 forward 5′- GGAGGCCCACACGCTGCTAC -3′ and JMJD3 reverse 5′- GCCAGTATGAAATGTCCTGCAGTGC -3′, EZH2(isoforms 1-5) forward 5′- GGGACAGTAAAAATGTGTCCTGC -3′ and EZH2 reverse 5′- TGCCAGCAGAGGTGTTACAGCAGTGC -3′, INK4A(isoforms 1/2) forward 5′- GAAGGTCCCTCAGACATCCCC -3′ and INK4A reverse 5′- CCCTGTAGGACCTCCGTGAC -3′, 18S rRNA forward 5′-CGGCGGCTTTGGTGACTCTAG -3′ and 18S rRNA reverse 5′-CGGCGGCTTTGGTGACTCTAG -3′. Relative expression levels of the four genes were normalized to the internal reference 18S RNA. The data were analyzed using the comparative threshold cycle ($2^{ΔCT}$) method.

**Western blotting**

Both cancer tissues and adjacent tissues were homogenized in radioimmunoprecipitation assay (RIPA) buffer containing the protease inhibitors phenylmethylsulfonylfluoride (100μg/mL), NaVO3 (1mmol/L) and dithiothreitol (DTT, 0.5mmol/L). Homogenates were centrifuged and supernatants were collected. Protein concentrations were determined with NanoDrop ND-1000 (Thermo Scientific, Wilmington, USA) and appropriately corrected. 50 μg of protein from each sample was resolved by reducing loading buffer and then separated with 8% sodiumdodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by electrophoretic transfer to a nitrocellulose (NC) membrane. The NC membrane was saturated with 5% skim milk in TBST (50 mM Tris-HCl, 150 mM NaCl, 1% Tween-20) and 1% BSA.
0.1% Tween-20) for 2 hours and then incubated with primary antibodies at 4 °C overnight. The primary antibodies used in this research included rabbit polyclonal antibodies UTX (1:1000, Abcam, Hongkong, China), JMJD3 (1:1500, Abcam, Hongkong, China), EZH2 (1:500, Santa cruz biotechnology, Hongkong, China), H3K27me3 (1:1500, Epigentek, Brooklyn, USA), H3 (1:2000, Sigma-Aldrich, St Louis, USA) and ACTIN (1:2500, Sigma, St Louis, USA). NC membrane was incubated with 1:5,000-diluted peroxidase-coupled goat anti-rabbit immunoglobulin G (IgG) (secondary antibody, Sigma, St Louis, USA) for 1 h after washing with TBST 3 times (5 min/time) at room temperature. After being washed with TBST four times, the NC membrane was exposed to enhanced chemiluminescence substrate (Pierce, Rockford, USA) for 5 min and then detection was performed with Fujifilm LAS-4000 (GE Healthcare, Bucks, United Kingdom).

Immunohistochemical analysis

After fixation in 4% formalin, cancer tissues and adjacent tissues in 63 RCC patients were dehydrated through an ascending series of graded ethanol, embedded in paraffin wax, and cut into 5 μm sections with a microtome. The endogenous peroxidase activity of sections was inhibited by treatment with 3% H2O2/methanol. Then, Antigen retrieval was performed for xylene-deparaffinized and dehydrated sections by heating the slides for 10 minutes in 0.01M citrate buffer (pH 6.0). Non-specific binding was blocked by incubating sections with 5% BSA in a humidified chamber. Sections were then incubated overnight at 4°C with 1:100 dilution of anti-UTX or anti-JMJD3 primary polyclonal rabbit antibodies (Abcam, Hongkong, China). After being washed in PBS twice, sections were treated with peroxidase-conjugated affinipure goat anti-rabbit IgG (ZSGB Bio, Beijing, China) at room temperature for 30 min and subsequently DAB kit (ZSGB Bio, Beijing, China) was used as a chromogen to visualize the peroxidase activity. The negative immunohistochemical control procedure included replacement of the primary antibodies by antibody diluents.

The protein expression scores of both UTX and JMJD3 were quantitated according to Wu et al [23]. Briefly, the proportion of UTX/JMJD3-expressing tumor cells was scored as follows: 0, no positive cells; 1, <5%; 2, 6%-25%; 3, 26%-50%; 4, 51%-75%; and 5, >75%. The staining intensity was graded according to the mean optical density: 0, no staining; 1, weak staining (light yellow); 2, moderate staining (yellow brown); and 3, strong staining (brown). Staining index was calculated as the multiplication of staining intensity score and the proportion of UTX/JMJD3-positive tumor cells.

Statistical analysis
All statistical analysis was carried out with the SPSS 17.0 statistical software package. The data in the qRT-PCR and immunohistochemical assays were analyzed with two-tailed paired-sample t test and Mann–Whitney U test ($\alpha=0.05$), and $P$ values of 0.05 or less were considered statistically significant between cancer tissue and adjacent one.

**Results**

**Patient clinical characteristics**

In total, 63 cancer tissues and adjacent tissues were available from patients who had undergone surgery. All the patients were treated with radical nephrectomy and didn't receive radiation or chemotherapy preoperatively. Most of patients (56/63) were at early stage (stage 1 and 2), and no lymph node metastasis was discovered in all patients. The overall 5-year survival rate for all patients in the studies was 100%. It means that early diagnosis and surgical removal of cancer tissue can achieve a good prognosis. All the patients' data see Table 1.

**The mRNA expressions of UTX and JMJD3 in cancer tissues and adjacent tissues of RCC patients**

The transcription levels of two H3K27 demethylases, H3K27 methyltransferase EZH2 and CDK4/CDK6 inhibitor $p16INK4a$ were determined by qRT-PCR in cancer tissues and adjacent tissues of 36 RCC patients. The results indicated that the mRNA levels of three proteins were significantly increased in cancer tissues than adjacent tissues ($p<0.05$, Figure 1). In addition, the expression of $p16INK4a$ was also unregulated in cancer tissues than adjacent ones ($p<0.001$, Figure 1).

**The protein contents of UTX and JMJD3 in cancer tissues and adjacent tissues of RCC patients**

To further prove these results of mRNA changes of UTX and JMJD3, the protein contents of two H3K27 demethylases were measured with western blotting. The contents of UTX and JMJD3 proteins were obviously increased in cancer tissue(C) compared to adjacent tissue (A) (Figure 2). With elevated levels of two H3K27 demethylases, the content of H3K27me3 was decreased in RCC cancer tissue although the expression of H3K27 methyltransferase EZH2 was also upregulated (Figure 2).

**The change of UTX and JMJD3 expression in cancer tissues and adjacent tissues of RCC patients analyzed by immunohistochemistry**
Expression and subcellular localization of two H3K27 demethylases were further determined by immunohistochemical analysis in 63 paraffin-embedded RCC cancer tissues and paired adjacent tissues. In renal tissue, two H3K27 demethylases were localized mainly in the nucleus. The UTX and JMJD3 protein contents in cancer tissues were higher than those in adjacent tissues (p < 0.01, Figure 3-5).

Discussion

It has been demonstrated that histone demethylases are closely related with tumor formation [24]. Several researches also indicated H3K9 demethylase JMJD1A was associated with RCC [25, 26]. Therefore, it is worthy of evaluating the roles of histone demethylases in RCC. Both UTX and JMJD3 are H3K27me3 demethylases which are essential for the expression of many genes including HOX through decreasing the H3K27me3 level [16, 17]. It has been demonstrated that there existed inactivating somatic mutations of UTX and reduced expression of JMJD3 in many cancers [21, 27]. Both UTX and JMJD3 can be considered as candidate tumor suppressors [28, 29]. Two H3K27me3 demethylases may be involved tumor suppression via oncogene-induced senescence (OIS) mechanism.

Human tumor suppressor genes INK4b-ARF-INK4a are located on 9p21, and encode three proteins, p16INK4a, p15INK4b and p14ARF. p16INK4a/p15INK4b are CDK4 / CDK6 inhibitors and block the retinoblastoma protein (Rb) phosphorylation and inactivation, which enhances the pRB-E2F signaling pathway. p14ARF inhibits the activity of p53-specific ubiquitin ligase murine double minute 2 (MDM2), which increases the p53 protein[30]. Three proteins are involved in senescence regulation and induce cell cycle arrest at the G0/G1 phase, which leads to cell senescence [31]. Cellular senescence is irreversible growth arrest, while OIS is an importantly preventive mechanism for pre-cancerous damage [32]. OIS can effectively block uncontrolled cell proliferation induced by DNA damage or oncogenic stimuli. The imbalance of OIS can lead to unlimited cell proliferation across the limitations of aging, eventually cancer development [33].

The INK4b-ARF-INK4a locus is regulated by many factors, in which histone modification is an important regulator [34]. Polycomb repressive complex2 (PRC2) containing EZH2 can bind the INK4b-ARF-INK4a locus and silence their gene expressions through increasing local H3K27me3 content, which promotes cell proliferation and reduces cell senescence [35, 36]. In contrast, JMJD3 binding to INK4b-ARF-INK4a locus can suppress the PRC2 occupancy and decrease H3K27me3 content, resulting the increased expressions of three proteins and promoting cell
senescence [29]. In primary Hodgkin's Lymphoma (HL), JMJD3 is over-expressed and induced by the Epstein-Barr virus (EBV) oncogene [37]. In our study, the H3K27me3 level was lower in cancer tissues compared to adjacent tissues accompany with increased JMJD3 expression (Figure 2). Consistent with the strong decrease in H3K27me3 level, the gene expression of p16INK4a was obviously higher in cancer tissue compared to adjacent one (Figure 1). Previous research has indicated that homozygous deletions of the INK4a/ARF locus could contribute to tumor progression in RCC [38]. These results mean that inactivation or expression down-regulation of p16INK4a is a later event in RCC progression.

On the other hand, UTX is also important for cell senescence in tumor suppression. Tumor suppressor Rb and its binding proteins are regulated by UTX catalyzing H3K27me3 demethylation [39]. UTX can occupy the promoter region of Rb and related gene Rbl2 (retinoblastoma-like protein 2) and increase their expressions, which reduces cell proliferation and increases cell senescence [40]. In Drosophila, UTX mutant cells showed tumor-like growth characteristics accompanying reducing Rb expression [41]. In our study, UTX expression was obviously higher in cancer tissues in mRNA and protein levels (Figure1, 3 and 4). Although all these three proteins are upregulated, the reduction of H3K27me3 level implies both H3K27 demethylases play more important role than H3K27 methyltransferase in regulating p16INK4a expression.

Previous studies have demonstrated that abnormality of H3K27 level or H3K27 methyltransferase EZH2 expression was associated with cancer development and prognosis [14, 42]. In our study, it was also proved upregulated expressions of two H3K27 demethylases UTX and JMJD3 were relevant to tumor suppression. We unexpectedly discovered two demethylases upregulation in cancer tissue instead of adjacent tissue. In fact, previous researches have been proved that JMJD3 was regulated in cancer tissue in many cancers, including prostate cancer and primary Hodgkin's Lymphoma [20, 37]. The researches on relationship between histone demethylases with cancer development would be contributed to molecular mechanism understanding and new therapy for RCC [43, 44]. The possible role of UTX and JMJD3 involved in RCC can be supposed as follows. The activation of oncogene leads to an increased binding of JMJD3 to region of the promoter for p16INK4a and transcriptional induction of the p16INK4a by demethylating H3K27me3 at the INK4A–ARF locus [31]. And then p16INK4a inhibits the RCC development via induction of cell cycle arrest. At present, the understanding of mechanism underlying cell senescence in tumor suppression is limited, so the roles of UTX and JMJD3 in RCC remain to be further studied.
Conclusion

In summary, our studies indicate that upregulated expressions of UTX and JMJD3 in cancer tissue are common characteristics in early stage of RCC patients which have good prognosis. Two H3K27 demethalyses may be inhibit cell proliferation in primary RCC though oncogene-induced senescence. These studies also imply that identification of regulated genes by UTX and JMJD3 in RCC development will be beneficial to the understanding of carcinogenesis and screen of RCC. Whether one or two H3K27 demethalyses can be considered as biomarker(s) in early diagnosis of RCC or prognostic evaluation need to be explored.

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Authors’ contributions

XG, YZ, AZ and XD were responsible for experimental design, data analysis and writing of manuscript. YS, XG and YW conducted these experiments including qRT-PCR, western blotting and immunohistochemical analysis. YS, WQ and AZ were responsible for collection and histological classification of clinical specimens. All authors have read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

References


41. Herz HM, Madden LD, Chen Z, Bolduc C, Buff E, Gupta R, Davuluri R, Shilatifard A, Hariharan IK, Bergmann A: The H3K27me3 demethylase dUTX is a suppressor of


**Figure legends**

**Figure 1** Real-time quantitative RT-PCR analysis of both H3K27 demethylases, H3K27 methyltransferase EZH2 and CDK4/CDK6 inhibitor p16INK4a. The relative mRNA expressions of *UTX* and *JMJD3* were higher in RCC cancer tissues than in paired adjacent tissues (n=36, P=0.004 and P=0.003 respectively). The mRNA content of *p16INK4a* was also upregulated in cancer tissues compared to adjacent tissues (P < 0.001). Although the expression of EZH2 was also upregulated, two H3K27 demethylases may be major determiners in H3K27me3 regulating target genes.

**Figure 2** Western blotting analysis of H3K27 methylation modified enzymes and H3K27me3 levels. The protein expressions of two H3K27 demethylases UTX and JMJD3 were higher in RCC cancer tissues (C) than in paired adjacent tissues (A). The relative H3K27me3 level was lower in RCC cancer tissues although the protein content of H3K27 methyltransferase EZH2 was increased.

**Figure 3** The protein expression changes of UTX and JMJD3 in RCC. The relative protein expressions of UTX and JMJD3 in RCC cancer tissue samples (C) were higher than that in the paired adjacent tissue samples (A) (n=63, P < 0.05).

**Figure 4** Immunohistochemical analysis of the expression of UTX. The protein expression of UTX was obviously higher in cancer tissues (C and D) that in adjacent tissues (A and B). The magnifications of A and C are 200x, and B and D are 400x.
Figure 5 Immunohistochemical analysis of the expression of JMJD3. The protein expression of JMJD3 was obviously higher in cancer tissues (C and D) than in adjacent tissues (A and B). The magnifications of A and C are 200×, and B and D are 400×.
Figure 3
Additional files provided with this submission:

Additional file 1: Table 1.doc, 39K
http://www.biomedcentral.com/imedia/1301687097713846/supp1.doc
Additional file 2: Figure S1.tif, 1175K
http://www.biomedcentral.com/imedia/3844805247138468/supp2.tif