Original Article

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Loss of SETD2, but not H3K36me3, correlates with aggressive clinicopathological features of clear cell renal cell carcinoma patients

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Summary **Recent studies facilitated by DNA sequencing identified the histone modifying gene *SETD2* as the second most frequent mutant gene in sporadic clear cell renal cell carcinoma (ccRCC) patients. SETD2 functions as a tumor suppressor in ccRCC. However, its clinical association and biological functions are not fully delineated. The aim of this study is to evaluate the clinical significance of SETD2 in ccRCC patients. SETD2 and its canonical histone modification product H3K36me3 were analyzed by immunohistochemistry (IHC) in 155 ccRCC patients from two independent cohorts retrospectively. Both SETD2 and H3K36me3 were heterogeneously stained and down-regulated in ccRCC tissues, compared with normal controls. The SETD2 protein deficiency rate was 34.07%, which is much higher than the reported *SETD2* gene inactive mutation rate. Furthermore, low SETD2 protein expression, but not H3K36me3 expression, was associated with the aggressive phenotype of ccRCC patients. In addition, cox multivariate analysis identified low SETD2 protein expression as an independent prognostic factor for overall survival of ccRCC patients. Consistently, using RNA-Seq data of ccRCC patients from The Cancer Genome Atlas, we validated our findings that low SETD2 mRNA expression is significantly associated with the aggressive phenotypes, and predicted a worse outcome for ccRCC patients. In conclusion, our study demonstrated a massive down-regulation of SETD2 protein in ccRCC, and identified SETD2 protein, but not H3K36me3, as an independent good prognostic marker, which warrants further study focusing on the non-methyltransferase role of SETD2 in kidney tumor biology.**

Keywords: SET domain-containing protein 2, clear cell renal cell carcinoma, H3K36me3, prognosis

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1. Introduction

Clear cell renal cell carcinoma (ccRCC) is the most common tumor originating from adult kidney, and is the most malignant subtype of all genitourinary tumors (*1*). Although the early diagnosis of kidney cancer has dramatically improved with the advancement of ultrasound scanning, the prognosis for advanced ccRCC remains poor, and the recurrence and mortality rate of ccRCC have been constantly rising (*2*).

The Von Hippel-Lindau (VHL) gene inactivation by mutation or methylation has been reported in up to 91% of patients with sporadic ccRCC (*3*). Loss of VHL protein, pVHL, leads to abnormal activation of the HIF pathway, which contributes to renal carcinogenesis (*4*). However, VHL gene mutation alone is insufficient to initiate renal cancer, suggesting additional gene mutations are required (*5-7*). Based on well-established VHL gene mutant status, systematic sequencing in recent studies have identified recurrent mutations in a number of genes located near the VHL gene in sporadic ccRCC (*8*), including PBRM1, SETD2, BAP1, KDM6A and JARID1c (*9*). Among which changes SETD2 mutations occur in 3-12% of sporadic ccRCC patients (*5,10*). However, according to our previous studies, we found a much higher SETD2 protein deficiency rate. We believe it is much more likely that the SETD2 mutation rate is underestimated, due to the heterogeneous nature of ccRCC.

SETD2 protein is a histone modifier, which is responsible for trimethylation of lysine-36 of histone H3, H3K36me3 (*11*). H3K36me3 is known to be associated with transcription activation as well as elongation (*9,11*). It is reported that SETD2 mutations have been found to be associated with an advanced clinical stage and poor prognosis in patients with primary ccRCC (*12,13*), which implied that SETD2 is a tumor suppressor. However, the SETD2 protein deficiency rate, its downstream targets and the underlying mechanisms of SETD2 in ccRCC remain unclear.

In this study, we use immunohistochemical staining (IHC) to retrospectively evaluate the association between SETD2, and its main functional product H3K36me3 expression levels and the clinicopathological features in patients with primary or metastatic ccRCC in two independent cohorts. Sequencing data and clinical information from the TCGA ccRCC project were extracted and analyzed to validate our findings.

2. Materials and Methods

2.1. *Patients and samples*

The study involved a total of 155 formalin-fixed, paraffin-embedded tissue samples, including 20 normal renal tissues and 135 ccRCC tissues, which were pathologically diagnosed and underwent radical nephrectomy in Qilu Hospital of Shandong University or Weihai Municipal Hospital from 2005 to 2013. Before sample collection for research purposes, patients' consent and approval from the Ethics Committee of Qilu Hospital and Weihai Municipal Hospital were obtained. For the purpose of survival analysis, more cases with advanced disease were enrolled. Those cases with a family history in first-degree relatives as judged by questioning at the time of admission for surgery were excluded. Patients who received adjuvant therapy, chemotherapy or radiotherapy were excluded from this study. The median age at diagnosis for the 135 subjects was 55 years (23-78 years). Of the 135 cases, 12.6% (n = 17) had lymph node metastasis, 9.6% (n = 13) had renal vein invasion and 10.4% (n = 14) had distant metastasis. Median follow-up time was 76 months (15-129 months). 18 subjects had experienced recurrence by the time of the last follow-up and 9 patients died of ccRCC. Patients' clinicopathological parameters were obtained from the patients' medical records in their hospitals and summarized in Table 1.

2.2. *Immunohistochemistry (IHC)*

For the IHC staining, 5-μm-thick sections, which contained representative histology of the ccRCC were prepared. Tumor adjacent normal renal tissues were used as controls. The sections were deparaffinized, followed by quenching the endogenous peroxidase activity using 3% hydrogen peroxide. Sections were autoclaved in 10 μM citrate buffer (pH 6.0) for 2 min for antigen retrival. 5% albumin was used to block non-specific binding. The primary antibody used in this study was anti-SETD2 antibody (1:150; ab184190, Abcam, Cambridge, MA, USA) and anti-H3K36me3 antibody (1:150; ab9050, Abcam, Cambridge, MA, USA). The sections were incubated in the primary antibody diluted solution at 4°C in a humidified chamber. After washing with PBS, sections were incubated with secondary antibody at 37℃ for 30 min. The immunoreactions were visualized using DAB Horseradish Peroxidase Color Development Kit (P0202, Beyotime, Haimen, China), and then counterstained with hematoxylin.

2.3. *Evaluation of IHC staining*

Immunoreactive activities were evaluated by two investigators, without clinical or pathological information of the slides. SETD2 and H3K36me3 expression were scored as follows: the extension of staining-positive tumor cells (0, 0-10%; 1, 11-30%; 2, 31-60%; 3, > 61%) and the staining intensities (0, no staining; 1, weak staining in nucleus; 2, moderate staining in nucleus; and 3, strong staining in nucleus). The immunoreactive scores were defined as the sum of extension and intensity. Cases with a score of ≥ 5 were defined as high expression, and cases of < 5 were defined as low expression. Moreover, for SETD2 gene status evaluation, it is defined as SETD2 protein deficiency if the extension of SETD2-staining-positive tumor cells is less than 60%, or the tumor cells are weakly stained or not stained.

2.4. *RNA-Seq data set*

The SETD2 expression profiles of ccRCC containing

25 normal renal tissues, 541 primary ccRCC tumors and the corresponding clinical information were acquired from The Cancer Genome Atlas Research Network (TCGA, *http://cancergenome.nih.nov*). Reads were aligned using STAR version 2.4.2 and gene expression was represented as FPKM (Fragments Per Kilobase of transcript per Million mapped reads).

2.5. *Statistical analysis*

Multiple group statistical comparisons were analyzed by one-way ANOVA, followed by post hoc test. Associations between the SETD2 expression levels and the clinicopathological parameters were analyzed with a chi-square test. Survival analysis was performed with Kaplan-Meier curves and log rank test. Multivariate Cox regression model was used to identify the independent prognostic factors. p value less than 0.05 was recognized as statistically significant. All analyses were performed using SPSS 18.0. software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. *The expression of SETD2 in ccRCC tissues*

SETD2 and its functional product H3K36me3 protein expression levels were tested in 135 cases of ccRCC FFPE specimens and 20 tumor adjacent normal renal tissues by an immunohistochemical approach. As shown in Figure 1, SETD2 and H3K36me3 restricted expression in the nucleus of the normal renal tubular cells in a homogeneous pattern, while they were heterogeneously stained in the nucleus of cancer cells. SETD2 was positively stained in all (20/20) normal renal tissues, however, the SETD2 protein deficiency rate in ccRCC tissues was 34.07% (46/135), based on the IHC staining evaluation. H3K36me3, SETD2's canonical protein product, showed a similar expression pattern with SETD2. However, using the same IHC evaluation method, no association between SETD2 and H3K36me3 expression levels was observed (Table 1).

3.2. *The association between SETD2 expression and the clinicopathological variables of ccRCC patients*

SETD2 gene inactive mutation is a major event along with the kidney cancer formation and progression. The association of its protein expression with the clinicopathological parameters of ccRCC patients was evaluated. As shown in Table 1, low SETD2 expression levels significantly correlated with higher primary tumor stage (p = 0.024), distant metastasis (p = 0.0001), higher Fuhrman Nuclear Grade (p = 0.018),

Table 1. Associations between patient characteristics and SETD2&H3K36me3 expression

Variables	Number. of patients	SETD2 expression			H3K36me3 expression		
		Low (n = 72)	High (n = 63)	p value	Low (n = 72)	High (n = 63)	p value
Sex							
Male	105	58	47	0.407	48	57	0.29
Female	30	14	16		17	13	
Age							
≤ 55	62	31	31	0.474	30	32	0.959
> 55	73	41	32		35	38	
p T stage							
T1-2	99	47	52	0.024	44	55	0.153
T3-4	36	25	11		21	15	
Lymph node metastasis							
N0	116	58	58	0.112	57	59	0.286
N1	17	12	5		6	11	
Distant metastasis							
M0	120	58	62	0.001	54	66	0.061
M1	14	13	1		10	4	
Fuhrman nuclear grade							
G1-2	79	36	43	0.018	38	41	0.944
G3-4	41	28	13		20	21	
Clincial stage							
I	66	26	40	0.001	32	34	0.09
II	25	16	9		7	18	
III	23	11	12		12	11	
IV	20	18	2		13	7	
Renal vein invasion							
None	119	60	59	0.069	58	61	0.859
Invasion	13	10	3		6	7	
H3K36me3 expression							
Low	65	40	25	0.066			
High	70	32	38				

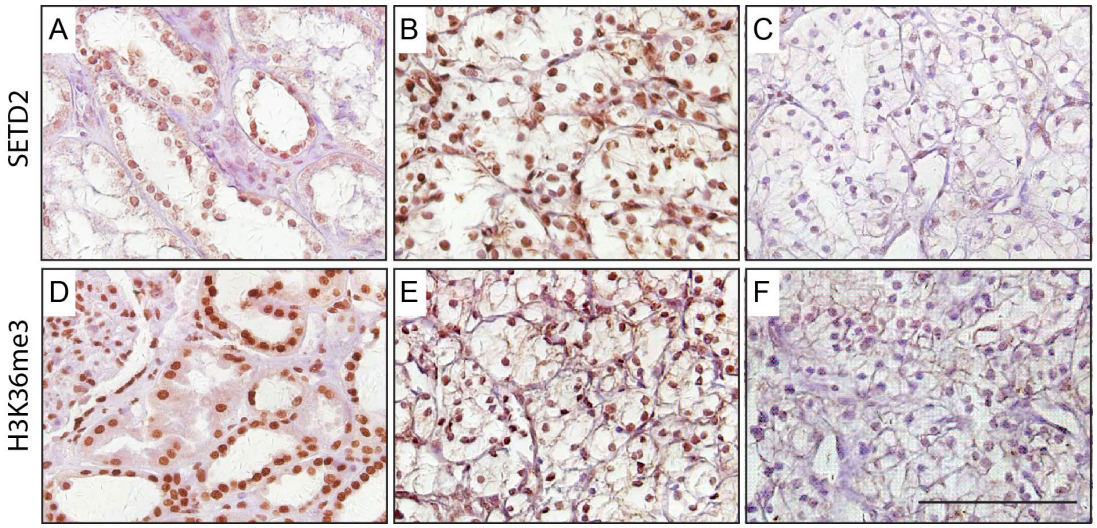

Figure 1. Immunohistochemical staining of SETD2 and H3K36me3 in ccRCC samples. (A,B) High SETD2 expression in ccRCC tumor tissues and adjacent normal renal tissues, which mainly localized in the nucleus. **(C)** Low SETD2 expression in ccRCC tissues. **(D,E)** High H3K36me3 expression in ccRCC tumor tissues and adjacent normal renal tisssues, with a similar expression pattern of SETD2 expression. **(F)** Low H3K36me3 expression in ccRCC tissues. Original magnification 200×; insert bar = 100 μm.

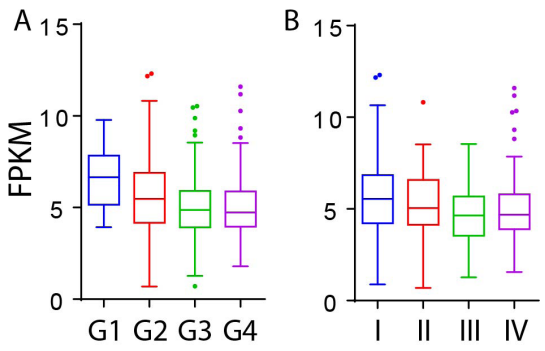

Figure 2. The clinical association of SETD2 mRNA expression in TCGA ccRCC RNA-Seq database (*n* = 541). (A) The low expression of SETD2 mRNA significantly associated with advanced Fuhrman Nuclear Grade ($p = 0.0015$, one-way ANOVA). **(B)** The low expression of SETD2 mRNA predicts advanced clinical stage ($p = 0.0004$, one-way ANOVA).

and advanced clinical stage ($p = 0.001$). There was no significant correlation of the SETD2 expression with gender, age or lymph node metastasis (all $p > 0.05$; Table 1). Of note, though a trend was observed between low SETD2 expression and renal vein invasion ($p = 0.069$), it failed to reach statistical significance, which may be due to the small sample size. On contrast, no significant association between H3K36me3 expression and the clinicopathological variables mentioned above was observed. In parallel, using an alternative approach to evaluate the clinical significance of SETD2 expression, TCGA ccRCC RNA-Seq data and the corresponding clinical information was extracted. Consistently, low SETD2 mRNA expression was significantly associated with high Fuhrman Nuclear Grade ($p = 0.0015$, Figure 2 A) and advanced clinical stage ($p = 0.0004$, Figure 2B).

3.3. *SETD2 expression and the postoperative survival of ccRCC patients*

Kaplan-Meier analysis compared by log rank test was used for survival analysis. As shown in Figure 3 A and B, low SETD2 expression was significantly associated with better overall survival of ccRCC patients ($p < 0.0001$, HR = 0.1143). However, H3K36me3 expression failed to show any correlation with patients' outcome. Consistently, survival analysis using the TCGA ccRCC RNA-Seq data indicates that low SETD2 mRNA expression was significantly correlated with Overall Survival ($p = 0.0044$, HR = 0.6493) and Progression-Free Survival ($p = 0.0030$, HR = 0.5796) of ccRCC patients (Figure 3 C and D). In addition, multivariate analysis by a Cox regression hazard model identified lymph node metastasis ($p = 0.0004$), distant metastasis ($p = 0.0003$), Fuhrman Nuclear Grade ($p = 0.0319$), advanced clinical stage ($p = 0.0277$), and SETD2 protein expression ($p = 0.0004$) as independent prognostic factors (Table 2).

4. Discussion

Recent sequencing efforts identified novel recurrent mutations on chromosome 3p21 in ccRCC, including SETD2, BAP1, PBRM1, and KDM6A, which are responsible for modifying histones and chromatin remodeling (*14*) and play an important role in kidney cancer formation and progression (*5,10,15,16*). Previous studies indicated that SETD2 mutation occurred in 3-12% of sporadic ccRCC patients, which ranked second of the most frequent gene mutations in ccRCC next to VHL gene mutation (*17*). In our present study, IHC staining revealed that SETD2 was

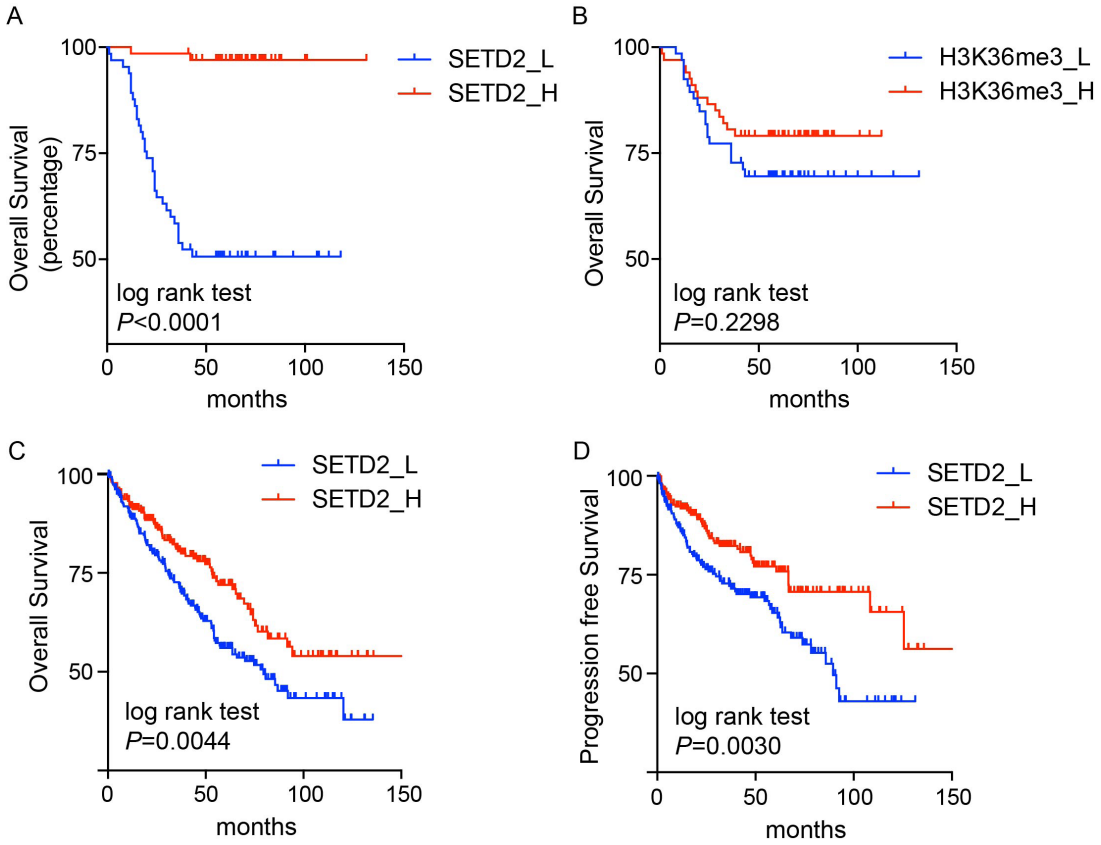

Figure 3. Kaplan-Meier curve analysis for survival based on SETD2 or H3K36me3 expression in Qilu-Weihai cohort (*n* = 135, A and B) or in TCGA ccRCC cohort (*n* = 541, C and D). (A) The high SETD2 protein expression predicts better overall survival ($p < 0.0001$). **(B)** No significant difference was observed between ccRCC patients with high or low H3K36me3 expression in terms of overall survival. The high expression of SETD2 mRNA predicts better Overall Survival ($p = 0.0044$, **C**) and Progression-free Survival ($p = 0.0030$, **D**). All *p* values calculated with log rank test.

Table 2. Multivariate analysis indicates SETD2 protein expression as an independent predictor for ccRCC patients overall survival (*n* = 135)

Items	*p* value	Hazard Ratio	95% confidence interval	
sex	0.2234	2.076	0.641	6.727
age	0.0586	0.402	0.156	1.034
T	0.7632	1.162	0.437	3.095
N	0.0004	8.590	2.601	28.375
M	0.0003	8.747	2.678	28.564
Fuhrman Nuclear Grade	0.0319	2.825	1.094	7.295
Clinical Stage	0.0277	4.821	1.189	19.552
Renal vein invasion	0.4640	1.408	0.564	3.515
SETD2 expression	0.0004	0.045	0.008	0.249
H3K36me3 expression	0.8018	0.902	0.403	2.017

positively expressed in the cell nucleus of all normal renal tubular tissues. However, its expression was lost in part of the cancer tissues. If we define the SETD2 protein deficiency as the SETD2-postitive rate is less than 60% of block tumor cells, or the tumor cells are weakly stained or not stained, then the SETD2 protein deficiency rate was 34.07%, determined by IHC, which was much higher than the *SETD2* gene mutation rates of 3-12%. There may be two reasons. First of all, ccRCC exhibited a heterogeneity nature, which easily could cause sampling bias. To date, most *SETD2* gene mutation status was based on multiple kinds of sequencing, which could only reflect the gene status of very circumscribed cancer lesions and potentially underestimate the mutation rates (*13,18-21*). In comparison, IHC could detect the protein expression of a whole block section, which is more objective. Second, the *SETD2* gene inactive mutation may not be the only reason leading to its protein deficiency, alternative ways exist, such as miR-106b-5p, which could target SETD2 and inactivate its function in ccRCC (*22*). Considering the important role of SETD2 protein in kidney cancer formation, we recommend further study focusing on the mechanism of SETD2 protein deficiency.

Numerous studies revealed that chromatin remodeling plays an important role in cancer development (*5,8*). SETD2 protein is responsible for H3K36me3, which may lead to chromatin accessibility changes (*23,24*) and hinder mismatch repair (MMR) (*25*), and eventually cause RNA processing defects and promote oncogenesis (*26*). Previous studies support SETD2's role as a tumor suppressor in ccRCC (*27*). In our present study, low SETD2 expression was found to be associated with an aggressive phenotype of ccRCC, and was identified as an independent prognostic factor in two independent ccRCC cohorts. Consistently, using the RNA-Seq data from the TCGA ccRCC cohort, we also found a significant association between SETD2 mRNA expression and the advanced clinical features of ccRCC patients.

Considering the close connection of H3K36me3 to SETD2, here we assessed H3K36me3 expression using IHC side by side. Previous study found a correlation coefficient between SETD2 and H3K36me3 in non-metastatic ccRCC specimens (*28*). In contrast, our study only observed a trend of H3K36me3-SETD2 correlation in advanced ccRCC patients, which failed to reach statistical significance. Consistently, a high-resolution profiling of H3K36me3 in metastatic ccRCC found that H3K36me3 is not significantly affected by monoallelic loss of SETD2 (*26*). Subsequently, we evaluated the association of H3K36me3 expression with the clinicopathologic parameters independently, and found no significant associations, except for a trend of low H3K36me3 towards distant metastasis. Consistently, Thai *et al*. from the Mayo Clinic found that H3K36me3-negative tumors had a worse outcome only in a low-risk SSIGN (stage, size, grade and necrosis) group, but not in a high-risk SSIGN group (*29*). In our study, for survival analysis, more cases at an advanced clinical stage were enrolled, which resemble the high-risk SSIGN group in the Mayo cohort. The above findings suggest a less important role for H3K36me3 in the progression of ccRCC at advanced stages. Considering the complex fact that besides SETD2, H3K36 demethylases, such as the JHDM3/JMJD2 family, were also important during the regulation of H3K36me3 levels, there is no need to be fussy about the controversy of the correlation of SETD2 and H3K36me3 expression (*28*). Further studies focusing on both methytransferase and demethylase of H3K36me3 are warranted to figure out the dynamic regulation of H3K36me3 during kidney cancer initiation and progression.

In conclusion, our study demonstrated that SETD2 protein was massively down-regulated in ccRCC, besides a SETD2 inactive mutation, multiple reasons contribute to its protein deficiency. Moreover, out study identified SETD2 protein, but not H3K36me3, as a good independent prognostic marker, and encourage further study to investigate its non-H3K36me3 role in kidney tumor biology.

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