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Decreased Expression of SETD2 Predicts Unfavorable Prognosis in Patients With Nonmetastatic Clear-Cell Renal Cell Carcinoma

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Abstract: DNA sequencing revealed that mutations in SETD2 occur in 3% to 12% of clear-cell renal cell carcinoma (ccRCC) cases and are associated with poor clinical outcome. In this study, we used an immunohistochemistry (IHC) assay to evaluate the impact of SETD2 loss, with expression of H3K36me3, a nonredundantly histone modification by SETD2, on recurrence and survival of nonmetastatic ccRCC patients after nephrectomy.

SETD2 and H3K36me3 were assessed in 192 nonmetastatic ccRCC patients enrolled retrospectively from a single institution. Kaplan–Meier and Cox regression analysis were used to associate prespecified SETD2/H3K36me3 score with overall survival (OS) and recurrence-free survival (RFS). And a nomogram was constructed to predict OS at 10 years.

Patients with low expression of SETD2 were prone to possess large tumor size and advanced pT stage. And low H3K36me3 expression was associated with larger tumor size. A prespecified combined score based on SETD2 and H3K36me3 expression remained an independent prognosticator for OS and RFS, which was associated with tumor size, pT stage, and sarcomatoid. Furthermore, using prespecified SETD2/H3K36me3 score could stratify nonmetastatic ccRCC patients into different risk subgroups, especially in patients dichotomized by pT stage and Fuhrman grade, respectively. Finally, the C-index for predicting OS increased from 0.727 to 0.747, after adding SETD2/H3K36me3 score to pT stage and Fuhrman grade.

The combined score based on expression of SETD2 and H3K36me3 using IHC could predict poor clinical outcomes in nonmetastatic ccRCC patients, and it may benefit preoperative risk stratification and guide treatment planning in the future.

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Abbreviations: ccRCC = clear-cell renal cell carcinoma, CI = confidence interval, CSS = cancer-specific survival, HIFs = hypoxia-inducible factors, HR = hazard ratio, IHC = immunohistochemistry, IRS = immunoreactivity score, OS = overall survival, RCC = renal cell carcinoma, RFS = recurrence-free survival, VHL = von Hippel-Lindau.

INTRODUCTION

The incidental diagnoses of renal cell carcinoma (RCC) have become frequent, as the widespread use of abdominal imaging, and ~60% patients are diagnosed at early stage with a low risk of cancer-specific death; however, ~30% of them would recur after surgery with poor 5-year survival and the mortality rates of RCC have been climbing steadily during the last decades.[1,2] The most common histological subtype (~70%) is clear-cell renal cell carcinoma (ccRCC), which contributes majority of RCC-related deaths.[3,4] Unlike papillary and chromophobe RCC, other 2 histological subtypes of RCC, the inactivation of von Hippel-Lindau (VHL) protein was found in most of ccRCC patients, this leading to deregulate the control of hypoxia-inducible factors (HIFs), then contributing to overexpress numerous hypoxia-regulated genes displaying a pronounced angiogenic phenotype.[5] However, *VHL* deletion in mice was deficient for tumorigenesis, suggesting additional mutations are required.[6] Recently, exome sequencing of ccRCC identified missense and truncating mutations in genes involved in histone modifying, such as *PRBM1*, a subunit of the PBAF SWI/SNF chromatin remodeling complex, *BAP1*, a histone deubiquitinase, and *SETD2*, a histone methyltransferase, suggesting epigenetic reprogramming emerged as central features of ccRCC.[7–10] Further analysis of the contribution of these remodeling genes in predicting disease progression might offer new views on the opportunities for disease management and treatment.

SETD2 belonged to a superfamily of lysine methyltransferase, which was a nonredundantly H3K36 trimethylation methyltransferase.[11] H3K36me3 was generally related with active transcription, although it was also associated with alternative splicing and transcriptional repression.[11] Furthermore, homozygous deletion of *SETD2* in mice resulted in embryonic lethality and vascular defects.[12] *SETD2* was a 2-hit tumor suppressor gene and was located on chromosome 3p, an area frequently deleted in ccRCC.[13] Besides the copy number loss, the *SETD2* mutations were associated with

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high-grade tumors in glioma.[14] Meanwhile, the mutations in *SETD2* occurred in 3% to 12% of sporadic ccRCC tumors, majority of mutations contributing to loss of the protein product or function.[7,8,10] Admittedly, the mechanisms of diallelic *SETD2* inactivation leading to ccRCC were still unclear. Recently, it was found loss of SETD2 promoted with renal cancer branched evolution via replication stress and impaired DNA repair.[15,16] Furthermore, a study about metastatic ccRCC suggested a decline in H3K36me3 was observed in distant metastases, caused by SETD2 copy number loss and mutations.[17] Taken together, these studies indicated that the loss of SETD2 and H3K36me3 might play a key role in pathogenesis and prognosis of ccRCC. Previous study has identified *SETD2* mutations were associated with a worse cancer-specific survival (CSS) in ccRCC patients,[18] however, the prognostic value of alteration of SETD2 expression, accompanied with H3K36me3 change, in nonmetastatic ccRCC is not well established.

Here, we used immunohistochemistry (IHC) assay to retrospectively assess expression of SETD2 and H3K36me3 in nonmetastatic ccRCC specimens. A combined score of SETD2 and H3K36me3 expression was developed, and then correlations with clinic outcomes and prognostic values in Cox regression models were analyzed. Finally, a nomogram based on multivariate analysis was constructed to predict overall survival (OS).

PATIENTS AND METHODS

Clinical Specimens

A total of 192 patients, from 2003 to 2004, who underwent radical or partial nephrectomy for nonmetastatic ccRCC, 25 patients for papillary RCC and 13 patients for chromophobe RCC at Zhongshan Hospital, Fudan University, Shanghai, China, were enrolled in this study. The database of ccRCC patients included baseline clinicopathologic factors and follow-up outcomes. The pT stage was resigned according to the American Joint Committee on Cancer 2010 TNM classification. The primary endpoint was OS with recurrence-free survival (RFS) as a secondary endpoint. OS and RFS were calculated from the day of surgery to the day of death and recurrence, respectively, or to the data of the last follow-up. The patients were excluded if larger necrotic and hemorrhagic areas were observed in samples hampering the obtainment of representative area in samples or receiving preoperative neoadjuvant therapy. Ethical approval was granted by the research medical ethics committee of Fudan University.

Tissue Microarray and Immunohistochemistry

Tissue microarrays were constructed as previously described.[19] Primary anti-SETD2 antibody (1:200; HPA04245, Sigma-Aldrich Corp, St Louis, MO) and anti-H3K36me3 (1:200; ab9050, Abcam, Cambridge, MA) were performed for IHC staining. The negative controls were performed without primary antibodies. Two pathologists blinded to the clinical data assessed the staining of each specimen. To avoid the interobserver variability, the mean value of scores was adapted for further analysis. The SETD2 staining was evaluated by semi-quantitative immunoreactivity score (IRS) system, which ranged from 0 to 30, deriving from the multiplication of intensity of immunohistochemical staining (0, no staining; 1, weak; 2, moderate; and 3, strong) and percentage of positive tumor cells (1 point for each 10% increment; the percentage of positive tumor cells ranged from 1 to 10). The nuclear H3K36me3 staining was evaluated by percentage of positive tumor cells (1 point for each 10% increment) ranged from 1 to 10. Less than medium value was considered as low expression.

Statistical Analysis

Clinicopathologic data were evaluated between patients stratified by SETD2 and H3K36me3 expression, respectively, using *t* tests for continuous variables and Chi-square tests for classified variables. Age and tumor size were modeled as continuous variables. The relationship between SETD2 and H3K36me3 staining was calculated by Chi-square test and Pearson correlation analysis. Meanwhile, the relationship between clinic characteristics and combined score based on SETD2 and H3K36me3 expression was analyzed by Kruskal–Wallis method. In addition, OS and RFS were estimated by Kaplan–Meier method and analyzed by log-rank test. Furthermore, the prespecified SETD2/H3K36me3 combined score was evaluated in multivariable Cox regression analysis adjusting by well-known prognostic variables. Finally, a nomogram for OS based on multivariable analysis was constructed and calibrated as previously described,[20] R software (''rms'' package, R Foundation for Statistical Computing, Vienna, Austria) was performed to construct and calibrate the nomogram. C-index analysis was preformed to compare the predictive accuracy of clinical outcomes by the parameters. Statistical analysis was preformed with SPSS statistics 22. All tests were 2 sided and P values < 0.05 were considered statistically significant.

RESULTS

Association SETD2 and H3K36ME3 Immunohistochemical Expression With Clinical and Pathologic Characteristics

SETD2 and H3K36me3 expression were evaluated by immunohistochemical staining analysis in 192 nonmetastatic ccRCC specimens. As shown in Figure 1A, SETD2 and H3K36me3 showed variable intensities in tumor tissues. The staining of SETD2 distributed in cytoplasm and nucleus, while the staining of H3K36me3 distributed in nucleus (Fig. 1A). Meanwhile, the expressions of SETD2 and H3K36me3 were much lower than the expressions in papillary RCC (n = 25) and chromophobe RCC (n = 13) specimens (Supplementary Figure 1A, http://links.lww.com/MD/A509). According to the IRS criterion, 100 (52.1%) and 106 (55.2%) were grouped as SETD2 and H3K36me3 low-expression, respectively. The clinical characteristics dichotomized by SETD2 and H3K36me3 are listed in Table 1. The specimens with larger tumor size tended to have low expression of SETD2 ($P = 0.005$) and H3K36me3 ($P = 0.014$). Meanwhile, the negative relationship of pT stage was observed with SETD2 expression ($P = 0.040$). We failed to observe the significant correlation with other well-known clinical characteristics in our study (Table 1). Moreover, coefficient correlation between expression of SETD2 and H3K36me3 was observed (Pearson r = 0.460, $P < 0.001$). Thus, we built a combined score based on the expression of SETD2 and H3K36me3 for further analysis, where 74 (38.5%) and 60 (31.3%) were both high expression and both low expression of SETD2 and H3K36me3, respectively.

Prognostic Value of SETD2/H3K36me3 Score for Clinical Outcomes of CCRCC Patients

At last follow-up, a mean duration of OS was 87.8 months (median = 106 months; range 7–120 months) and RFS was 86.6 months (median = 106 months; range 2–120 months). The

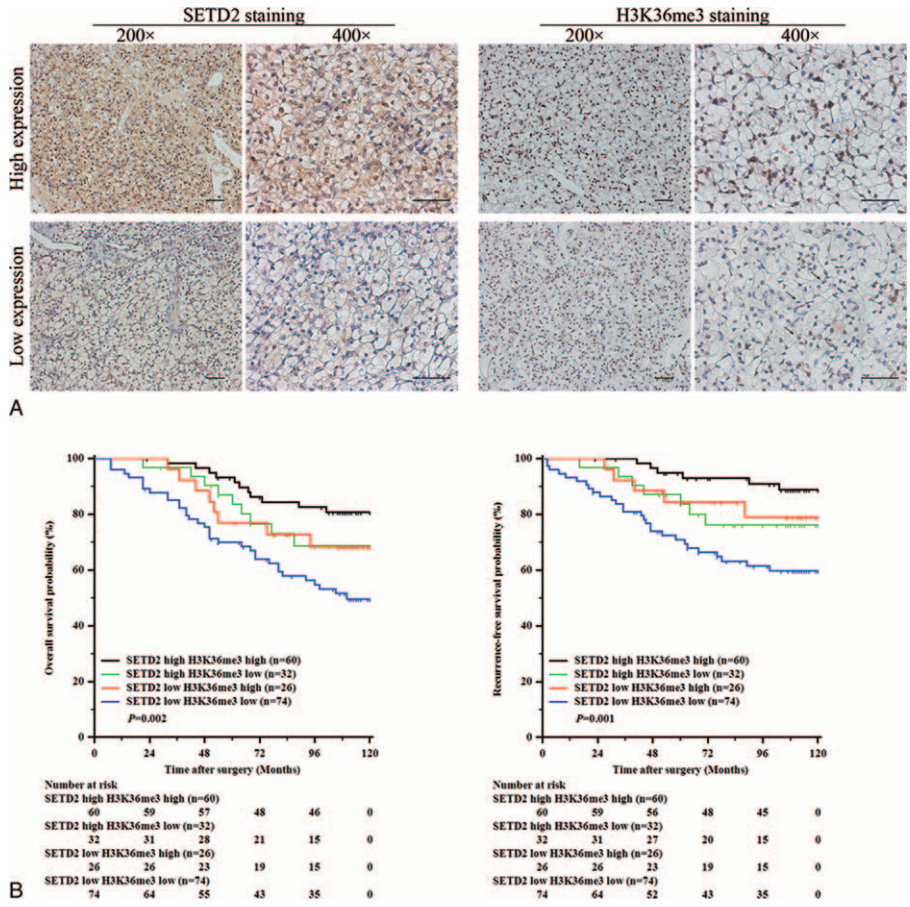

FIGURE 1. SETD2 and H3K36me3 immunohistochemical expression in nonmetastasis ccRCC specimens and estimated clinical outcomes subgrouped by SETD2/H3K36me3 expression. (A) Representative SETD2 and H3K36me3 immunohistochemical (IHC) images of nonmetastasis ccRCC specimens. (200× and 400×). Black arrows showed negative nuclear H3K36me3 staining, and Red arrows showed positive nuclear H3K36me3 stainings. (B) Kaplan–Meier analysis of OS and RFS subgrouped by a combined score based on SETD2 and H3K36me3 expression. Scale bar: 50 μm.

nonmetastatic ccRCC patients could be stratified dichotomized by SETD2 and H3K36me3 expression, respectively, in OS analysis (log rank $P = 0.001$, hazard ratio [HR] = 2.32; 95% confidence interval [CI] = 1.37–3.94; $P = 0.002$ for SETD2 and log rank $P = 0.002$; HR = 2.26; 95% CI = 1.32–3.86; $P = 0.003$ for H3K36me3) and in RFS analysis (log rank $P = 0.002$; HR = 2.71; 95% CI = 1.43–5.12; $P = 0.002$ for SETD2 and log rank $P = 0.001$; HR = 3.07; 95% CI = 1.56–6.03; $P = 0.001$) (Table 2 and Supplementary Figure 1B and C, http://links.lww.com/MD/A509). Furthermore, in 4 subgroups, patients with both low SETD2 and H3K36me3 expression were more likely to have poor survival (HR for both low vs. both high = 3.31; 95% CI = 1.68–6.49, $P = 0.001$) and suffer early recurrence (HR for both low vs. both high = 4.77; 95% CI = 1.98–11.5; $P = 0.001$) (Fig. 1B and Table 2). Considered the patients with SETD2 low H3K36me3 high or SETD2 high H3K36me3 low experienced similar survival and recurrence and the limited specimens in these 2 subgroups, we combined these either-low patients as new subgroup for further analysis (HR for either low vs. both high = 1.80; 95% CI = 0.85–3.84; $P = 0.128$ for OS, HR for either low vs. both high = 2.31; 95% CI = 0.87–6.13; $P = 0.094$ for RFS) (Table 2). Moreover, the prespecified SETD2/H3K36me3 score was related with tumor size ($P = 0.003$), pT stage ($P = 0.043$), and sarcomatoid ($P = 0.004$) (Table 3).

Multivariate Analysis of Prespecified SETD2/H3K36me3 Score With OS and RFS

To assess the robustness value of combination score, multivariate Cox regression analysis was performed to derive risk evaluation correlated of OS and RFS with clinicopathologic characteristics. Along with well-established prognosticators (pT stage, Fuhrman grade, necrosis, ECOG-PS, microvascular invasion (MVI) and sarcomatoid), prespecified SETD2/H3K36me3 score remained an independent prognostic factor for OS (HR for both low vs. both high = 2.40; 95% CI = 1.18–4.86; $P = 0.016$) and RFS (HR for both low vs. both high = 3.31; 95% CI = 1.33–8.25; $P = 0.011$) (Table 2). Meanwhile, in C-index analysis, the value of SETD2 was 0.61 for OS and 0.62 for RFS, the value of H3K36me3 was 0.60 for OS and 0.63 for RFS, and the value of combined score improved to 0.64 for OS and 0.67 for RFS.

Impact of Prespecified SETD2/H3K36me3 on OS and RFS Dichotomized by Pt Stage and Fuhrman Grade

After multivariate analysis, pT stage, Fuhrman grade, and prespecified SETD2/H3K36me3 score remained to be incomplete. Thus, we further analyzed the impact of prespecified

TABLE 1. Associations Between Patient Characteristics and Expression of SETD2 and H3K36me3

	Patients	SETD2 Expression			H3K36me3 Expression		
Characteristics	**Total (%)**	**Low (n = 100)**	**High (n = 92)**	***P*-Value**	**Low (n = 106)**	**High (n = 86)**	***P*-Value**
Age (years)†				0.489			0.816
Mean	55.1	54.5	55.7		54.9	55.3	
Median	54	54	54		54	55	
IQR	47–63	47–62	47.5–64.5		47–65	47–63	
Gender				0.815			0.667
Male	132 (68.7)	70	62		71	61	
Female	60 (31.2)	30	30		35	25	
Tumor size (cm)§				0.005*			0.014*
Mean	4.5	5.0	4.0		4.9	4.0	
Median	4	4	3.5		4	3.5	
IQR	3–5.5	3–6	2.4–5		3–6	2.5–5	
pT stage				0.040*			0.405
pT1a	68 (35.4)	26	42		33	35	
pT1b	54 (28.1)	30	24		28	26	
pT2a	14 (7.3)	7	7		9	5	
pT2b	4 (2.1)	3	1		3	1	
pT3	52 (27.1)	34	18		22	19	
Fuhrman grade				0.487			0.275
1	31 (16.1)	15	16		15	16	
2	84 (43.7)	43	41		50	34	
3	53 (27.6)	26	27		25	28	
4	24 (12.5)	16	8		16	8	
Necrosis				0.586			0.268
Absent	148 (77.1)	75	73		78	70	
Present	44 (22.9)	25	19		28	16	
ECOG-PS				0.856			0.134
0	165 (85.9)	86	79		87	78	
≥1	27 (14.1)	14	13		19	8	
MVI				0.252			0.284
Absent	153 (79.7)	76	77		81	72	
Present	39 (20.3)	24	15		25	14	
Sarcomatoid				0.075			0.122
Absent	178 (92.7)	89	89		95	83	
Present	14 (7.3)	11	3		11	3	
H3K36me3				<0.001*			
Low	106 (55.2)	74	32				
High	86 (44.8)	26	60				

ECOG-PS = Eastern Cooperative Oncology Group performance status, HR = high-risk, IQR = interquartile range, IR = intermediate-risk, LR = low-risk, MVI = microvascular invasion.

† The results were calculated by *t* test.

§ The results were calculated by Mann–Whitney test.

* $P < 0.05$ is considered statistically significant.

SETD2/H3K36me3 score dichotomized by pT stage and Fuhrman grade, respectively. For all patients, the 10-year risk of death of prespecified SETD2/H3K36me3 score of both high, either high and both low were 19.2% versus 31.5% versus 50.4%, and 10-year risk of recurrence were 11.1% versus 22.6% versus 40.3%, respectively. Meanwhile, prespecified SETD2/H3K36me3 score could stratify patients into 3 different risk groups for subgroups dichotomized by pT stage (ratio of death: 15.6% vs. 22.9% vs. 35.9 in early pT stage and 32.7% vs. 65.9% vs. 74.2% in advanced pT stage; ratio of recurrence: 13.0% vs. 24.7% vs. 34.3% in low grade and 26.6% vs. 47.1% vs. 68.9% in high grade) and Fuhrman grade (ratio of death: 9.1% vs. 16.3% vs. 25.6% in early pT stage and 21.4% vs. 48.1% vs. 65.4% in advanced pT stage; ratio of recurrence: 7.1% vs. 14.3% vs. 26.6% in low grade and 15.8% vs. 41.8% vs. 56.9% in high grade), where both low expression of SETD2 and H3K36me3 indicating significant hazard risk of death and recurrence in each subgroups (Fig. 2A and B).

Nomogram for OS Based on pT Stage, Fuhrman Grade, and Prespecified SETD2/H3K36me3 Score

At last, we tried to construct a nomogram to predict OS for 10 years after nephrectomy with the pT stage, Fuhrman grade, and prespecified SETD2/H3K36me3 score based on the results

TABLE 2. Univariate and Multivariate Cox Regression Analysis of Overall Survival and Recurrence-Free Survival of Nonmetastasis ccRCC Patients

	Overall Survival (n = 192, Events = 46)		Recurrence-Free Survival (n = 192, Events = 63)	
Characteristic	**HR (95% CI)**	***P*-Value**	**HR (95% CI)**	***P*-Value**
(a) Univariate Cox regression analysis				
Age (year)†	1.02 (0.99–1.04)	0.112	1.01 (0.99–1.04)	0.364
Gender (male vs. female)	0.85 (0.50–1.42)	0.528	0.72 (0.40–1.32)	0.292
Tumor size (cm)†	1.18 (1.10–1.27)	<0.001*	1.17 (1.08–1.28)	<0.001*
pT stage (III vs. I + II)	3.47 (2.12–5.68)	<0.001*	3.78 (2.12–6.73)	<0.001*
Fuhrman grade (3 + 4 vs. 1 + 2)	2.53 (1.54–4.17)	<0.001*	2.99 (1.64–5.42)	<0.001*
Necrosis (present vs. absent)	1.87 (1.11–3.16)	0.019*	2.16 (1.19–3.93)	0.012*
ECOG-PS (≥1 vs. 0)	2.24 (1.27–3.93)	0.006*	2.87 (1.54–5.37)	0.001*
MVI (present vs. absent)	2.13 (1.25–3.61)	0.005*	2.17 (1.18–4.02)	0.014*
Sarcomatoid (present vs. absent)	4.91 (2.61–9.22)	<0.001*	5.42 (2.69–10.9)	<0.001*
SETD2 (low vs. high)	2.32 (1.37–3.94)	0.002*	2.71 (1.43–5.12)	0.002*
H3K36me3 (low vs. high)	2.26 (1.32–3.86)	0.003*	3.07 (1.56–6.03)	0.001*
Combination of SETD2 and H3K36me3				
SETD2 high H3K36me3 low vs. both high	1.76 (0.73–4.24)	0.207	2.49 (0.84–7.38)	0.101
SETD2 low H3K36me3 high vs. both high	1.85 (0.75–4.58)	0.185	2.10 (064–6.83)	0.222
Both low vs. both high	3.31 (1.68–6.49)	0.001*	4.77 (1.98–11.5)	0.001*
Combination of SETD2 and H3K36me3				
Either low vs. both high	1.80 (0.85–3.84)	0.128	2.31 (0.87–6.13)	0.094
Both low vs. both high	3.31 (1.68–6.49)	0.001*	4.77 (1.98–11.5)	0.001*
(b) Multivariate Cox regression analysis				
pT stage (III vs. I + II)	2.67 (1.57–4.53)	<0.001*	2.62 (1.41–4.88)	0.003*
Fuhrman grade (3 + 4 vs. 1 + 2)	2.15 (1.27–3.65)	0.005*	2.42 (1.28–4.56)	0.007*
Necrosis (present vs. absent)	1.01 (0.56–1.84)	0.963	1.12 (0.55–2.25)	0.757
ECOG-PS (≥1 vs. 0)	1.38 (0.75–2.52)	0.299	1.74 (0.89–3.38)	0.107
MVI (present vs. absent)	1.30 (0.69–2.43)	0.416	1.16 (0.55–2.44)	0.706
Sarcomatoid (present vs. absent)	2.19 (0.95–5.08)	0.069	2.21 (0.83–5.90)	0.115
Combination of SETD2 and H3K36me3				
Either low vs. both high	2.12 (0.98–4.61)	0.059	2.67 (0.98–7.27)	0.056
Both low vs. both high	2.40 (1.18–4.86)	0.016*	3.31 (1.33–8.25)	0.011*

95% CI = 95% confidence interval, ECOG-PS = Eastern Cooperative Oncology Group performance status, HR = hazard ratio, MVI = microvas-microvascular invasion.

† The results are modeled as continuous variables.

* $P < 0.05$ is considered statistically significant.

raising from multivariate analysis of OS (Fig. 3A). The calibration plots of the nomogram are shown for 10-year predictions (Fig. 3B). The C-index of pT stage and Fuhrman grade were 0.672 and 0.653 and improved to 0.715 and 0.707, respectively, after adding SETD2/H3K36me3 score. The C-index of pT stage and Fuhrman grade was 0.727 and improved to 0.747 when SETD2/H3K36me3 score was added.

DISCUSSION

As function in chromatin modulating, which is essential for gene transcriptional regulation, and location in close proximity at VHL resides, PBRM1, BAP1, and SETD2 were paid more attention on addressing the clinical and pathologic significance.[21] Considered the tight connection between SETD2 and H3K36me3, here, we constructed a combined score based on expression of SETD2 and H3K36m3 and tried to divide nonmetastatic ccRCC patients into different risk subgroups. Here, our prespecified SETD2/H3K36me3 score could stratify the nonmetastatic ccRCC patients into different risks, where patients with both low expression of SETD2 and H3K36me3 had worse OS and earlier recurrence. Meanwhile, it is an independent marker of prognosis in nonmetastatic ccRCC.

Besides the loss of copy number, the majority of mutations in *SETD2*, unlike *BAP1*, is nonsense or frameshift truncating and invariably causes loss of the protein product or function.[18] Meanwhile, assessment of SETD2 expression could identify tumors with lack of *SETD2* mutation but with declined expression of SETD2, due to epigenetic silencing, such as miRNAs.[22,23] And considered the high expense of sequencing and relatively small sample sizes that were deficiently powered to analysis in some unique subgroups,[24] assessment the expression of SETD2 using IHC assay might be an affordable, reproducibly and straightforward tool for widespread use.

Recently, an H3K36me3 expression study in ccRCC found SETD2 copy number loss alone failed to have a significantly proportional effect on change of H3K36me3, suggesting monoallelic loss may be deficient to alter SETD2 methyltransferase activity and subtle alteration of SETD2 expression may have a cooperative biological effect in tumors through loss of other

TABLE 3. Associations Between Patient Characteristics and SETD2/H3K36me3 Score

	SETD2/H3K36me3 Score			
Characteristics	**Both Low (n = 74)**	**Either Low (n = 58)**	**Both High (n = 60)**	***P*-Value**
Age (years)†				0.681
Mean	54.2	56.1	55.2	
Median	53.5	55	55	
IQR	47–62	49–67	46.5–63	
Gender				0.249
Male	53	35	44	
Female	21	23	16	
Tumor size (cm)†				0.003*
Mean	5.4	3.8	4.04	
Median	4.3	3.9	3.4	
IQR	3–7	2.5–5	2.4–5.3	
pT stage				0.043*
pT1	37	43	42	
pT2	9	4	5	
pT3	28	11	13	
Fuhrman grade				0.088
1	10	10	11	
2	31	31	22	
3	18	15	20	
4	15	2	7	
Necrosis				0.489
Absent	54	45	49	
Present	20	13	11	
ECOG-PS				0.315
0	63	47	55	
≥1	11	11	5	
MVI				0.297
Absent	55	47	51	
Present	19	11	9	
Sarcomatoid				0.004*
Absent	63	58	57	
Present	11	0	3	

ECOG-PS = Eastern Cooperative Oncology Group performance status, IQR = interquartile range, MVI = microvascular invasion.
† The results were calculated by Kruskal–Wallis test.
* $P < 0.05$ is considered statistically significant.

chromosome 3p tumor suppressors.[15,17] Meanwhile, besides SETD2, H3K36 demethylases (JHDM3/JMJD2 family) were also involved in the orchestration of H3K36 methylation, which were important to retain the H3K36me3 level.[25] Thus, assessment of SETD2 expression alone may be unilateral, and analysis of prognostic value of patients with expression of SETD2 and H3K36me3 might provide more precise decentralized management of ccRCC patients. In our prespecified score, ~30% patients had an asymmetric expression and these patients had an intermediate risk level of death and recurrence (Table 2). However, as the limited specimens in SETD2 high H3K36me3 low and SETD2 low H3K36me3 high subgroups, we combined them as a new subgroup, which might underestimate the prognostic value of these 2 subgroups, thus, further studies are warranted to value the HR of these subgroups, respectively, to assess the different prognostic value between them.

Disruption of chromatin biology has become an emerging issue for a new pathobiology underlying oncogenesis, however, the alteration of these chromatin modulating genes contributing to the pathogenesis of ccRCC were less known.[26] SETD2-dependent H3K36me3 was commonly associated with active transcription, more importantly, was required for homologous recombination repair, DNA mismatch repair and genome stability, and cells lacking SETD2 displayed microsatellite instability and an elevated spontaneous mutation frequency.[16,27] In human acute leukemia, loss of SETD2, company with global loss of H3K36me3, contributed to both initiation and progression in conjunction with chromosomal translocations.[28,29] Thus, disruption of SETD2/H3K36me3 might accelerate the progress of ccRCC initiation and development via elevating mutation frequency, and studies on them would likely lead to development of precise prognostic and predictive system and novel therapeutic interventions for ccRCC patients.

There were some limitations of our study that warrant further discussion. Firstly, given the heterogeneous nature of ccRCC and the population of our study, our conclusion might be overestimated and noncomprehensive based on single region of tumor tissues and small population. Thus, further validated in external heterogeneous cohorts were warranted. Secondly, although IHC was a cost-effective and straightforward tool,

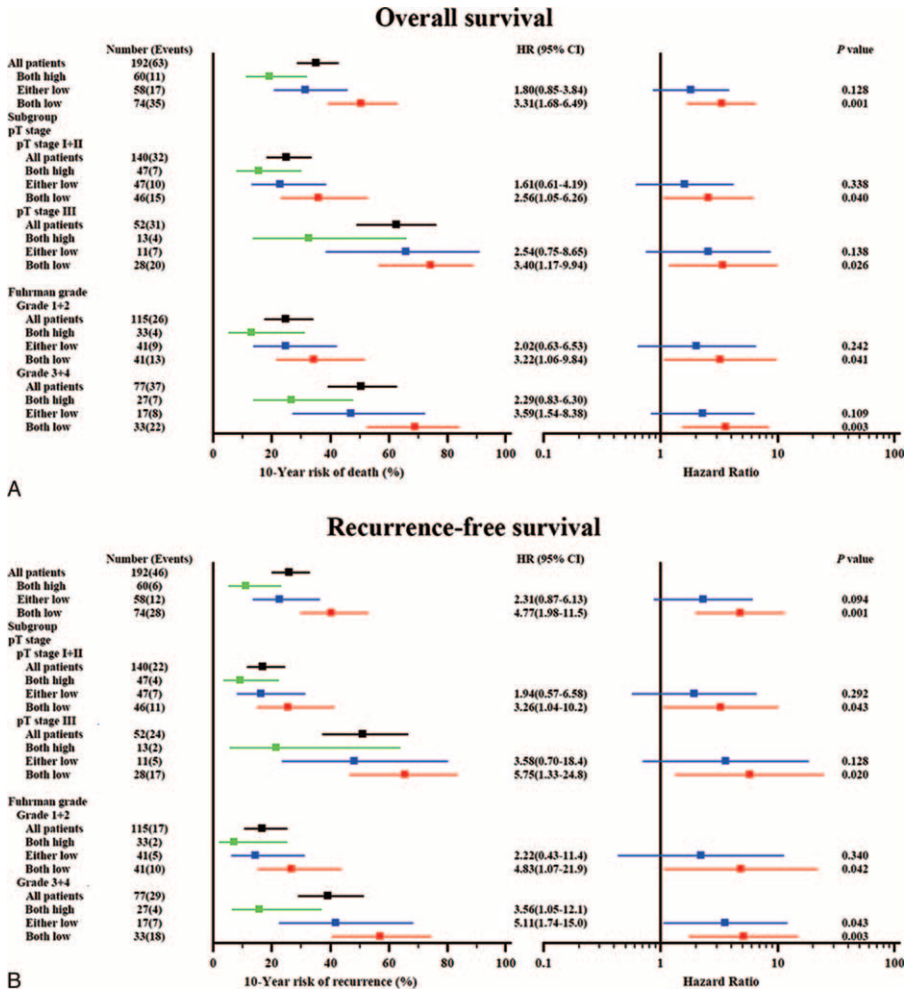

FIGURE 2. Association of prespecified SETD2/H3K36me3 score with OS and RFS in patients dichotomized by pT stages and Fuhrman grades. (A) Association of prespecified SETD2/H3K36me3 score with OS dichotomized by pT stages and Fuhrman grades. (B) Association of prespecified SETD2/H3K36me3 score with RFS dichotomized by pT stages and Fuhrman grades. The black lines represented the total patients; the green lines represented patients with both high expression of SETD2 and H3K36me3; the blue lines represented patients with either high expression of SETD2 and H3K36me3; the red lines represented patients with both low expression of SETD2 and H3K36me3.

the results of IHC could be inconsistent due to variability in observers. We used mean score based on IHC scores from 2 pathologists to maximize result reliability. Further validations were necessary to confirm the prognostic value of SETD2/H3K36me3 using IHC in ccRCC patients. Thirdly, in our study, we focused on nonmetastatic patients; however, considered the significant relationship with declined expression of H3K36me3 and metastasis status, more study is necessary to determine the

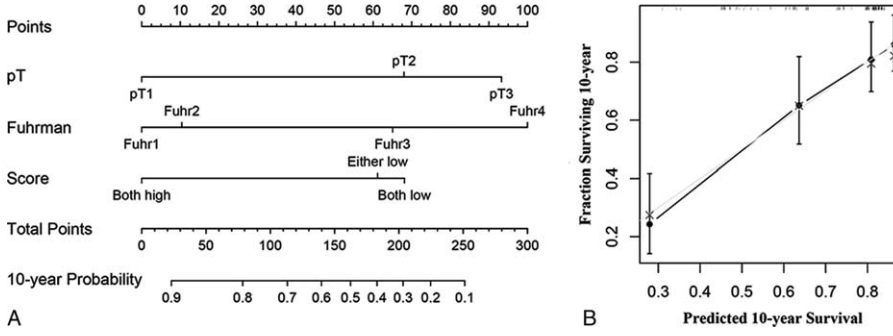

FIGURE 3. Nomogram and calibration plots for the prediction of OS in patients with nonmetastatic ccRCC. (A) Nomogram for prediction OS at 10 years after nephrectomy. (B) Calibration plots for predicting OS at 10 years after nephrectomy. Gray line means ideal nomogram; circles means apparent predictive accuracy; blue X means bootstrap-corrected estimates; vertical bars means 95% CIs.

prevalence of SETD2 mutations in metastatic lesions and potential prognostic value for metastasis ccRCC patients. Finally, though we focused on the correlation between SETD2 and H3K36me3, other factor such as H3K36 demethylases were not fully assessed in our present study. Further studies were warranted to evaluate the function and prognostic value of these demethylases in the progression of ccRCC.

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