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Original Article

SET7 interacts with HDAC6 and suppresses the development of colon cancer through inactivation of HDAC6

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Abstract: SET7 is the first lysine methyltransferase and plays vital roles in tumorigenesis. This study aims to seek clinical value of SET7 in colorectal cancer (CRC) patients, along with its biological impact on cell proliferation and migration. In patients with CRC, the expression of SET7 in cancer tissue was significantly lower than that in adjacent tissue, and down-regulated SET7 was closely correlated with poor prognosis. Loss-of-function and gain-of-function studies indicated that SET7 inhibited cell proliferation and migration by acting on HDAC6 substrate in colon cancer cells. Besides, the co-immunoprecipitation assay showed that SET7 and HDAC6 can interact reciprocally. The interaction effect between SET7 and HDAC6 could significantly reduce cell viability, scratch healing rate, and migrated cells in colon cancer cells. Instead of acting on each endogenous expression, the results demonstrated that the level of acetylated α-tubulin was greatly decreased in HDAC6 overexpression group, while significantly increased in SET7 overexpressed group. However, changes were partly restored in both SET7 and HDAC6-transfected group. On the contrary, the expression of acetylated α-tubulin protein was significantly increased in HDAC6 knockdown group, but higher in both HDAC6 and SET7 silencing group. These results indicated that SET7 played a role in tumor suppression via increasing levels of acetylated-α-tubulin mediated by HDAC6. In addition, the interaction effect significantly decreased the ratios of p-ERK/ERK, which indicated that it may partly suppress ERK signaling pathway. In conclusion, SET7 is a promising therapeutic target for preventing metastasis and improving prognosis in colon cancer.

Keywords: Colon cancer, SET7/9, histone deacetylase 6, α-tubulin, deacetylation, acetylation

Introduction

Colorectal cancer (CRC) rank the second in the most common cancers in female and the third in male. In 2018, the global incidence of CRC was about 1.85 million accompanied with 880,792 deaths worldwide [1]. Although the treatment of CRC has made great progress in the past 10 years, there are still a significant number of patients suffered local recurrence or distant metastasis [2]. Thus, it is of great significance to prevent metastasis and explore new therapeutic strategies to treat colorectal cancer.

Lysine methylation modification plays a vital role in regulating histone function and affects the function of gene activation or inhibition by methylation of specific lysine residues [3, 4]. Researches showed that lysine methyltransferases contain a highly conserved SET domain, which has been discovered in the Drosophila proteins Su (var) 3-9, Enhancer-of-zeste and Trithorax [5, 6]. SET7 (also known as SET7/9 and SETD7), a histone methyltransferase, contains two SET domains with two different lysine methylation functions [7]. The effects of SET7 in cancer development are controversial. Previous studies have shown that SET7 promoted tumor growth in non-small cell lung cancer [8] and breast cancer [9], but had tumor suppressor effects in gastric cancer [7] and acute myeloid leukemia [10]. Recently, the latest research showed that resveratrol regulated the effect of p53 by influencing SET7 expression in colorectal cancer [11]. However, there is no related reports on SET7 alterations in colon cancer, and the specific mechanism in colon cancer remains unclear.

Histone acetylation and deacetylation are modified by histone acetyltransferases and deacetylation enzyme, which play critical roles in gene expression and transcription. HDAC6 belongs to the histone deacetylases (HDACs) family, which contains 18 members that are divided into four categories based on their homology to yeast deacetylases in mammals [12]. In addition to maintaining the deacetylation balance of histones, HDAC6 also acted on some non-histone substrates, such as α-tubulin, cortactin, and HSP90 [13-15]. α-tubulin was the first identified non-histone substrate of HDAC6 and its reversible deacetylation effect on α-tubulin can affect the function and stability of microtubules, which are closely related to cell motility [15, 16].

In the present study, we found that the expression of SET7 decreased in most colon cancer tissues, and was closely related to poor prognosis in CRC patients. Results of loss-of-function and gain-of-function studies indicated that SET7 played a role in tumor suppression by suppressing deacetylating activity of HDAC6 partly through ERK signaling pathway in colon cancer cells.

Materials and methods

Immunohistochemistry

Paraffin sections of 54 CRC tissues and adjacent tissues (interval between October 2011 and June 2013) were collected from the department of pathology of the Second Xiangya Hospital. The study was approved by the Medical Ethics Association of the Second Xiangya Hospital and all patients signed informed consent forms. Details of the immunohistochemical (IHC) score were reported previously [17].

Cell culture and transfection

HCT116 and SW480 cells obtained from American Type Culture Collection (ATCC, Manassas, VA) were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS, GIBCO, USA) and penicillin/streptomycin (Invitrogen, USA) at 37°C in a humidified 5% CO_2 atmosphere.

HCT116 and SW480 cells (2×10^5/well) were plated in 6-well culture dishes treated with 1 μg of HDAC6, SET7 or $pcDNA_2$ plasmid using Lipofectamine reagent respectively (Invitrogen, Carlsbad, CA). Cells were harvested 48 hours later for western blot analysis, only low-passage cells (P<10) were used for experiments [18].

Reagents and antibodies

Anti-HDAC6 (7558), Anti-SET7 (2825), anti-phospho-ERK (3192), anti-ERK (4695), anti-α-Tubulin (2125), anti-Ac-α-Tubulin (5335), anti-HA-Tag (2367) and anti-DYKDDDDK Tag (8146) antibodies were purchased from Cell Signaling Technology. Anti-SET7 (NB100-56664SS), Anti-HA agarose beads (A-2095) and anti-Flag agarose beads (A-2220) were purchased from Sigma.

Cell proliferation assay

Cell proliferation was measured using Cell Counting Kit-8 assay (Bimake, USA). Cells (3×10^3/well) treated with plasmid/siRNAs respectively in Opti-MEM for 48 h, then the CCK-8 reagent was added (20 μl/well) and incubated for 2 h at 37°C. The value of the absorbance was measured at a wavelength of 450 nm using a microplate reader, and the experiment was repeated thrice.

Wound-healing and transwell assays

When HCT-116 and SW480 grew to fused, the wound was created by scratching the cell monolayer with a pipette tip. Cells treated with plasmid/siRNAs respectively in Opti-MEM and migrated for 48 hours at 37°C. In the cell migration experiment, 5×10^4 HCT-116 cell treated with relevant plasmid was seeded in the upper chamber containing Opti-mem, and DMEM medium supplemented with 10% FBS placed in the low chamber. After 48 hours of incubation, non-migrating cells in the upper chamber were wiped with a cotton swab, and the remaining cells were stained with 0.5% crystal violet. After 15 minutes, the crystal violet was washed with double-distilled H_2O. Randomly selected eight fields under the microscope for positive cell counting, and averaged the total number of cells.

Immunoprecipitation and western blot assay

HCT116 and SW480 cells were transfected with SET7, HDAC6 or $pcDNA_2$ plasmid respectively, preparation of whole cell lysates and immunoblotting were performed as described-

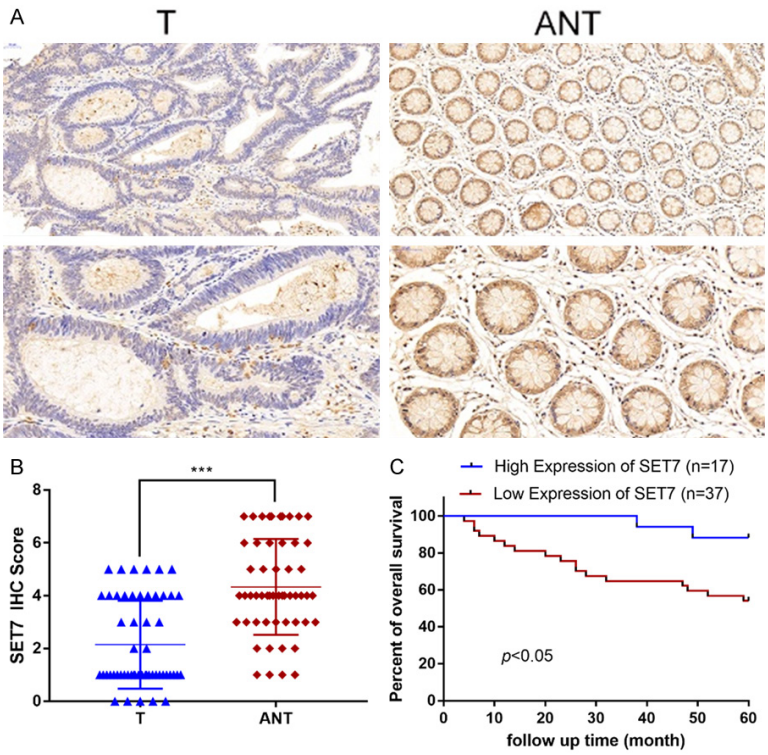


Figure 1. SET7 was down-regulated and correlated with poor prognosis in CRC patients. A. Representative immunohistochemistry photographs (upper: 200× and lower: 400×) of SET7 expression in CRC patients. The expression of SET7 was significantly lower in tumor tissue (T) than adjacent normal tissue (ANT). B. The semi-quantitative immunohistochemistry results indicated that expression of SET7 in tumor tissue was notably lower than that in adjacent normal tissue (2.15 vs 4.33, P<0.0001). C. Survival curve analysis by SET7 status (n = 54). The vertical axis represents the percentage survival rate; the horizontal axis represents the survival days. The red line showed that patients with low SET7 expression had a shorter overall survival time (median = 43 months) compared to the blue line (median = 58 months, *P*<0.05). The data shown represent mean ± SD from a representative experiment. **P*<0.05.

previously [17]. 1 mg total lysates were incubated with the appropriate antibody-conjugated beads (2 μg) for 4 hours at cold room. Immunocomplexes were washed four times with NETN buffer and were eluted by boiling for 5 minutes in SDS loading buffer. Bound proteins were separated by SDS-PAGE and immunoblotted with indicated antibodies [18].

Small interfering RNA assay

When HCT116 cell was at 60% confluence, HDAC6 and SET7 siRNA (10 uM, GenePharma, Shanghai, China) were transfected with LipofectamineRNAi-MAX (ThermoFisher Scientific) according to the instructions. After transfected with HDAC6-siRNA for 24 h, the cell was treated with SET7-siRNA for another 24 h and then proceeded to protein isolation. The validated siRNAs sequences are as following: Human HDAC6 siRNAs: CATCCCATCCTGAATATCCTTCTC; CCTCACTGATCAGGCCATATTC; Human SET7 siRNAs: GCCAGGGAGTTTACACTTA; CCTGGACGATGACGGATTA.

Statistical analysis

Statistical analysis was performed using SPSS 23.0 software (Chicago, IL, USA), and data were expressed as mean ± standard deviation. T-tests was carried out between two groups, while one-way analysis of variance was conducted among multiple groups. Differences were considered significant if **P*<0.05, ***P*<0.01, ****P*<0.0001, respectively.

Results

SET7 was down-regulated and correlated with poor prognosis in CRC patients

The expression of SET7 collected from 54 CRC patients were qualified by immunohistochemistry. As illustrated in **Figure 1A**, SET7 was expressed mainly in nucleus. The semi-quantitative results of immunohistochemistry showed that level of SET7 in cancer tissues was significantly lower than that in adjacent tissues (2.15 vs 4.33, P<0.0001) (**Figure 1B**). Meanwhile, the strong expression of SET7 was more common in noncancerous tissue (68.5%, 37/54) than cancer tissue (33.3%, 18/54) in CRC patients. Interestingly, we observed a significantly correlation between level of SET7 and tumor site, but not with other parameters such as age, gender, tumor size, lymph node invasion, differentiation grade, TMN clinical stage (**Table 1**). According to univariate analysis tumor size, depth of tumor invasion, lymph node invasion, distant metastasis, TMN clinical stage and high

Table 1. Relevance between SET7 expression and clinicopathologic features of CRC patients

clinicopathologic features	No. of patients	SET7 expression		*P*-value
		Low (n)	High (n)	
Gender				0.700
Male	28	18	10	
Female	26	18	8	
Age				0.776
<60	27	17	10	
≥60	27	18	9	
Tumor size				0.561
<5 cm	24	15	9	
≥5 cm	30	21	9	
Tumor site				0.048
Colon Carcinoma	44	32	12	
Rectum Carcinoma	10	4	6	
Depth of tumor invasion				1.000
T1-T2	15	10	5	
T3-T4	39	26	13	
Lymph node invasion				0.690
Absent	34	22	12	
Present	20	14	6	
Distant metastasis				0.142
Absent	50	32	18	
Present	4	4	0	
TMN clinical stages				0.370
I	11	9	2	
II	23	15	8	
III	16	10	6	
IV	4	4	0	
Differentiation grade				0.311
Well	3	3	0	
moderately	35	24	11	
Poorly	16	9	7	

SET7 expression were all significantly associated with patients' survival (**Table 2**). Besides, survival curve results showed that patients with low SET7 expression had a shorter survival time (median = 43 months) compared to those with high SET7 expression (median = 58 months, $P<0.05$, **Figure 1C**).

SET7 inhibited cell proliferation and migration in colon cancer cells treated with HDAC6

Cell proliferation and migration are essential for cancer metastasis [19]. Researches have demonstrated that HDAC6 promoted proliferation and migration in colon cancer [17, 20]. However, the role of SET7 in colon cancer remains unknown and their interaction effect in colon cancer metastasis are undiscovered. Results showed that over-expression of HDAC6 increased cell proliferation and migration, which consistent with previous studies [17]. Over-expression of SET7 significantly reduced cell proliferation and migration, while up-regulated SET7 in HDAC6-overexpression cells can reverse the promotion effect induced by HDAC6 and inhibit cell proliferation and migration as well in colon cancer cells (**Figure 2A-C**).

Contrary to the over-expression results, silencing HDAC6 suppressed cell proliferation and migration, whereas reducing expression of SET7 prominently promoted cell proliferation and migration and these effects were still existed in both SET7 and HDAC6 silencing group (**Figure 2D**, **2E**). These gain-of-function and loss-of-function studies above suggested that SET7 played an inhibiting effect on the development of colon cancer by acting on HDAC6.

Interaction between SET7 and HDAC6

SET7 or HDAC6 over-expression and knockdown effect were confirmed by western blotting in colon cancer cell (**Figure 3A**, **3B**). Since HDAC6 played an oncogenic role in colon cancer and down-regulated SET7 correlated with poor prognosis in CRC, we further explored the interaction effect between SET7 and HDAC6 by performing a co-immunoprecipitation assay. As the results showed that SET7 and HDAC6 interacted reciprocally and made no effects on each endogenous expression (**Figures 3C**, **3D** and S1).

SET7 suppressed deacetylating activity of HDAC6 and increased levels of acetylated-α-tubulin partly through ERK signaling pathway

We have demonstrated that interaction between SET7 with HDAC6 made no effects on endogenous HDAC6 expression. Considering that α-tubulin is the major substrate of HDAC6, and its deacetylation effect is important for the

Table 2. Univariable analysis of overall survival (OS) and clinicopathologic variables in CRC patients

Clinicopathologic features	No. of patients	overall survival		*P*-value
		Death (n)	Alive (n)	
Gender				0.627
Male	28	9	19	
Female	26	10	16	
Age				0.393
<60	27	11	16	
≥60	27	8	19	
Tumor size				0.011
<5 cm	24	4	20	
≥5 cm	30	15	15	
Tumor site				0.724
Colon Carcinoma	44	15	29	
Rectum Carcinoma	10	4	6	
Depth of tumor invasion				0.006
T1-T2	15	1	14	
T3-T4	39	18	21	
Lymph node invasion				0.019
Absent	34	8	26	
Present	20	11	9	
Distant metastasis				0.005
Absent	50	15	35	
Present	4	4	0	
TMN clinical stages				<0.001
I	11	1	10	
II	23	20	3	
III	16	7	9	
IV	4	4	0	
Differentiation grade				0.692
Well	3	1	2	
moderately	35	11	24	
Poorly	16	7	9	
SET7 expression				0.015
Low	37	17	20	
High	17	2	15	

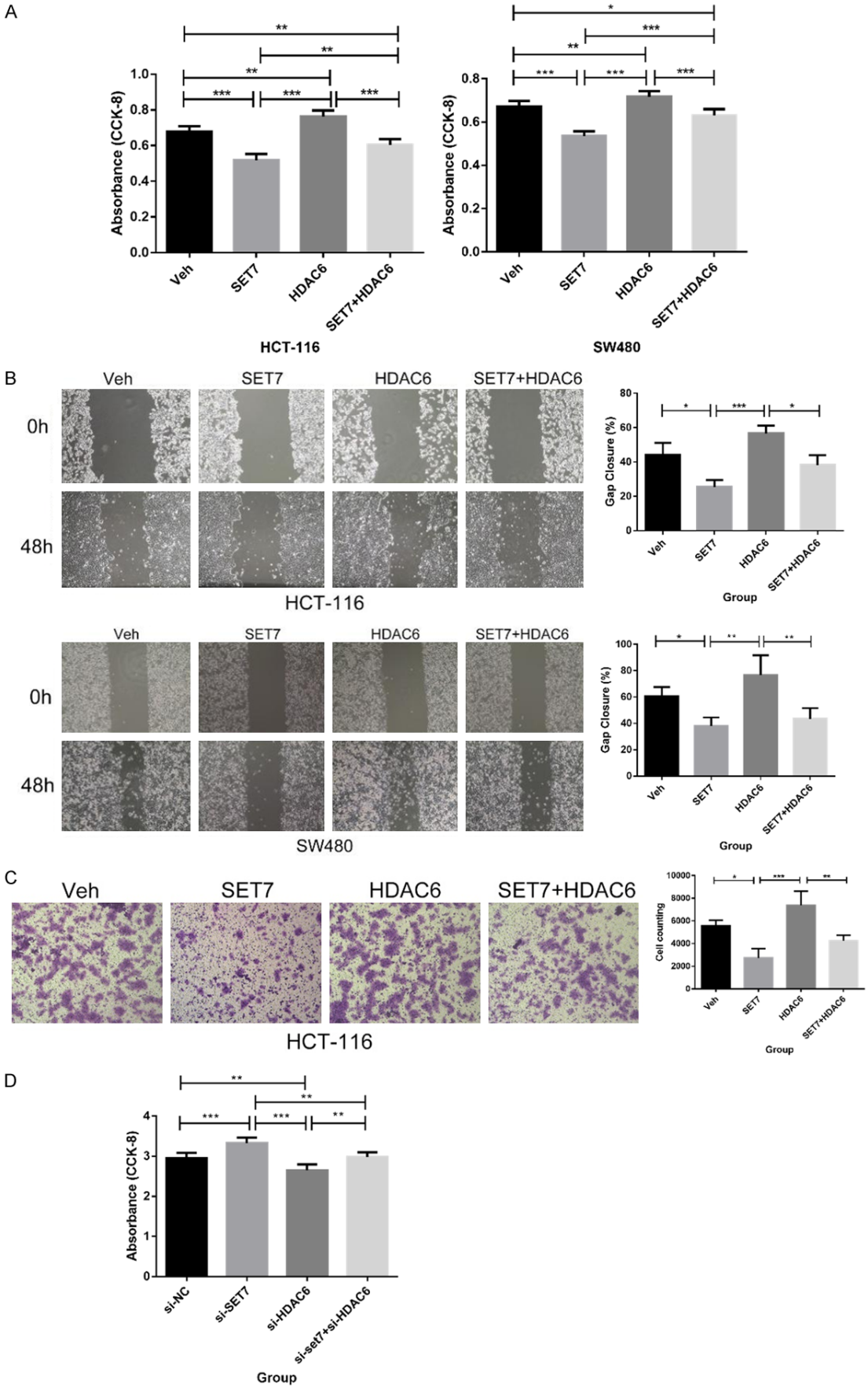
movement of tumor cells. So, we further explore whether SET7 could affect the deacetylation of α-tubulin mediated by HDAC6. The results demonstrated that the level of acetylated α-tubulin was greatly decreased in HDAC6 over-expression group, while significantly increased in SET7 over-expressed group. However, changes were partly restored in both SET7 and HDAC6-transfected group (**Figure 4A**). On the contrary, the expression of acetylated α-tubulin protein was significantly increased in HDAC6 knockdown group, but decreased in the SET7 silencing group and totally restored in both HDAC6 and SET7 silencing group (**Figure 4B**). These results above indicated that SET7 may play an inhibitory effect on the activity of HDAC6.

It has been demonstrated that HDAC6 exerted its function through regulation of the MAPK/ERK signaling pathway in colon cancer [17]. Consistent with our previous results, over-expression of HDAC6 indeed increased the level of p-ERK, but up-regulation of SET7 in HDAC6-overexpression cells inversely decreased p-ERK expression (**Figure 4C**, **4D**). The results suggested that SET7 may suppress deacetylating activity of HDAC6 partly through the ERK signaling pathway in colon cancer cells.

Discussion

In this study, we observed SET7 reduction and was significantly associated with shorter overall survival in CRC patients. Results on SET7 and its interacting protein HDAC6 demonstrated that SET7 inhibited cell proliferation and migration via acting on HDAC6 substrate. Furthermore, SET7 increased levels of acetylated-α-tubulin by suppressing deacetylation activity of HDAC6, and this effect was partly through inactivating ERK signaling pathway, which indicated that SET7 has a tumor suppressor function in colon cancer.

Research has indicated that HDAC6, as a tubulin deacetylase, could decrease the expression of acetylated α-tubulin by deacetylating α-tubulin and regulating microtubule-dependent cell motility [21]. Previous studies have reported that the expression of Ac-α-tubulin were increased in head and neck squamous cell carcinoma [22], breast cancer [23], pancreatic cancer [24], but decreased in cylindromatosis [25], and multiple myeloma [26]. In addition, it has been proved that HDAC6 could decrease microtubules stability by deacetylating α-tubulin in TGF-β-induced epithelial-to-mesenchymal transition in epithelial cells [27]. In our current study, we show-


A
Absorbance (CCK-8)
Veh
SET7
HDAC6
SET7+HDAC6
HCT-116
SW480
B
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Gap Closure (%)
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D
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si-SET7
si-HDAC6
si-set7+si-HDAC6

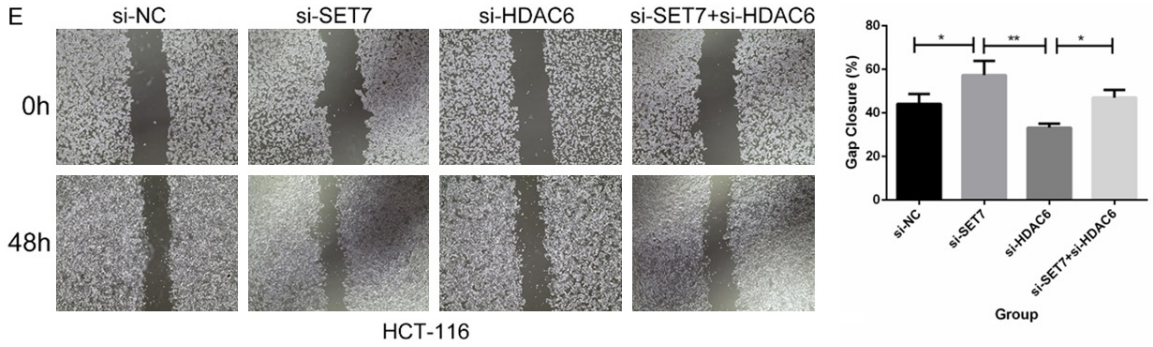

Figure 2. SET7 inhibited cell proliferation and migration in colon cancer cells treated with HDAC6. A. The proliferation in SW480 and HCT116 cells transfected with pc-DNA2.0, Flag-SET7 and HA-HDAC6 plasmid were detected by CCK-8 assays respectively. B, C. The potential migration ability in SW480 and HCT116 cells after transfection were respectively detected by wound healing and transwell assays. D, E. The proliferation and migration ability targeting si-HDAC6 and si-SET7 were detected by CCK-8 assays and wound healing assays respectively in HCT116 cell. Veh and NC served as control.

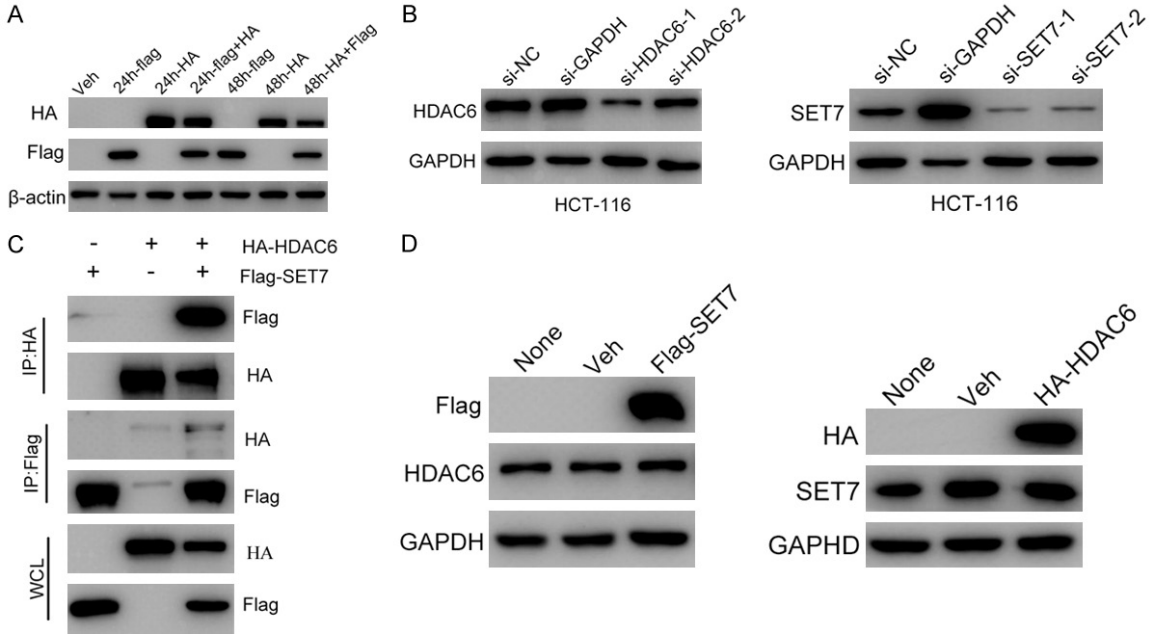

Figure 3. Interaction between SET7 and HDAC6. A. HCT116 cell was transfected with pc-DNA2.0, Flag-SET7 and HA-HDAC6 plasmid, and the over-expression effect was detected by western blotting for 24 and 48 hours before harvesting. B. HCT116 transfected with siRNA targeting si-NC, si-SET7, si-HDAC6, and the siRNA-depletion efficiency was detected by western blotting for 48 hours before harvesting. C. Western blotting analysis of whole cell lysates (WCLs) and immunoprecipitates (IP) derived from HCT116 cells transfected with Flag-SET7 and HA-HDAC6 constructs for 24 hours before harvesting. D. The effect of over-expression Flag-SET7 and HA-HDAC6 on each endogenous expression were detected by western blotting for 48 hours before harvesting. NOTE: there is 5 samples in this member. (si-NC, si-GAPDH, si-SET7-1, si-SET7-2, si-SET7-3). As si-SET7-3 did not played a silencing effect, so we cut it.

ed that SET7 inhibited cell proliferation and migration in colon cancer cell by interacting with HDAC6, and partially reversed the deacetylation effect mediated by HDAC6. Altogether, the results above suggest that SET7 inhibits the growth of colon cancer cells by regulating the deacetylation of HDAC6.

HDAC6 has been shown to play important roles in promoting the development of colon cancer

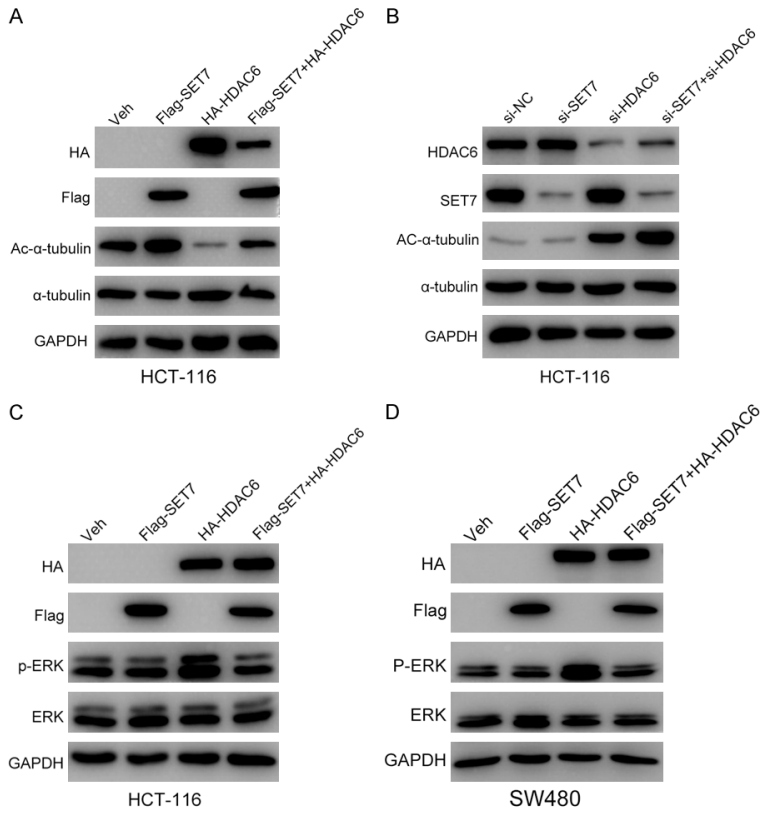

Figure 4. SET7 suppressed deacetylating activity of HDAC6 and increased levels of acetylated-α-tubulin partly through ERK signaling pathway. (A, B) Effects of SET7 and HDAC6 up-regulation or down-regulation on levels of acetylated-α-tubulin in colon cancer cell were analyzed by western blotting for 48 hours before harvesting. (C, D) The expression of p-ERK and ERK were detected in HCT116 and SW480 cells treated with Flag-SET7 and HA-HDAC6 plasmid for 48 hours before harvesting. α-tubulin served as the loading control. NOTE: we do the silencing effect on five groups: None, si-NC, si-SET7, si-HDAC6, si-SET7+si-SET7. But we only used the later four groups (si-NC, si-SET7, si-HDAC6, si-SET7+si-SET7) to detected the expression of AC-α-tubulin/α-tubulin/GAPDH. So, when we try to make a whole membrane, we cut the first band (group None: only cell without any treatment). NOTE: like (B). We also do the over-expression effect on five groups: None, over-expression-Veh, over-expression-SET7, over-expression-HDAC6, over-expression-SET7+over-expression-SET7. But we only used the later four groups to detected the expression of P-ERK/ERK/GAPDH. So, when we try to make a whole membrane, we also cut the first band (group None: only cell without any treatment).

by activating MAPK/ERK signaling pathway [17, 26]. In addition, HDAC6 selective inhibitors exerted an inhibitor effect on cell proliferation and migration via suppressing MAPK/ERK signaling pathway in colon cancer [28, 29]. There have been no reports on the role of SET7 in MAPK/ERK signaling pathways, our study first reported that SET7 could inhibit HDAC6-mediated activation of ERK signaling pathway, and combined with its effect on HDAC6-mediated α-tubulin deacetylation. Therefore, we speculate that SET7 exerts antitumor effect by inhibiting the deacetylation of HDAC6 and it may serve as a potential diagnostic biomarker and a therapeutic target in colon cancer.

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Disclosure of conflict of interest

None.

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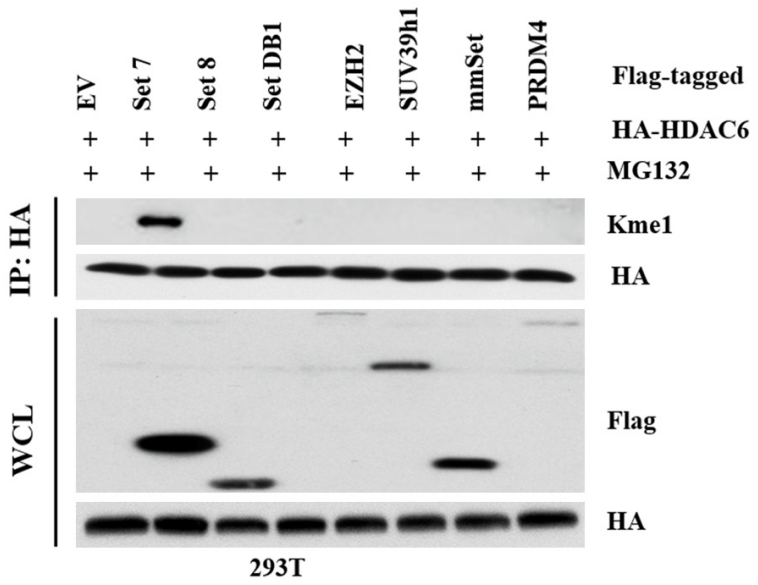

Figure S1. NOTE: lysine methyltransferases interacted with HDAC6. Lysine methyltransferases were labelled with Flag tag and HDAC6 was labelled with HA tag. WCL: whole cell lysates; MG132 is a proteasome inhibitor; EV: Empty Vech.