

Histone deacetylase 6 is overexpressed and promotes tumor growth of colon cancer through regulation of the MAPK/ERK signal pathway

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Purpose: To investigate the expression of histone deacetylase 6 (HDAC6) in colon cancer and its role in colon cancer cell growth and migration.

Materials and methods: We detected the expression of HDAC6 in a colon cancer tissue chip using immunochemical staining, and analyzed the difference in HDAC6 expression between cancer and adjacent noncancerous tissues. Then, we explored the relationship between HDAC6 expression and patients' clinicopathological characteristics and prognoses. In addition, the role of HDAC6 in colon cancer cell growth and migration, as well as its potential related signal pathway, through HDAC6 knockdown was explored.

Results: The immunochemical score of HDAC6 expression was higher in cancer tissue than in the adjacent noncancerous tissue (4.54 vs 3.08, $P < 0.005$); similarly, as well as the rate of high HDAC6 expression was higher in cancer tissue than in the adjacent noncancerous tissue (71.1% vs 40.9%, $P < 0.001$). Patients showing high HDAC6 expression had a shorter overall survival time. Additionally, Cox regression analysis showed that high HDAC6 expression was an independent risk factor for poor prognosis. HDAC6 knockdown decreased cell viability, colony formation, and number of migrated colon cancer cells (HCT116 and HT29); the expression of p-MEK, p-ERK, and p-AKT was also decreased, but had no influence on MEK, ERK, and AKT expression.

Conclusion: HDAC6 is highly expressed in colon cancer and associated with a poor prognosis. HDAC6 knockdown inhibits colon cancer cell growth and migration, partly through the MAPK/ERK pathway.

Keywords: colon cancer, cell growth and migration, histone deacetylase 6, MAPK/ERK pathway, CRC prognosis

Introduction

Colorectal cancer (CRC) is one of the most common malignant tumors worldwide, ranked third in cancer incidence and second in mortality.¹⁻³ Although surgery, chemotherapy, radiotherapy, and biological target therapy have been used in the treatment of colorectal cancer and have greatly improved the prognosis of CRC patients, even today over 50% of patients die due to tumor recurrence or metastasis.⁴ What's more, the underlying mechanism of the disease remains largely undefined. Therefore, further research is required to explore the pathogenic mechanism of CRC and new therapeutic strategies to combat it.

Acetylation and deacetylation play important roles in tumorigenesis, and deacetylation is mediated by histone deacetylases (HDACs). HDACs alter the

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acetylation status of histone and non-histone proteins to regulate various cellular events, such as proliferation, differentiation, apoptosis, autophagy, angiogenesis and tumor immunity, in tumor cells. HDACs can be divided into four classes based on their homology to yeast HDACs: class I, class II, class III and class VI. In addition, class II can be further divided into IIa and IIb.^{5,6}

Histone deacetylase 6 (HDAC6) is a unique Class IIb HDACs, in that it is a predominant cytoplasmic protein with two deacetylase domains, and it has been demonstrated to promote tumor growth in many human cancers including gastrointestinal cancers.^{7,8} In our previous study, we found that HDAC6 knockdown could suppress proliferation, migration, and invasion in HCT116 cells.⁹ Other research has found that HDAC6-selective inhibitors could inhibit colon cancer cell growth, proliferation and invasion.^{10–15} However, the expression of HDAC6 in colon cancer tissue and its relationship with patients' clinicopathologic characteristics remain largely unknown.

In the present study, we first examined the expression of HDAC6 in clinical colon cancer patients and explored the association between HDAC6 expression and patients' clinical features. Then we investigated the effects of HDAC6 on the cellular biological behavior of colon cancer cells and presented evidence of HDAC6 interacting with the MAPK/ERK signaling pathway. The results of this study may provide a better understanding of the role HDAC6 plays in colon cancer, as well as a rationale for clinical investigations or for the application of HDAC6-selective inhibitors in CRC patients.

Materials and methods

Immunohistochemistry

Human tissue microarrays were purchased from Shanghai Outdo Biotech, (catalog no. HCol-Ade180Sur-07, Shanghai, China). Demographic and clinicopathological data, including clinical stage (according to the American Joint Committee on Cancer staging system) and survival data were provided by the manufacturer. Tissue sections were deparaffinized, soaked in 0.01 M sodium citrate buffer and boiled in a microwave oven for 5 min at 500 W to retrieve antigens. After treatment with 3% hydrogen peroxide to inactivate endogenous peroxidase and blocking with normal goat serum to reduce non-specific staining, the sections were incubated with anti-HDAC6 antibody (7558; Cell Signaling Technology, USA) at a dilution of 1:400 overnight at 4 °C. Tissue sections were immunohistochemically stained using Envision reagents (Envision+Dual

link/HRP; Dako Denmark). All sections were counterstained with hematoxylin. The immunohistochemical (IHC) score was assessed with the Fromowitz standard:¹⁶ 0–5%=0; 5–25%=1; 26–50%=2; 51–75%=3; >75%=4. The staining intensity was graded as follows: absent or faint blush=0; weak=1; moderate=2; strong=3. The final IHC score was calculated by adding the percentage and intensity scores. Thus, the minimum score was 0, the maximum score was 7, and the grading was as follows: 0–1=(-), 2–3=(+), 4–5=(++), 6–7=(+++). A final score ≤ 3 was considered low expression, while and ≥ 4 was considered high expression. Two experienced pathologists, blinded to all clinicopathological information on the patients, independently evaluated the immunostaining scores. The cases eliciting disagreement were reevaluated jointly for consensus.

Cell lines and cell culture

Human colon cancer HCT116 and HT29 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in DMEM medium (Life Technologies, CA) containing 10% fetal bovine serum (FBS), 100 units of penicillin and 100 mg/ml streptomycin. The cells were maintained in a 5% CO₂ culture incubator at 37 °C.

Reagents and antibodies

Anti-HDAC6 (7558), anti-phospho-s473-AKT (9018), anti-AKT (2938), anti-phospho-MEK (9154), anti-MEK (8727), anti-phospho-ERK (3192), anti-ERK (4695) and anti-Ac- α -Tubulin (5335) antibodies were purchased from Cell Signaling Technology. Anti-Tubulin (SC-73242) antibody was purchased from Santa Cruz. All antibodies were used in 1:1,000 dilutions in 5% non-fat milk for western blot.

Plasmids

ShScramble, shHDAC6-1, and shHDAC6-2 were described previously.⁹ Lipofectamine 2,000 Transfection Reagent (Thermo Fisher Scientific, USA) was used for transfection according to the protocol recommended by the manufacturer. A 293FT cell line was to package and amplify the lentivirus. The media containing viruses were collected 48 hrs and 72 hrs after transfection. After filtering through 0.45 μ M filters, viruses were used to infect cells in the presence of 4 μ g/mL polybrene (Sigma-Aldrich). At 48 hrs post-infection, cells were split and selected using 1 μ g/mL puromycin (Sigma-Aldrich) for three days to eliminate the uninfected cells before harvesting.

Cell viability assay

Cell treated with sh-Scramble, shHDAC6-A, and shHDAC6-B were respectively plated at 3,000 cells/well in 96-well plates and cultured in a 5% CO₂ culture incubator at 37 °C. After 24 hrs, 48 hrs, 72 hrs, and 96 hrs, cell viability was detected with the Cell Titer-Glo Luminescent Cell Viability Assay kit according to the manufacturer's instructions (Promega).

Colony formation assays

HCT116 and HT29 cells treated with sh-Scramble, shHDAC6-A, and shHDAC6-B were respectively plated at a density of 1,000 cells/well in 6-well culture dishes and allowed to grow undisturbed for 10 days. Cells were stained with crystal violet and the colony numbers were counted.

Transwell assay

HCT116 cells and HT29 cells treated with sh-Scramble, shHDAC6-A, and shHDAC6-B were respectively plated at a density of 5×10^4 cells in an 8- μ m, 24-well plate chamber insert (Corning Life Sciences, catalog no. 3422), with 100 μ L serum-free medium at the top of the insert and DMEM medium (Gibco) containing 10% FBS (500 μ L) at the bottom. Cells were incubated for 24 h and fixed with 4% paraformaldehyde for 15 min. After washing with PBS, cells at the top of the insert were scraped with a cotton swab. Cells adhering to the bottom were stained with 0.5% crystal violet blue for 15 min and then washed with double-distilled H₂O. The positively stained cells were counted in eight random fields under the microscope, and the average value of the eight fields was expressed. All assays were performed in triplicate.

Western blot assay

Cells treated with sh-Scramble, shHDAC6-A, and shHDAC6-B were harvested and washed once using cold PBS. The cell pellets were lysed in EBC buffer (50 mM Tris pH 7.5, 120 mM NaCl, 0.5% NP40) supplemented with protease inhibitors (Roche) and phosphatase inhibitors (Calbio chem). The lysates were cleared by centrifugation and then quantified with a Beckman Coulter DU-800 spectrophotometer (Beckman Coulter) using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, CA). The lysates were eluted by boiling for 5 mins in SDS loading buffer. Bound proteins were resolved by SDS-PAGE and immunoblotted with the indicated antibodies.

Statistical analysis

Data were analyzed by with the SPSS 23.0 software (Chicago, IL, USA). The data were expressed as the mean and SD and compared by ANOVA unless stated otherwise. HDAC6 expression in tumor tissues and matched non-tumor tissues was analyzed with the paired Student's *t*-test. The association between HDAC6 expression and various clinicopathological parameters was evaluated with the Chi-Square test. The Cox proportional hazard regression model was used for univariate and multivariate analyses to determine the effects of the clinicopathological variables and HDAC6 expression on patient survival. Only variables with *P*-values <0.1 in the univariate analysis were included in the multivariate analysis.

Results

HDAC6 was overexpressed in colon cancer tissues and associated with poor prognosis

Immunohistochemical staining was performed on a tissue microarray containing 180 cores/spots consisting of paired colon cancer tissue and corresponding adjacent non-neoplastic mucosa tissue from 90 patients (Figure 1A). Among the 90 pairs, 7 pairs were excluded due to a lack of either detectable tumor cells or normal colon epithelial cells, so that a total of 83 pairs were enrolled for analysis. As illustrated in Figure 1B, HDAC6 was expressed in both the cytoplasm and nucleus (but mostly in the cytoplasm), and cytoplasmic HDAC6 staining was evaluated in the study. Our results showed that the IHC score of HDAC6 expression in the cancer tissue was higher than in the matching noncancerous tissue (4.54 vs 3.08, *P*<0.005) (Figure 1B, right). Out of the analyzed cases of colon cancer, 71.1% (59/83) showed strong cytoplasmic HDAC6 staining, which was significantly higher than that in noncancerous tissue (40.9%, 34/83). We then explored the correlation between HDAC6 expression and the clinicopathological characteristics of colon cancer patients. The data showed that the HDAC6 expression levels were associated with tumor size, but not with other parameters such as age, gender, tumor site, lymph node invasion, differentiation grade, JACC clinical stage, etc (Table 1). Univariate analysis revealed that tumor size, lymph node invasion, JACC clinical stage, vascular invasion, and high HDAC6 expression were significantly associated with patients' survival (Table 2). Furthermore, multivariate Cox regression analysis confirmed lymph

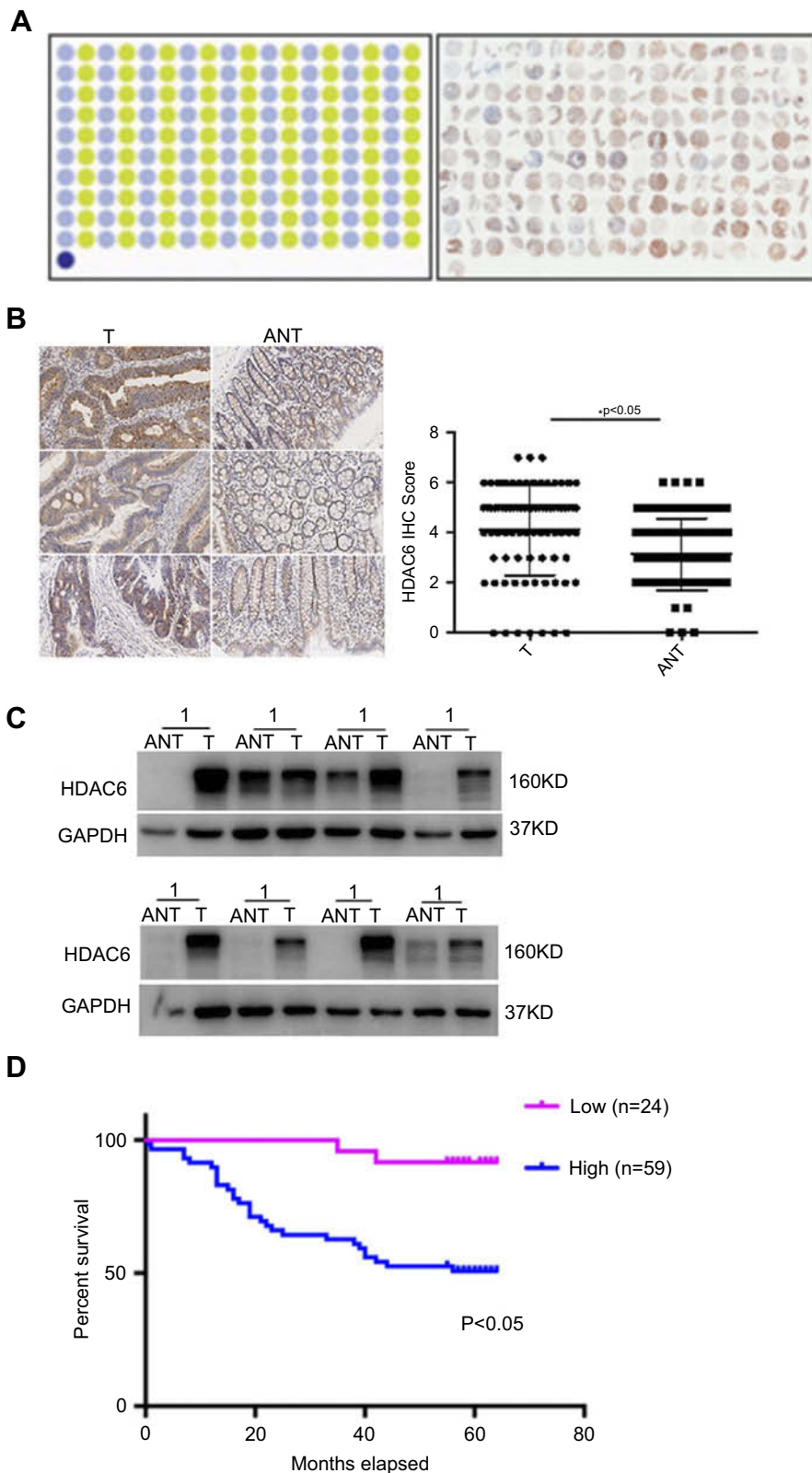


Figure 1 HDAC6 was overexpressed in colon cancer tissues and associated with poor prognosis. **(A)** Human CRC tissues were detected by Super Chips microarrays. **(B)** The tumor sections underwent IHC staining using antibodies against HDAC6. **(C)** HDAC6 expression in 8 cases of human CRC tissues and their matched adjacent normal tissues by western blot analysis. **(D)** Kaplan-Meier survival analysis by HDAC6 status (n=83). The vertical axis represents the percentage survival rate; the horizontal axis represents the survival days. The blue line indicates that the patients with higher HDAC6 expression had a worse overall survival than the red line. The overall median survival (OS) time was 55 months for the HDAC6-positive group and 58 months for the HDAC6-negative group. The data shown represent mean \pm SD from a representative experiment. **Note:** * $P < 0.05$.

Table I Relationship between HDAC6 expression and clinicopathologic features of CRC patients

Clinicopathologic features	No. of patients	HDAC6 expression		P-Value
		Low (n)	High (n)	
Gender				0.944
Male	42	12	30	
Female	41	12	29	
Age				0.644
<60	28	9	19	
≥60	55	15	40	
Tumor size				0.036
<5 cm	37	15	22	
≥5 cm	46	9	37	
Tumor site				0.487
Left Hemicolon Carcinoma	40	13	27	
Right Hemicolon Carcinoma	43	11	32	
Gross classification of tumors				0.288
Protrude type	35	10	25	
Infiltrating type	16	7	9	
Ulcerative type	32	7	25	
Depth of tumor invasion				0.312
T1-T2	12	2	10	
T3-T4	71	22	49	
Lymph node invasion				0.734
Absent	30	8	22	
Present	53	16	37	
Distant metastasis				0.361
Absent	81	24	57	
Present	2	0	2	
AJCC clinical stages				0.704
I	10	3	7	
II	41	13	28	
III	29	8	21	
IV	3	0	3	
Differentiation grade				0.549
Well	3	0	3	
Moderately	66	21	45	
Poorly	13	3	10	
Undifferentiated	1	0	1	
Tumor pathological type				0.476
Adenocarcinoma	74	21	53	
Mucous carcinoma	7	3	4	
Undifferentiated carcinoma	2	0	2	
Vascular invasion				0.667
Absent	76	23	54	
Present	7	1	6	

Abbreviations: AJCC, American Joint Committee on Cancer; CRC, colorectal cancer; HDAC6, histone deacetylase 6.

Table 2 Univariable and multivariable analyses of OS and clinicopathologic variables in CRC

Clinicopathologic features	No.	Univariable analysis	Multivariable analysis	
		P-Value	95% CI	P-Value
Gender		0.294		
Male	42			
Female	41			
Age		0.238		
<60	28			
≥60	55			
Tumor size		0.028	0.630 (0.273–1.454)	0.279
<5 cm	37			
≥5 cm	46			
Tumor site		0.165		
Left Hemicolon Carcinoma	40			
Right Hemicolon Carcinoma	43			
Gross classification of tumors		0.302		
Protrude type	35			
Infiltrating type	16			
Ulcerative type	32			
Depth of tumor invasion		0.521		
T1-T2	12			
T3-T4	71			
Lymph node invasion		0.001	1.060 (0.413–2.716)	0.904
Absent	30			
Present	53			
Distant metastasis		0.137		
Absent	81			
Present	2			
AJCC clinical stages		0.001	2.567 (1.314–5.016)	0.006
I	10			
II	41			
III	29			
IV	3			
Differentiation grade		0.067		
Well	3			
Moderately	66			
Poorly	13			
Undifferentiated	1			
Tumor pathological type		0.165		
Adenocarcinoma	74			
Mucous carcinoma	7			
Undifferentiated carcinoma	2			
Vascular invasion		0.001	3.822 (1.413–10.340)	0.004
Absent	76			
Present	7			

(Continued)

Table 2 (Continued).

Clinicopathologic features	No.	Univariable analysis	Multivariable analysis	
		P-Value	95% CI	P-Value
HDAC6 expression		<0.001	6.207 (1.442–26.708)	0.014
Low	24			
High	59			

Abbreviations: CRC, colorectal cancer; HDAC6, histone deacetylase 6; IHC, immunohistochemical; OS, overall survival.

node invasion, vascular invasion, and high HDAC6 expression as independent risk factors for poor survival (Table 2). Kaplan-Meier analysis demonstrated that those patients with high HDAC6 expression had a shorter overall survival time (median=55 months) compared to patients with low HDAC6 expression (median=58 months, $P<0.05$, Figure 1D). We also detected HDAC6 expression in 8 colon cancer pairs using western blot, and the results showed that HDAC6 was up-regulated compared with adjacent noncancerous tissue (Figure 1C).

HDAC6 knockdown inhibits colon cancer cell proliferation, migration, and invasion

Since HDAC6 was overexpressed in colon cancer and correlated with poor prognosis, we next determined whether HDAC6 played an oncogenic role in colon cancer. We investigated the biological effects of HDAC6 knockdown in two colon cancer cell lines (HCT116 and HT29) using shRNA (Figure 2A). The results showed that HDAC6 knockdown significantly decreased cell viability and the colony formation number, suggesting cell growth inhibition by HDAC6 knockdown (Figure 2B–C)

Cell migration and invasion are essential to cancer metastasis.¹⁷ Thus, we further investigated the potential impact of HDAC6 knockdown on the metastatic properties of colon cancer cells using transwell assays, and found that HDAC6 knockdown with either of shRNA significantly impaired cell migration potential (Figure 2D). The results demonstrated that HDAC6 suppression may inhibit colon cancer metastasis and invasion.

HDAC6 exert its function partly through the MAPK/ERK signaling pathway

Mitogen-activated protein kinases (MAPKs) consisting of ERK, JNK and p38 are a major class of serine-threonine kinases that form the main cell proliferation and migration signaling pathway from the cell surface to the nucleus.^{18,19}

MAPK/ERK signaling pathways play critical roles in tumor proliferation, migration, and invasion.²⁰ A growing body of evidence indicated that activation of the MAPK/ERK pathway promotes CRC progression and inhibition of this pathway may be used to treat CRC.^{20,21}

We therefore determined whether HDAC6 knockdown had an effect on the MAPK/ERK signaling pathway, and the results showed that HDAC6 knockdown could decrease the expression of p-MEK, p-ERK and p-AKT, but had no obvious effect on MEK, ERK and, AKT expression in either cell line (Figure 3). These data suggest that HDAC6 knockdown exerts its function partly through the MAPK/ERK signaling pathway in colon cancer cells.

Discussion

In the present study, we found that HDAC6 is highly expressed in colon cancer, and its high expression is associated with poor prognosis. To the best of our knowledge, this is the first study reporting on the expression levels of HDAC6 in colon cancer, as well as on its association with patients' clinical characteristics. We further certified the oncogenic role of HDAC6 in colon cancer via cell experiments. The results of the present study help to better understand the mechanism of colon cancer development, and provide a rational basis for the clinical application of HDAC6-selective inhibitors in the treatment of colon cancer.

HDAC6 is a unique Class IIb histone deacetylase that plays vital roles in many human cancers. In gastrointestinal cancers, the role of HDAC6 varies with the cancer type and tumor microenvironment. For example, HDAC6 is an oncogene in esophageal cancer, while it can both an oncogene and a tumor suppressor in hepatocellular and gastric cancer.^{22–24} Our study showed that HDAC6 was highly expressed in colon cancer tissues, and a high expression of HDAC6 predicted a shorter overall survival. Furthermore, HDAC6 knockdown could inhibit tumor growth and migration. Several previous studies

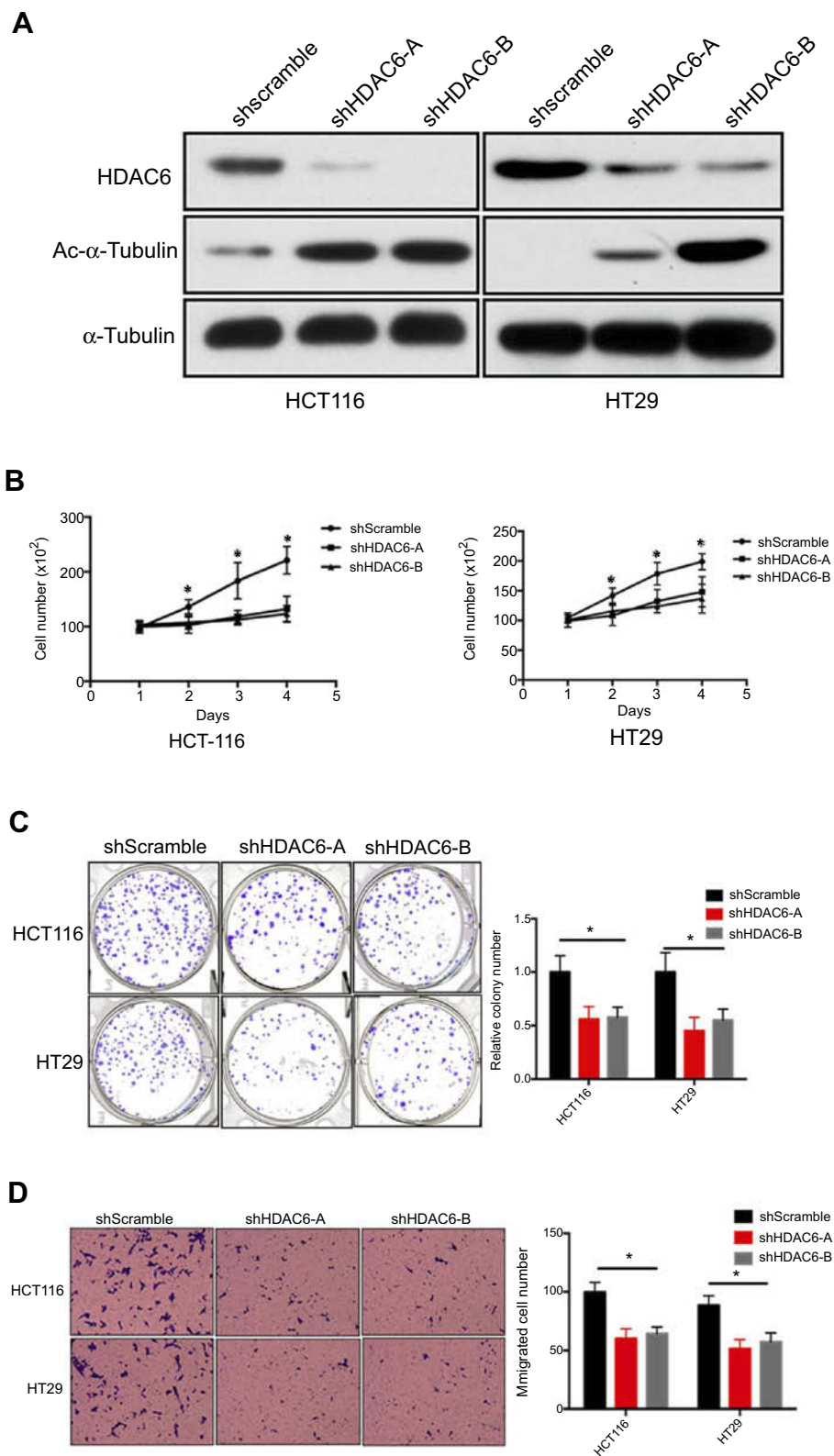


Figure 2 HDAC6 knockdown inhibits colon cancer cell proliferation, migration and, invasion (**A**) HCT116 and HT29 cells were transfected with shRNA specifically targeting shHDAC6-A, shHDAC6-B, shscramble, and the shRNA-depletion efficiency was detected by Western blot. (**B and C**) The proliferative ability of the indicated cells detected by cell viability and colony formation assays. (**D**) The potential migration ability of HCT116 and HT29 cells after transfection was detected by transwell assays. Data are represented as mean \pm SD from three independent experiments. The *P*-values were calculated using the unpaired Student's *t*-test.

Note: **P*<0.05.

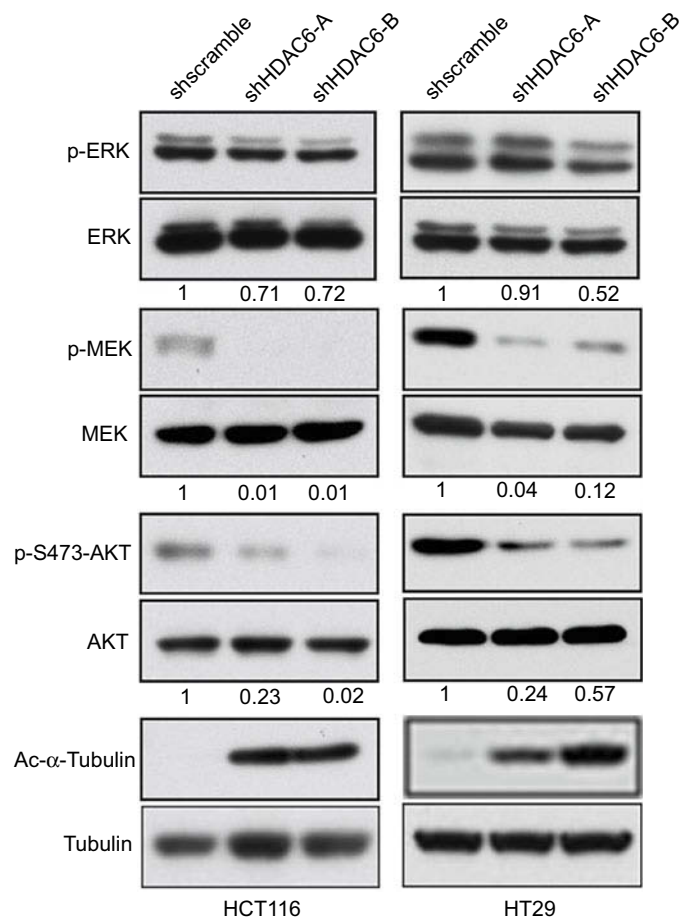


Figure 3 HDAC6 exerts its function partly through the MAPK/ERK signaling pathway. Western blotting analyses of HCT116 and HT29 cells after transfection with shHDAC6-A, shHDAC6-B and shscramble. ERK, p-ERK, MEK, p-MEK, AKT, and p-AKT levels were evaluated. The numbers below the blots indicate the fraction of protein remaining normalized to tubulin and to the amount detected in cells expressing scrambled shRNA. Tubulin served as the loading control. The results are representative of assays performed in triplicate.

have shown that HDAC6 inhibitors can inhibit the growth of colon cancer cell lines (HCT116, HT29, Caco-2).^{10,11,13,25,26} However, more recent research reported that a HDAC inhibitor (TriA) showed no effect on migration in colon cancer cells induced by TPA (a tumor promoter). Differently from previous studies, this latest study focused on the extent to which gene promoter methylation induced by reactive oxygen species facilitated the migration and invasion of colon cancer cells but not on the effect of histone deacetylases, which are two different mechanisms of tumorigenesis. Overall, the results indicated that methylation was also one of the factors responsible for colon cancer metastasis, which may serve as a therapeutic tool in the future.²⁷

Previous studies have demonstrated that HDAC6 plays an important role in various cellular processes, such as proliferation, differentiation, apoptosis, autophagy, and angiogenesis, and in the underlying signal pathways involved, including

MAPK/ERK, TGF- β , mTOR, NF- κ B, etc.^{28–31} Since HDAC6-selective inhibitors showed a suppressive effect on colon cancer cells via the MAPK/ERK signal pathway, and since the MAPK/ERK pathway plays an important role in colon cancer development,^{32,33} we investigated the role of HDAC6 knockdown on the MAPK/ERK pathway in colon cancer cell lines. As the results showed, HDAC6 knockdown significantly decreased the levels of p-ERK, p-MEK and p-AKT, while no obvious change was noticed in MEK, ERK and AKT expression, suggesting that HDAC6 exerts its function partly through the MAPK/ERK signal pathway, which is consistent with the results obtained with HDAC6-selective inhibitors.

Additionally, MAPKs are regulated by reactive oxygen species, which were proposed to be involved in tumor metastasis by triggering mitochondrial dysfunction and DNA damage.³⁴ Accumulating evidence suggests that an increase in reactive oxygen species initiates the p38

MAPK signaling pathway and triggers the apoptosis cascade in colon cancer. Therefore, treatment with ROS scavengers or MAPK inhibitors would abolish this effect in cancer cells.^{35–38} Altogether, measures that target the ROS-induced activation of p38 MAPK provide new insights into the molecular mechanisms that may be of therapeutic interest for the treatment of colon cancer.

Conclusion

In conclusion, our study indicates that HDAC6 plays a critical role in regulating tumor growth and metastasis in colon cancer. This effect maybe partly mediated by the HDAC6-mediated inactivation of MAPK/ERK pathways. Thus, HDAC6 may be a valuable prognostic marker, and targeting HDAC6 may serve as a potential therapeutic approach that could effectively inhibit tumor growth and metastasis in colon cancer patients.

Institutional review board statement

The study was approved by the Ethics Committee of the Second Xiangya Hospital, Central South University, Changsha, China.

Institutional animal care and use committee statement

No animal was used in the present study.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

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