Original Article
High expression of HDAC6 correlates with poor prognosis in colon cancer

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Abstract: Objectives: To elaborate the correlation between the expression of HDAC6 and tumor progression as well as prognosis in colon cancer patients. Methods: We chose a tissue microarray containing 90 colon cancer specimens with more than 5 years follow-up information. The expression level of HDAC6 was investigated by immunohistochemistry using a specific antibody. The experimental data, as well as relationship between HDAC6 expression and clinical pathological parameters were statistically analyzed by SPSS software. Results: HDAC6, expressed specifically in the cytoplasm, was obviously higher in colon cancer tissues than para-carcinoma tissues (P = 0.000). The expression of HDAC6 was negatively correlated with tumor size in colon cancer significantly (r = -0.213, P = 0.048). Survival analysis showed patients with HDAC6 over-expression had worse prognosis (18.2% vs 47.0%, P = 0.013) and HDAC6 was an independent prognostic factor (P = 0.033). Survival analysis of groups based on the tumor size indicated that patients with HDAC6 over-expression had worse prognosis among colon cancer groups bearing tumor diameter ≤5.5 cm (18.8% vs 51.4%, P = 0.006). However, there was no obvious correlation among groups bearing tumor diameter >5.5 cm patients (P = 0.532). In addition, the correlation analysis between HDAC6 and mismatch repair gene family showed that there was a negative correlation between HDAC6 expression and MSH2 expression (r = -0.205, P = 0.060). Conclusions: HDAC6 may specifically shorten the survival time of the colon patients bearing smaller-size tumor through promoting cell growth and proliferation; on the contrary, it had no effect on patients developing bigger-size tumor for the accomplishment of tumor cell growth and proliferation. At the same time, we also speculate that HDAC6 may promote colon cancer progression by inhibiting the gene repair function of patients.

Keywords: HDAC6, colon cancer, tissue microarray, immunohistochemistry, prognosis, targeted therapy

Colorectal cancer (CRC) is one of the third most mortal tumors in the world [1]. Although continual innovative therapy strategies had been investigated in the past 20 years, there were still no effective methods to improve the prognosis of CRC patients. Researchers have to get a further insight into CRC, so as to identify novel targets for prediction and therapy.

The acetylation and deacetylation of histone is one of the most important regulations of gene expression. Under normal circumstances, the dissociation of the DNA and histone octamer catalyzed by histone acetylation and the following exposure of nucleosome structure can assist in the binding of many transcription factors to specific DNA binding sites, and then activated gene transcription [2]. Conversely, histone deacetylases (HDAC) suppressed gene

transcription through the deacetylation of histone. HDAC has been regarded as an important cancer drug targets through deacetylation to regulate expression of multiple tumor associated protein [3]. So far, there are 18 members of HDAC family in human, divided into 4 categories [4] and HDAC6 belongs to the class II HDAC. Studies about the correlation between HDAC6 and cancer were not much, and drew contradictory conclusions. Lv Z et al [5] showed HDAC6 was a bona fide tumor suppressor gene in hepatocellular carcinomas (HCC). HDAC6 was downregulated in HCCs compared to para-carcinomas (P = 0.025). Thus, the recurrence rate was higher (P = 0.006) as well as disease-free survival rate was lower (P = 0.047) in patients with HDAC6 down-regulation. Moreover, knockdown of HDAC6 could promote the migration and proliferation of human umbilical vein endo-

thelial cells, also inhibit apoptosis of hepatocellular carcinoma and promote proliferation of hepatocellular carcinoma under hypoxia. However, Hou H [6] demonstrated the carcinogenic ability in prostatic foamy gland carcinoma. They found HDAC6 was an independent prognostic factor (P = 0.007) with significant negative correlation with the prognosis of prostate cancer patients (P<0.05). Depletion of HDAC6 could suppress the migration and invasion of prostate cell line IA8. In addition, the relationship between HDAC6 and prognosis in breast cancer was more complicated. HDAC6 expression was positively correlated with luminal B-type and ER-positive breast cancer patients, respectively (P<0.05), and had no correlation among all breast cancer patients (P = 0.194) [7]. These investigations indicated that HDAC6 plays different biological function and mechanism in diverse cancers.

In order to elucidate the correlation between HDAC6 and the development of colon cancer, we chose a tissue array containing 90 colon cancer specimens which had immunohistochemical data about some mismatch repair genes (MLH1, MSH2, MSH6, PMS2) and follow-up information. We elaborated the specific expression pattern of HDAC6 by IHC, and then speculated the biological function and molecular mechanism in colon cancer.

Materials and methods

Colon cancer specimens

Colon cancer tissue array (TMA) (HCol-Ade180-Sur-06) was obtained from Shanghai Outdo Biotech Co., Ltd, contained 90 carcinoma tissues and paired para-carcinoma tissues (1.5-cm away from the loci of carcinoma tissues). TMA was produced by Shanghai Outdo Biotech Co., Ltd: all donor paraffin-embedded sections were resected and stained by hematoxylin-eosin (HE). Then, the pathologist labelled typical pathological sites on HE slices. Using tissue microarray instrument (Beecher Instruments. Inc.) drilled a block on the blank recipient paraffin (diameter was 1.5 mm), and then set the target tissue core according to the position on the tag. A 180-point tissue array was produced eventually. Subsequently, slicer (Leica, Germany) took continuous slices from tissue section to thickness of 4-5 um. Slices were

attached to anti-off microslides (HCol-Ade-180Sur-06).

The follow-up of colon cancer patients: The operation time was from July 2006 to May 2008 with eventual follow-up time in August 2014, which followed 7-8 years. During this follow-up time, 54 patients were died of colon cancer, with a median follow-up time of 28.0 months (5-93 months); 36 patients were still alive, with a median follow-up time of 91.5 months (87-94 months). All patients were clinical pathologically diagnosed as colon cancer, which shared immunohistochemistry data of some mismatch repair genes (MLH1, MSH2, MSH6, PMS2) and received no extra treatment before surgery.

Immunohistochemistry

Immunohistochemistry two-step assay by DAKO Auto Stainer LinK48: Antigen retrieval was performed in citrate buffer. The tissue sections were blocked with goat serum and subsequently incubated with anti-HDAC6 (1:6000, 12834-1-AP, proteintech Ltd) at 4°C overnight, then incubated with secondary antibody (HRP-labeled anti-mouse antibody, DAKO). Following PBS rinsing, visualizing using diaminobenzidine (DAB) system and hematoxylin re-dying, images were obtained under a light microscope after eventual dehydration, transparent and mounting. Randomly 3 high-power fields were chosen under optical microscope and calculated the positive cells under the selective field of more than 3 × 100 cells. The positive staining rate was defined according to the proportion of positively stained cancer cells. Statistically stratified by positive staining rate: 100% as the high expression group, <100% as the low expression group.

Statistical analysis

Including all 88 colon cancer tissues and 84 para-carcinoma tissues, immunohistochemistry data were analyzed by SPSS except off-point cases.

Difference of HDAC6 expression in carcinoma tissues and para-carcinoma tissues were analyzed by paired Wilcoxon test. The correlation between HDAC6 expression and clinical pathological parameters of colon cancer was calculated by Spearman correlation analysis. Thus,

Table 1. Clinical information and correlation analysis of HDAC6 in colon cancer cases

Clinical parameters	Total number (88 cases)	HDAC6 low expression (66 cases)	HDAC6 high expression (22 cases)	Coefficient	*P* value
Gender				-0.044	0.683
Male	45	33	12		
Female	42	32	10		
Lost	1	1	0		
Age				-0.060	0.591
≤60	16	12	4		
>60	67	51	16		
Lost	5	3	2		
Tumor size				-0.213	0.048
≤5.5 cm	51	35	16		
>5.5 cm	36	30	6		
Lost	1	1	0		
Pathological grade				0.165	0.124
I	3	1	2		
II	43	35	8		
III	42	30	12		
T stage				-0.125	0.268
T1	1	1	0		
T2	7	6	1		
T3	60	42	18		
T4	13	11	2		
Lost	7	6	1		
N regional lymph node metastasis				0.016	0.879
N0	57	42	15		
N1	22	18	4		
N2	9	6	3		
M Remote metastasis				0.011	0.921
M0	86	64	22		
M1	2	2	0		
cTNM stage				0.027	0.808
1	8	7	1		
2	47	33	14		
3	29	22	7		
4	2	2	0		
Lost	2	2	0		

univariate analysis between HDAC3 and survival time was estimated using the Kaplan-Meier method and the log-rank test. Then, statistically significant variables in Univariate analysis would be included in COX multivariate regression survival analysis. In addition, the correlation between HDAC6 expression and mismatch repair genes (MLH1, MSH2, MSH6, PMS2) was calculated by Pearson correlation analysis. P<0.05 was considered to be statistically significant.

Results

Clinical data of colon cancer cases

Ruling out off-point experimental cases, the clinical data of remaining 88 colon cancer cases was as follow: 45 males, 42 females and 1 gender loss. The ages were from 24 to 90 and tumor size was from 1.5 cm to 15 cm, respectively. Besides, the TNM stage was 8 in stage 1, 47 in stage 2, 29 in stage 3, 2 in stage 4 and 2
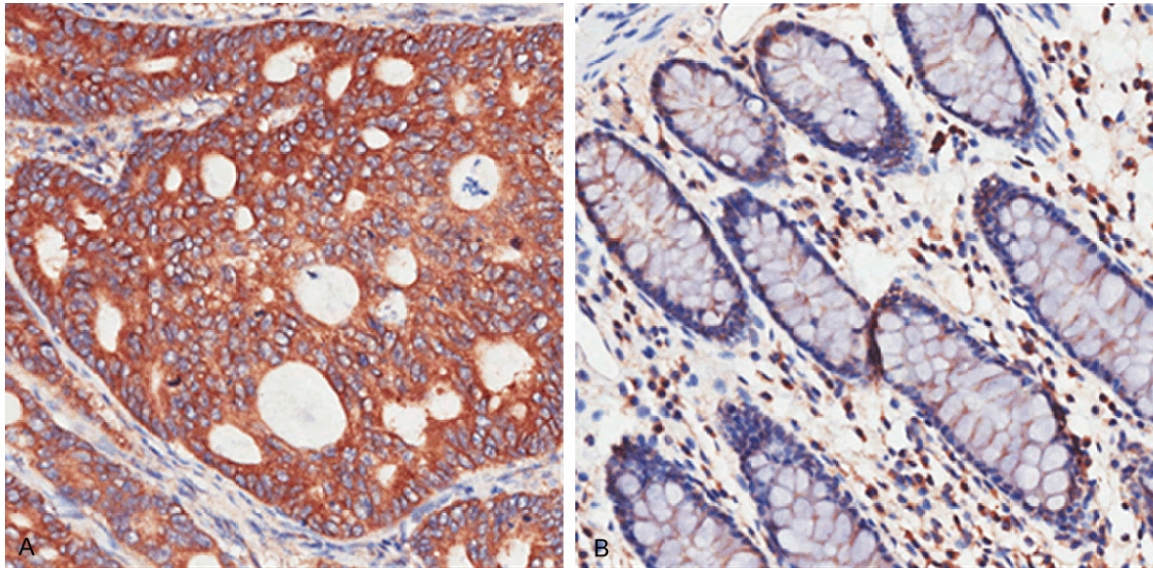
Figure 1. Immunohistochemistry of HDAC6: HDAC6 was specifically expressed in the cytoplasm of both colon cancer tissue and para-carcinoma cells. The positive staining rate of HDAC6 in colon carcinoma tissues (A) was significantly higher than that in para-carcinoma tissues (B). (Magnification times: ×200).

Table 2. Analysis of difference of HDAC6 expression in colon cancer tissues and para-carcinoma tissues

Sample	HDAC6 expression	Number	P value
Colon cancer tissues	84.0% ± 9.9%	84	0.000
Adjacent non-cancerous tissues	44.1% ± 19.2%	84	

missing clinical TNM stage. Among all cases, 31 cases have regional lymph node metastasis and 2 have distant metastasis. The detailed clinical information was showed in **Table 1**.

Expression pattern of HDAC6 in colon cancer

The results of Immunohistochemistry indicated that HDAC6 was localized in the cytoplasm in all colon cancer specimens. The representative pictures of the immunohistochemistry were shown in **Figure 1**. The data analyzed by Wilcoxon test revealed that positive staining rate of HDAC6 was higher in colon canner tissues than that in para-carcinoma tissues (P = 0.000). The analysis was showed in **Table 2**. The ratio of high expression of HDAC6, which was defined of 100% positive staining, was 25% in cancer tissues; however, only 1 specimen was positive in para-carcinoma and accounted for 1% merely. Spearman correlation analysis showed HDAC6 expression in colon cancer tissues was negatively correlated with the tumor size (r = -0.213, P = 0.048).

There was no correlation with gender, age, pathological grade, T, N and M stage. The analysis was showed in **Table 2**.

The relationships between HDAC6 expression and colon cancer prognosis

Kaplan-Meier analysis and log-rank test were applied to determine the association between HDAC6 expression and prognosis of all colon cancer patients. The results showed HDAC6 expression was significantly negatively correlated with the prognosis of colon cancer patients. In another word, patients with higher HDAC6 expression showed significantly worse overall survival than those with lower HDAC6 expression (18.2% vs 47.0%, P = 0.013). At the same time, N, M stage and clinical stage (P<0.05, data not show) exhibited the same negative correlation. The analysis was showed in **Figure 2**. Subsequently, we involved the 4 prognostic indexes into COX multivariate regression survival analysis, the result revealed only HDAC6 expression was an independent prognostic factor (P = 0.033). Detailed analysis was shown in **Table 3**.

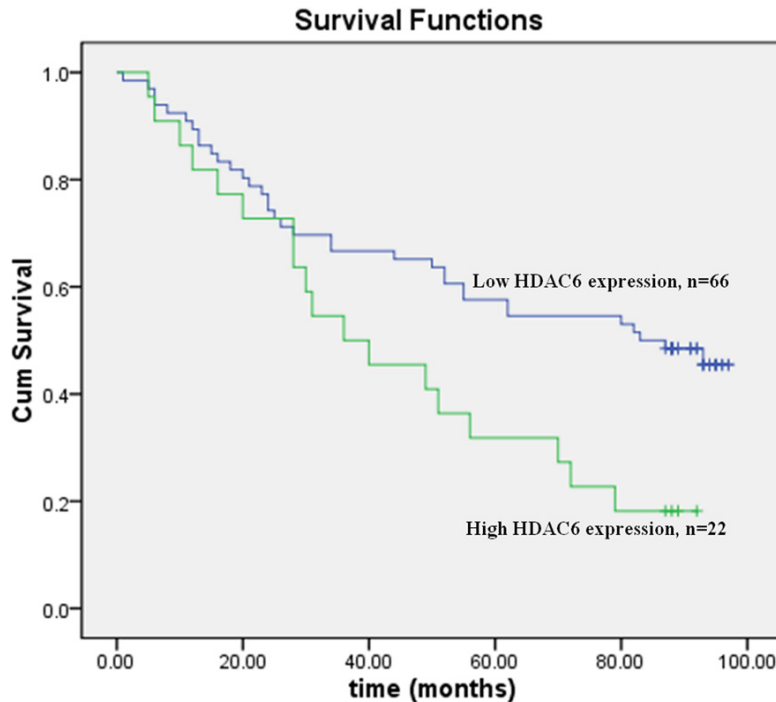Patients with colon cancer were further divided into two groups according the tumor size (tumor

Survival Functions

Figure 2. Relationship between HDAC6 expression and prognosis of overall colon cancer patients: HDAC6 was significantly reversely correlated with the prognosis of colon cancer patients. Patients with high HDAC6 expression showed a significantly worse prognosis than those with low HDAC6 expression (18.2% vs 47.0%, P = 0.013).

Table 3. Analysis of independent prognostic factor in colon cancer patients by cox multivariate analysis variables

	B	SE	Wald	df	P-value	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
HDAC6 expression	.659	.309	4.544	1	.033	1.933	1.054	3.542
N	.173	.364	.225	1	.635	1.189	.582	2.427
M	1.600	.872	3.366	1	.067	4.954	.896	27.381
cTNM stage	.411	.417	.970	1	.325	1.508	.666	3.413

diameter ≤5.5 cm group and diameter >5.5 cm group) in the consideration of significantly negative correlation between HDAC6 and tumor size. The correlation analysis between HDAC6 and prognosis of each group was estimated using the Kaplan-Meier method and the log-rank test. The prognosis of patients with higher HDAC6 expression was significantly worse than those with lower HDAC6 expression (18.8% vs 51.4%, P = 0.006) in the smaller tumor-size groups. However, among the bigger tumor-size groups, HDAC6 expression did not significantly affect the patient's prognosis (P = 0.532). Detailed results were shown in **Figure 3**.

The relationship between HDAC6 expression and mismatch repair genes (MLH1, MSH2, MSH6, PMS2)

The correlation analysis of HDAC6 expression and mismatch repair genes (MLH1, MSH2, MSH6, PMS2) was analyzed by Pearson correlation analysis. The results indicated there existed some negative correlation between HDAC6 and MSH2, although with no significant *P* value (r = -0.205, P = 0.060). However, there was almost no correlation with the other 3 mismatch repair genes (MLH1, P = 0.328; MSH6, P = 0.143; PMS2, P = 0.549). Detailed analysis was shown in **Table 4**.

Discussions

As a member of the class II HDACs, HDAC6 was firstly cloned from mouse and human in 1999 [8, 9]. HDAC6 contains two functional tandem deacetylase domains and each domain has complete biological function and activates HDAC6 [10]. Studies about correlation between HDAC6 and cancer were not so much, and got contradictory conclusions [5-7]. However, the correlation between HDAC6 and colon cancer had not been reported yet. In order to study the correlation between HDAC6 and the tumor development and prognosis of colon cancer patients and elucidate the potential molecular mechanism, we deliberately chose 90 colon cancer cases with more than 5 years' follow-up information. Meanwhile, we shared extra mismatch repair genes (MLH1, MSH2, MSH6, PMS2) with immunohistochemical data. Immunohistochemical technique and statistical analysis were applied to study the expression and clinical value of this protein in colon cancer patients.
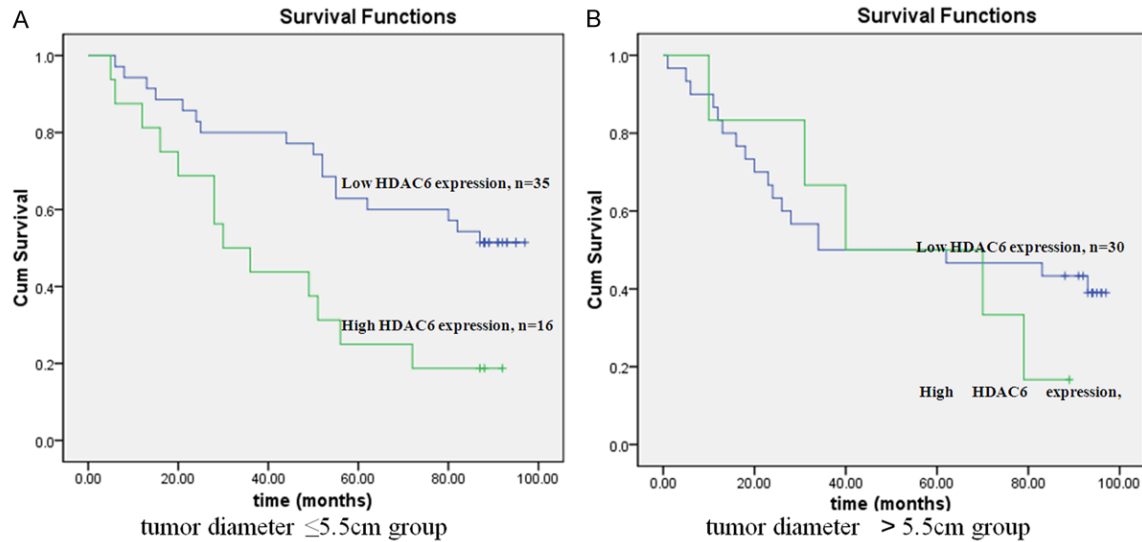
Figure 3. Relationship between HDAC6 expression and prognosis of different tumor-size patients in colon cancer: The prognosis of patients with high HDAC6 expression was significantly worse than those with low HDAC6 expression (18.8% vs 51.4%, P = 0.006) in the smaller tumor-size groups (A). However, among the bigger tumor-size groups, HDAC6 expression did not significantly affect the patient's prognosis (P = 0.532) (B).

Table 4. Relationship analysis between HDAC6 expression and mismatch repair genes (MLH1, MSH2, MSH6, PMS2)

		MLH1 expression	MSH2 expression	MSH6 expression	PMS2 expression
HDAC6 expression	Pearson Correlation	.107	-.205	-.157	.066
	Sig. (2-tailed)	.328	.060	.143	.549
	N	86	85	88	86

Experimental results show HDAC6 was specially expressed in cytoplasm and the positive rate was significantly higher in colon cancer tissue than para-carcinoma tissue, which indicated HDAC6 was a bona fide oncogene. However, clinical parameters analysis showed that the expression of HDAC6 was negatively correlated with tumor size in colon cancer patients significantly (r = -0.213, P = 0.048). In another word, patients with smaller tumors expressed higher HDAC6. We speculated that HDAC6 could promote colon cancer progression of patients especially those with smaller tumors through promoting cell growth and proliferation. However, it had no obvious role in bigger tumor-size colon cancer patients for the accomplishment of tumor cell growth and proliferation. In order to confirm this hypothesis, univariate analysis between HDAC3 expression and survival time was estimated. The results showed patients with HDAC6 overexpression had worse prognosis (18.2% vs 47.0%, P = 0.013) and HDAC6

was an independent prognostic factor (P = 0.033). Meanwhile, Survival analysis according to the tumor size indicated patients with HDAC6 overexpression had worse prognosis of colon cancer patients with tumor diameter ≤5.5 cm (18.8% vs 51.4%, P = 0.006), showed no effect on patients bearing tumor diameter >5.5 cm (P = 0.052). This conclusion further verified HDAC6 played the cancer promoting role in primarily smaller colon tumors. It comes to the hypothesis that the survival of the colon cancer patients could be greatly improved possibly by targeted treatment at early stage of tumor growth among patients with high HDAC6 expression. On the other hand, patients with larger tumors might not benefit from targeting therapy of HDAC6.

Recently, the suppressive activities of HDAC inhibitors have been confirmed in tumor cell migration, invasion and metastasis, which become a research hotspot of cancer targeted

therapy [11, 12]. Seidel C et al identified two novel HDAC6 inhibitors could reduce cell proliferation, colony forming in prostate cancer cells and increase the acetylation levels of α-tubulin and HSP90 [13]. Kaliszczak et al [14] indicated HDAC6 inhibitor C1A inhibited proliferation and induced apoptosis of colon cancer cell line; the inhibitor decreased the growth of colon tumors up to 78% in mouse. These therapeutic effect provided research foundation for C1A, which could be applied to clinical therapy.

There were fewer articles reporting the molecular mechanism of HDAC6. Previous study confirmed the interaction between MSH2 and HDAC6 in vivo. They reported HDAC6 sequentially deacetylates and ubiquitinates MSH2, leading to MSH2 degradation, reduces cellular sensitivity to damaging agents and decreases cellular DNA mismatch repair activities [15]. Mismatch repair (MMR), an important tumor-suppressive gene, is a major genome maintenance system that ensures genetic stability by correcting DNA biosynthetic errors and suppresses tumor development indirectly. We analyzed the immunohistochemical data of mostly frequent mismatch repair genes (MLH1, MSH2, MSH6, PMS2) in human colon cancers and the correlation between their expression and HDAC6. The results indicated there were almost no correlation with the MLH1, MSH6, PMS2 and some negative correlation between HDAC6 and MSH2 with no significant P value (r = -0.205, P = 0.060) in colon cancer. Combined with previous reports, we speculated HDAC6 could promote tumor development and decrease survival rate through inhibiting mismatch repair gene function in colon cancer patients. However, the unremarkable correlation between HDAC6 and MLH2 might lie in the involvement and effect of other genes.

In conclusion, we first found out the correlation between HDAC6 and prognosis of colon cancer patients, and speculated the potential molecular mechanism. Based on the obvious findings, we will next construct HDAC6 overexpressed as well as knockdown colon cancer cell lines. At the same time, further studies are needed to investigate HDAC6 signaling networks and molecular mechanism of targeted therapy with different cancer drugs and HDAC6 inhibitors.

Disclosure of conflict of interest

None.

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