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Original Article

Expression of HDAC9 in gastric cancer and its clinical significance

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Abstract: Our experiments mainly studied the expression of HDAC9 and the clinical index of gastric cancer patients. The data showed that HDAC9 was localized in the nucleus. The expression was significantly increased in cancer tissues compared to para-carcinoma tissues (73.25% ± 23.01% VS 59.74% ± 22.61%, P=0.000) with no correlation of each other (P=0.672). At the same time, we also found that the expression of ki67 was positively correlated with HDAC9 in gastric cancer tissues (r=0.404, P=0.000). Subsequently clinical parameters correlation analysis confirmed that HDAC9 expression in cancer tissues was not significantly correlated with any clinical factor (P>0.050), while HDAC9 expression in adjacent tissues was negatively correlated with M staging (r=-0.223, P=0.048). Survival analysis revealed that HDAC9 expression in cancer tissues was not associated with the prognosis (P=0.835), while HDAC9 expression in para-carcinoma tissues showed significant correlation with the survival time (P=0.002) and was the only independent prognostic factor (P=0.002). Thus, we speculated that HDAC9 may act two completely different biological functions in cancer tissues and adjacent tissues through different gene networks, respectively. The expression of HDAC9 in gastric cancer tissues might promote cell proliferation, thus could indirectly reduce the patients' prognosis. While the expression of HDAC9 in para-carcinoma tissues could prevent tumor cell from distant metastasizing, then improved the prognosis of patients directly. Further studies were needed to investigate HDAC9 signaling networks in gastric cancer cells, normal gastric epithelial cells and metaplastic cells.

Keywords: Tissue microarray, immunohistochemistry, HDAC9, gastric cancer, prognosis

Introduction

Gastric cancer is one of the most common diagnosed cancers in the world, especially in the developing countries [1]. Though the incidence rate of gastric cancer was decreased in recent decades in China, there were still over 1,000,000 newly diagnosed invasive gastric cancer cases in 2015 [2]. Therefore, the characterization of the molecular mechanism involved in development and progression of gastric cancer could be helpful to identify the molecular profiles underlying aggressiveness, clinical behavior, and response to therapy as well as to better classify the subsets of gastric cancer patients with different prognosis and/or clinical outcome.

Histone deacetylases (HDACs) is emerging as important epigenetic therapeutic targets in cancer treatment. HDACs regulate the acetylation status of histone protein and a variety of non-histone proteins including transcription factors and cytoskeletal elements by acetylating or deacetylating the lysine residues within the NH2-terminal tail. To our knowledge, HDACs has been detected and classified into four types based on their structure and homology.HDAC9 belongs to Class II and is mapped on chromosome 7p21, a region that is related to several human cancers [3]. HDAC9 is known to target non-histone proteins, such as forkhead box protein 3, ataxia telangiectasia group D-complementing protein (ATDC), which are members of pathways implicated in carcinogenesis [4, 5].

HDAC9 regulates a wide variety of normal and abnormal physiological functions, including suppression of the sonic hedgehog signaling pathway, mediating the effect of neuronelicited electrical activity, regulating activity-dependent

Table 1. Difference of HDAC9 expression in gastric cancer tissues and para-carcinoma tissues

Histology	No.	Expression score	*P*-value
Gastric cancer tissue	77	73.25% ± 23.01%	0.000
Adjacent normal tissue	77	59.74% ± 22.61%	

Mean ± Std. Deviation.

Table 2. Correlation between HDAC9 expression in gastric cancer tissues and in para-carcinoma tissues

		HDAC9 expression in para-carcinoma tissue
HDAC9 expression in cancer tissue	Correlation Coefficient	0.049
	P-value	0.672
	N	77

Table 3. Correlation between the expression of HDAC9 and ki67 in gastric cancer

		Ki67 expression in cancer tissue
HDAC9 expression in cancer tissue	Correlation Coefficient	0.404
	P-value	0.000
	N	96
HDAC9 expression in para-carcinoma tissue	Correlation Coefficient	0.047
	P-value	0.683
	N	78

gene expression and dendritic growth in developing cortical neurons, regulating the expression of fatty acid synthase enzyme and possibly regulating Ras-mediated epigenetic silencing of Fas [6-9]. HDAC9 shows an ambivalent role in different cancers, suggesting tissue- and tumor-specific expression patterns of HDAC9 [10-15]. However, its role in gastric cancer is still unclear.

We here examined the immunohistochemical expression of HDAC9 in a tissue microarray containing 100 gastric cancer tissues and 80 adjacent para-carcinoma tissues and analyzed associations between its expression levels and pathologic factors to investigate the potential role of HDAC9 in gastric carcinogenesis.

Materials and methods

Clinical materials

Gastric cancer tissue microarray (HStmA180-Su08) was obtained from Shanghai Outdo Biotech Co., Ltd. Formalin-fixed paraffin embedded tissue samples from 100 patients were used to construct tissue microarray (TMA), in which there were 80 samples with their adjacent para-carcinoma tissues (the para-carcinoma tissue was 1.5 cm away from its cancer tissue). The TMA contained well-documented clinicopathological information, including patients' gender, age, tumor size, tumor differentiation, T stage, N stage, distant metastasis and clinical stage. The patients' clinical information can be found in **Table 4**.

The follow-up information of gastric cancer patients were shown as follows: the operation time was from July 2006 to April 2007 and the eventual follow-up time in July 2015, which followed 99-108 months. During this follow-up time, 71 patients were died of gastric cancer, with a median overall survival time of 20 months (1-99 months); 26 patients were still alive, with a median follow-up time of 100 months (99-107 months); 3 patients were lost to follow-up in August 2013, and they were included in the survival group when statistical analysis was conducted. All patients were clinicopathologically diagnosed as gastric cancer and received no extra treatment before surgery.

Immunohistochemistry

Tissue sections were incubated in EDTA to retrieve antigen. To remove the endogenous peroxidase activity, sections were treated with freshly prepared 3% hydrogen peroxide in methanol. Tissue sections were blocked with goat serum and subsequently incubated with primary antibody which anti-HDAC9 (1:100000, ab109446, Abcam) at 4°C overnight. The sections were incubated with secondary antibody (HRP-labeled anti-mouse antibody, DAKO), then washed with PBS, visualized using diaminobenzidine (DAB) system and hematoxylin redying. Three visual fields from different areas of each specimen were chosen at random and more than 100 cells in each area were calculated for the immunohistochemistry evaluation. HDAC9 expression was scored according

Table 4. Correlation between clinicopathological parameters and HDAC9 expression in gastric cancer and para-carcinoma

Clinical parameters	Expression of HDAC9 in gastric cancer		*P* value	Expression of HDAC9 in para-carcinoma		*P* value
	Low	High		Low	High	
Gender			0.857			0.308
Male	12	50		23	24	
Female	6	29		13	20	
Age			0.059			0.983
≤60	6	26		11	14	
>60	12	52		23	30	
Lost		1		2		
Tumor size			0.493			0.282
≤5 cm	8	41		16	27	
>5 cm	10	36		20	17	
Lost		2				
Grade			0.322			0.610
II	2	13		6	7	
III	14	58		26	31	
IV	2	8		4	6	
T stage			0.623			0.315
T1	1	6		3	3	
T2	1	6		2	3	
T3	11	47		27	22	
T4	3	11		2	9	
Lost	2	9		2	7	
N stage			0.088			0.732
N0-N2	14	51		27	29	
N3	4	26		8	14	
Lost		2		1	1	
M stage			0.745			0.048
M0	18	70		30	41	
M1	0	8		6	2	
Lost		1			1	
cTNM stage			0.145			0.394
1	0	10		3	4	
2	10	20		12	16	
3	8	39		14	21	
4	0	8		6	2	
Lost		2		1	1	

to the percentage of positive cells. HDAC9 expression was classified as follows: ≤55% low expression, >55% high expression.

Statistical analysis

The positive rate of immunohistochemical staining was statistically analyzed. The differences of HDAC9 expression in carcinoma tissues and para-carcinoma tissues were analyzed by paired T test. The correlations between HDAC9 expression in cancer tissues and para-carcinoma tissues, as well as ki67 expression were analyzed by Pearson's correlation. The correlations between the expression of HDAC9 and the clinicopathological parameters of gastric cancer patients were calculated by Spearman's correlation analysis. The relationships between the overall survival time of patients and HDAC9 expression as well as clinical data were analyzed by 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. P< 0.05 was considered to be statistically significant.

Results

Expression of HDAC9 in gastric cancer tissues and para-carcinoma tissues

As the immunohistochemistry results shown in **Figure 1** and **Table 1**, HDAC9 specifically located in nuclear in both gastric cancer tissues and para-carcinoma tissues. The specimens which positive rate >55% of HDAC9 were divided into high expression group. There were 79 specimens with HDAC9 high expression in the nuclear of cancer tissues, account for 79.00%; while there were 48 specimens with HDAC9 high expression in the nuclear of para-carcinoma tissues, account for 60.00%. Paired T-Test analysis indicated that: the positive rate of HDAC9 in gastric cancer tissues was significantly higher than that in para-carcinoma tissues (73.25% ± 23.01% VS 59.74% ± 22.61%, P=0.000). The representative pictures of the immunohistochemistry were shown in **Figure 1**.

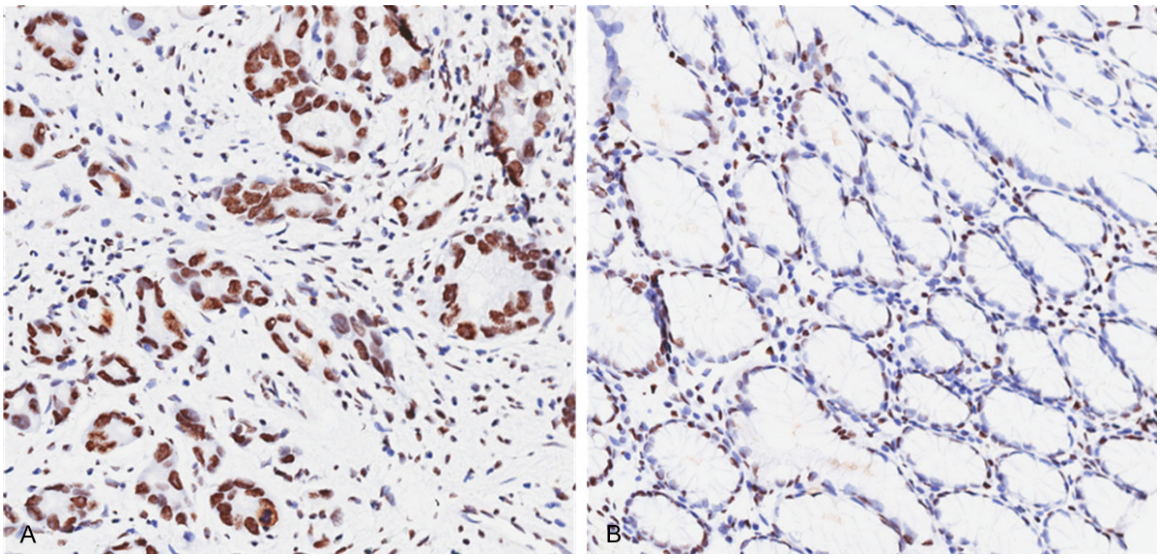

Figure 1. Immunohistochemistry of HDAC9: HDAC9 was specifically expressed in nuclei. The positive staining rate of HDAC9 in gastric cancer tissues (A) was significantly higher than that in para-carcinoma tissues (B). (Original magnification: ×200).

Spearman's correlation analysis was applied to study the correlation between the expression of HDAC9 in cancer tissues and para-carcinoma tissues. The results indicated no correlation between them (P=0.672). Detailed analysis results were shown in **Table 2**.

Correlation between the expression of HDAC9 and ki67 in gastric cancer

To test if HDAC9 correlated with tumor cell proliferation, we analyzed the correlation between the expression of HDAC9 and ki67, which was commonly present in proliferating tumor cells and absent in quiescent cells. The results indicated that the expression of HDAC9 was significantly correlated with ki67 in gastric cancer tissues (r=0.404, P=0.000); but on the other hand, there was no relationship between HDAC9 expression in para-carcinoma tissues and ki67 in cancer tissues (P=0.683). Analysis results were shown in **Table 3**.

Relationship between clinicopathological parameters and HDAC9 expression in gastric cancer patients

The relationships between HDAC9 expression and clinicopathological parameters of gastric cancer patients were evaluated by Spearman's analysis (**Table 4**). The results showed that HDAC9 expression in gastric cancer tissues wasn't associated with any clinical factor (P>0.050), while the expression of HDAC9 in para-carcinoma tissues was negatively correlated with distant metastasis (r=-0.223, P= 0.048).

HDAC9 expression in para-carcinoma tissues positively correlated with the prognosis

Kaplan-Meier analysis and log-rank test were applied to determine the association between HDAC9 expression, clinical parameters and the prognosis of gastric cancer patients respectively. The results showed that the expression of HDAC9 in cancer tissues was not correlated with the prognosis of gastric cancer patients (P=0.835), while HDAC9 expression in para-carcinoma tissues was positively correlated with the overall survival time of the patients (34.1% VS 11.1%, P=0.002). Detailed analysis results were shown in **Figure 2**. In addition, tumor size, pathological grade, T staging, N staging, M staging and clinical staging were all significantly negatively correlated with the patients' prognosis (P<0.050), while gender and age were not correlated (P≥0.050).

Subsequently, COX multi-factors analysis showed that HDAC9 expression in para-carcinoma tissues was the only independent predict factor (P=0.002). The detailed analysis results were shown in **Table 5**.

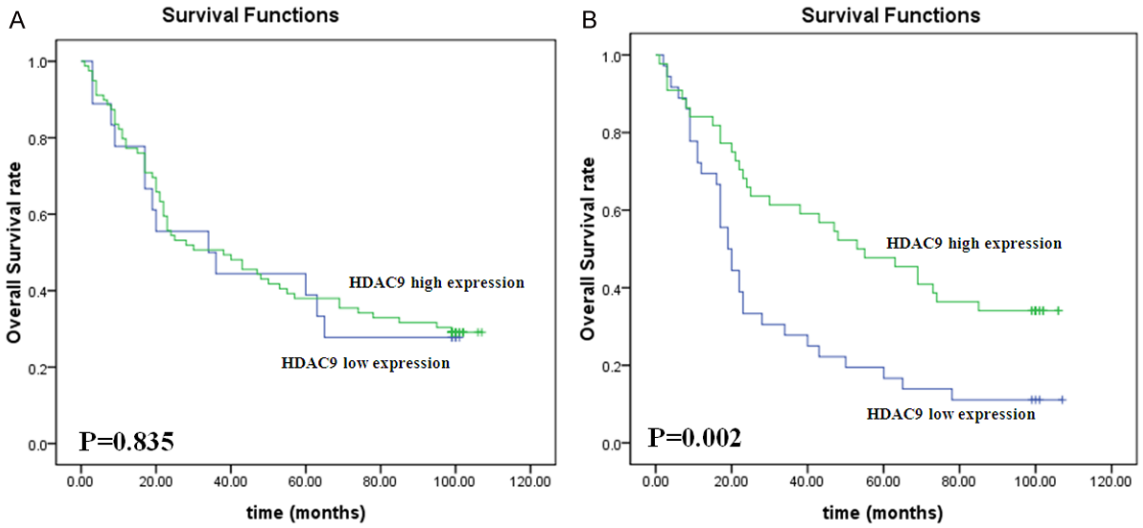

Figure 2. Kaplan-Meier survival curves dependent on (A) HDAC9 expression in gastric cancer tissues (B) HDAC9 expression in para-carcinoma tissues. *P* values were calculated with log-rank test.

Table 5. Independent prognostic factor in gastric cancer patients by Cox multivariate analysis

Variables in the Equation								
	B	SE	Wald	df	*P*-value	Exp (B)	95.0% CI for Exp (B)	
							Lower	Upper
HDAC9 in para-carcinoma	-1.057	0.343	9.485	1	0.002	0.347	0.177	0.681
Tumor size	-0.116	0.345	0.112	1	0.738	0.891	0.453	1.752
Grade	0.495	0.299	2.749	1	0.097	1.641	0.914	2.948
T staging	0.542	0.333	2.644	1	0.104	1.720	0.895	3.306
N staging	0.126	0.184	0.465	1	0.495	1.134	0.790	1.628
M staging	0.210	0.700	0.090	1	0.764	1.234	0.313	4.861
cTNM staging	0.726	0.376	3.733	1	0.053	2.067	0.990	4.319

Discussion

Regarding the important role of HDACs in DNA modification and gene expression regulation, accumulating evidence suggests that HDACs also play important roles in various cancers. As a member of HDACs family, the researches on the expression of HDAC9 and the prognosis of cancer were contradictory, in which the most studies showed the carcinogenicity of HDAC9. For example, there was a significant negative correlation between HDAC9 expression and the disease-free survival time of childhood acute lymphoblastic leukemia patients [16], and HDAC9 could promote cell proliferation of breast cancer and osteosarcoma [12, 17]. On the other hand, there were some opposite results. For example, Okudela et al. found lower HDAC9 expression in lung cancer cells comparing to the normal tissues, and high expression of HDAC9 could inhibit the growth of lung cancer cells, which indicating HDAC9 might be a tumor suppressor [15]. To our knowledge, there was no report about the relationship between HDAC9 expression and gastric cancer.

In this study, we firstly researched the relationship between HDAC9 and gastric cancer. Our results showed that HDAC9 was localized in the nucleus and the expression of HDAC9 in gastric cancer tissues was significantly higher than that in the adjacent tissues (73.25% ± 23.01% VS 59.74% ± 22.61%, P=0.000) with no correlation of each other (P=0.672). At the same time, we also found that the expression of ki67 was positively correlated with HDAC9 in gastric cancer tissues; but were completely unrelated with HDAC9 expression in adjacent

tissues. We speculated that HDAC9 might perform different biological functions in cancer tissues and adjacent tissues of patients with gastric cancer. Subsequently clinical parameters correlation analysis confirmed that HDAC9 expression in cancer tissues was not correlated with any clinical factor (P>0.050), while HDAC9 expression in adjacent tissues was significantly negatively correlated with M staging (r=-0.223, P=0.048). Survival analysis showed that the expression of HDAC9 in cancer tissues was not correlated with the survival time (P=0.835), while the prognosis of patients with high HDAC9 expression in para-carcinoma tissues was significantly better (34.1% VS 11.1%, P=0.002) and it was also the independent prognostic factor (P=0.002). Thus, we speculated that the mechanism of HDAC9 in gastric cancer was complex, and may act two completely different biological functions in cancer tissues and adjacent tissues through different gene networks, respectively. On the one hand, the expression of HDAC9 in gastric cancer tissues might promote cell proliferation, thus could indirectly reduce the patients' prognosis. On the other hand, the expression of HDAC9 in para-carcinoma tissues could inhibit tumor cell from distant metastasizing, then improved the prognosis of patients.

Many of the previous literatures on HDAC9 suggested it's carcinogenicity in cancers. For example, HDAC9 expression promoted proliferation, growth and development of retinoblastoma cell in vivo, while knockdown of it resulted in cell G1 phase arresting, thus inhibited tumor cell growth and development [11]. Rastogi et al. found that high HDAC9 expression promoted cancer cell growth via inhibiting the expression of MEF2D, a survival factor that could perform pro-apoptosis function in oral squamous cell carcinoma by activating orphan nuclear hormone receptor gene NR4A1/Nur77, thus decreased the patients' survival time [10]. Above all, we assumed that the expression of HDAC9 in gastric cancer tissues may promote tumor progress through the similar gene network, though it might not be the main regulatory member and could not directly affect patients' prognosis.

On the other hand, some reports showed that HDAC9 acted as a tumor suppressor through preventing the combination of ATDC and p53. ATDC was an important oncogene, which could inhibit the activity of p53 by binding itself to p53 and inhibit the transcription of downstream genes such as p21, then promoted the growth and proliferation of cancer cells. HDAC9 could weaken its combination with p53 through acetylating the lysine residues of ATDC, thus restored the cancer suppressive ability of p53 [5]. However, the expression of ATDC was significantly higher in gastric cancer tissues with almost no expression in adjacent tissues [18]. Thus we assumed that the expression of HDAC9 in para-carcinoma tissues may suppress gastric cancer via other gene regulative pathway but not ATDC. Another important function of HDAC9 was to control the fate of regulatory T (Treg) cells. HDAC9 could simultaneously inhibit the activity of Treg cells and the transformation of the normal T cells to Treg cells in various autoimmune diseases [4]. As we known before, Treg cells could inhibit the immune response of the host, thus enhanced tumor immune escape in some cancers [19]. We speculated that HDAC9 expression in para-carcinoma tissues could enhance the immune-mediated tumor cells killing ability of the host by decreasing the number of Treg cells, subsequently reduced the risk of distant metastasis and improved the prognosis of patients in gastric cancer.

These results provided the potential role of HDAC9 in gastric cancer. From these, we believed that not all cancer patients could benefit from the treatment of HDAC9 inhibitors, sometimes even be injured. Therefore, we must be more cautious about the clinical application of HDAC inhibitors, taking more researches. Further studies are needed to investigate HDAC9 signaling networks in gastric cancer cells, normal gastric epithelial cells and metaplastic cells.

Disclosure of conflict of interest

None.

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