

## Original Article

# Histone deacetylases 3 (HDAC3) is highly expressed in cervical cancer and inhibited by siRNA

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**Abstract:** Objective: To detect the expression of histone deacetylase 3 (HDAC3) in cervical cancer tissue and investigate the inhibition of HDAC3 by Small Interference RNA (siRNA) on the effect of HDAC3 in Hela cell line of cervical cancer. Methods: The expression of HDAC3 in 12 specimens of normal cervical tissues, 21 specimens of moderate to severe cervical intraepithelial neoplasia and 64 specimens of cervical carcinoma were measured by reverse transcription-Polymerase Chain Reaction (RT-PCR) and immunohistochemistry (IHC). RT-PCR and Western blotting were used to detect the expression of HDAC3 in Hela after transfected with siRNA and control RNA. Results: The expression levels of HDAC3 mRNA in normal cervical epithelia, CIN II~III and cervical cancer were (0.110±0.020), (0.756±0.065) and (0.310±0.021) respectively. The rate of HDAC3 positive expression in normal cervical epithelia, CIN II~III and cervical cancer were 8% (1/12), 38% (8/21), 94% (60/64) respectively. Significant down-regulation of the expression of mRNA and protein levels of HDAC3 was found following transfection of the HDAC3 siRNA. The results of IHC revealed that there were relationship between the expression of HDAC3 protein and the degree of tumor differentiation, myometrial invasion and lymph node metastasis. Conclusions: HDAC3 may play an important role in the course of cervical cancer. The in vitro transient transfection of synthesized HDAC3-siRNA on the Hela cell line can down-regulate the expression of mRNA and protein levels in the cells. siRNA is likely to be a new way to treating the cervical cancer.

**Keywords:** Cervical carcinoma, histone deacetylase 3 (HDAC3), Hela cell lines, small Interference RNA (siRNA)

## Introduction

Cervical carcinoma is one of the most widely female genital neoplasms and occupies leading causes of female deaths in developing country. Cervical carcinoma is characterized as substantial morbidity, easily metastasis and easily recurrence [1]. As popularization of gynecologic examination rising incidence of cervical carcinoma is detected by examination, which brings huge pressure on family and society. Although a multitude of studies had carried out to investigate the happen of cervical carcinoma, the exact mechanism remains to be elucidated. Molecular biotechnology had revealed the complex process in formation of cervical carcinoma, which involves a variety of tumor related molecular [2]. Histone deacetylases (HDACs), a potential target against many tumors, is associated with regulation of gene expression in a slice of tumor cells [3, 4]. HDACs

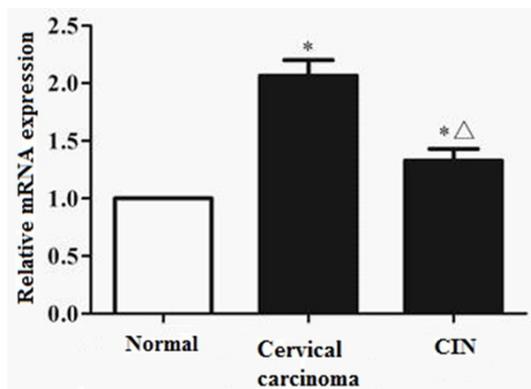
isoforms are known that could be classified into four classes in which HDAC3 belongs to I type [5]. Up to now few studies have investigated the association between HDAC3 expression and pathological characteristic of cervical carcinoma thereby in this study we detected HDAC3 expression by RT-PCR and immunohistochemical to get comparisons in normal cervical tissue, cervical intraepithelial neoplasia tissue (CIN) and cervical carcinoma tissues respectively. Subsequently using RNA interference we transfected HDAC3 is into Hela cells to determine if the interference with HDAC3 could inhibit Hela cells.

## Materials and methods

### Tissue sample

64 pathological specimens that had been diagnosed as cervical carcinoma were selected

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**Figure 1.** Expressions of mRNA in different cervical specimens: A: normal cervical specimens; B: CIN (II~III grade) specimens; C: cervical carcinoma specimens. Every experiment was repeated three times. \*, cervical carcinoma specimens vs. normal cervical specimens,  $P < 0.05$ ;  $\Delta$ , CIN (II~III grade) specimens vs. normal cervical specimens,  $P < 0.05$ . Error bars represent S.D.

from outpatient surgery and receiving operation between Jan 2012 and Dec 2013. The ages of patients selected ranged from 26 to 71 years (mean,  $46.8 \pm 11.6$  years). In the same period, 21 pathological specimens that had been pathologically confirmed as CIN were also enrolled with ages ranging from 24 to 65 years (mean,  $41.7 \pm 10.9$  years). 12 cervical specimens drawing from hysterectomy due to uterine benign disease were selected as control group, of which ages ranged from 28 to 61 years (mean,  $45.8 \pm 9.2$  years). None of the patients had received any tumor-specific therapy including operation, radiotherapy or chemotherapy before obtaining specimens and didn't contain other tumors.

### Cell culture and treatment

Human cervical carcinoma cell lines HeLa (from shanghai institute of cell biology) were cultured in DMEM (Gibco, USA) with 10% fetal bovine serum (Gibco) and all cells were maintained at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$ .

### siRNA interference

HeLa cells were plated at density of  $1 \times 10^5$  cells per well into six-well plate with 2 ml DMEM containing 10% fetal bovine serum and incubated until they reached 90% confluence. Simultaneously, mixture containing 0.4  $\mu\text{g}$  DNA, DNA-condensation buffer and Buffer EC were incubated for 3~5 min at  $37^\circ\text{C}$ . After

centrifugation 10  $\mu\text{l}$  Effectance Transfection Reagent was added and vibrated for 10 s. After removal of medium well was washed with PBS then the mixture and 1.6 ml DMEM were put into wells. 6 h after incubation at  $37^\circ\text{C}$  in a 5%  $\text{CO}_2$  humidified incubator, the serum-free medium was discarded and replaced with complete medium.

### Semi-quantitative RT-PCR

Cells were harvested 48 h after transfection and wells were added in 500  $\mu\text{l}$  Trizol. Spectrophotometer was used to measure the ratio of A260/280 in the 260/280 nm wavelengths. According to the protocol RNA was extracted and subsequently was reverse transcribed into cDNA. Using semi-quantitative RT-PCR to amplify cDNA in which PCR primer sequence of HDAC3: F: 5'-CGTCCGAAATGTGC-3', R: 5'-GAAGTTCCTCACTATGG-3'; fragment length augmented was 432 bp. Gel Imaging System scanned detached DNA fragment.

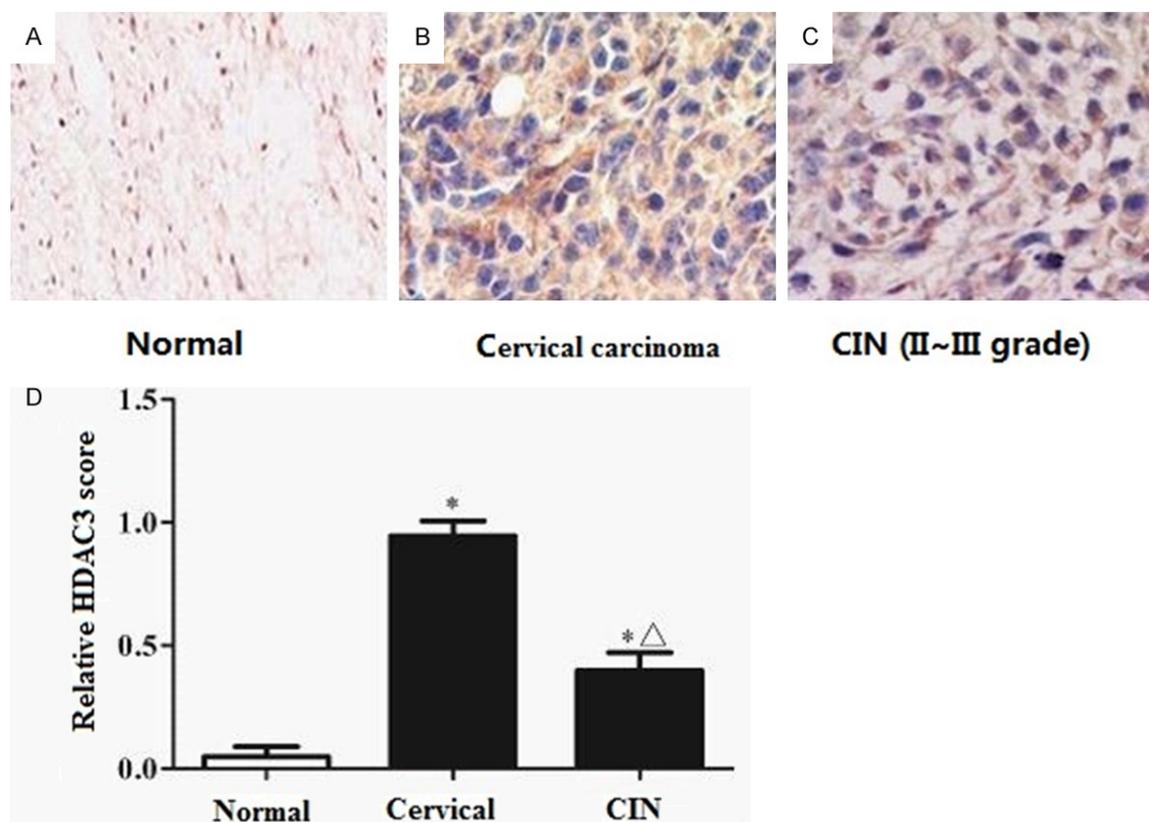
### Immunohistochemical staining and evaluation immunoreactivity

Tissue samples were cut into 5  $\mu\text{m}$  slices and the slides were processed by microwave heating in 0.01 M sodium citrate (pH 6.0) for antigen retrieval. After blockage with 5% milk slides were incubated with primary antibodies for 24 h. The following secondary antibodies were added and then coloration with DAB and counterstain with Mayer's haematoxylin for 30 seconds.

### Western blotting

Proteins were extracted using Protein Lysis Buffer on ice for 30 min then was quantified by BCA quantification kit-rapid. After  $100^\circ\text{C}$  coction with 5  $\times$  loading buffer, proteins were transferred to SDS-PAGE electrophoresis for 40 min. After SDS/PAGE, proteins were transferred to a PVDF membrane and then filters were blocked with 5% BSA dissolved in PBS containing 0.18% Tween-20 by incubation overnight at  $4^\circ\text{C}$ . After washing in PBS-Tween-20, the filters were probed with primary antibodies (HDAC3: 1:1000;  $\beta$ -actin: 1:5000) and then following secondary antibodies (1:5000) incubation for 2 h. Having been wash twice proteins were detected using enhanced chemiluminescence (ECL).

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**Figure 2.** HDAC3 expression positively correlates with cervical cancer malignancy: HDAC3 in expression was detected with Immunohistochemistry using rabbit anti-HDAC3. HDAC3 expression was different in A: normal cervical specimens; B: cervical carcinoma specimens; C: CIN (II~III grade) specimens. D: Plot representation of scores according to immunohistochemical expression of HDAC3 in different cervical specimens. A total of 64 specimens were analyzed. The scores were calculated by intensity × percentage of stained cells. \*, cervical carcinoma specimens vs. normal cervical specimens,  $P < 0.05$ ; △, CIN (II~III grade) specimens vs. normal cervical specimens,  $P < 0.05$ . Error bars represent S.D.

### Statistical analysis

Data was analyzed using SPSS software (version 16.0). Continuous variables were expressed as mean ± SD and mean differences between two groups were compared using Student's t-test. ANOVA was used to analyze comparison between multiple samples.  $P < 0.05$  was considered as statistically significant.

### Results

#### Comparison of HDAC3 mRNA expression in three groups of cervical specimens

Results of semi-quantitative RT-PCR demonstrated that the expression of HDAC3 mRNA in cervical carcinoma specimens was more than CIN (II~III grade) specimens ( $0.756 \pm 0.065$  vs.

$0.310 \pm 0.021$ ,  $P < 0.01$ ) and normal cervical specimens ( $0.756 \pm 0.065$  vs.  $0.110 \pm 0.020$ ,  $P < 0.01$ ). In the other hand, mRNA expression level of CIN (II~III grade) specimens was higher than normal specimens ( $0.310 \pm 0.021$  vs.  $0.110 \pm 0.020$ ,  $P < 0.01$ ). Representative comparison of stripes was shown as **Figure 1**.

#### Comparison of HDAC3 proteins expression in three groups of cervical specimens

Proteins expressed in three groups of cervical specimens were detected using immunohistochemical staining. The highest expression level of HDAC3 was detected in cervical carcinoma specimens, next in the CIN (II~III grade) specimens and the lowest expression level in the normal cervical specimens (positive rates were 98%, 38% and 8% respectively,  $P < 0.05$ , **Figure 2**).

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**Table 1.** Clinical and pathological characteristics of cervical cancer sufferers (n=64)

Clinical and pathological characteristics		Total cases (n)	Cases of HDAC3 up-regulated expression (n)	Positive rate (%)	P value
Age	< 50 year	28	25	89.3	0.446
	≥ 50 year	36	34	94.4	
Clinical stage	≤ Ib2	33	24	72.7	0.479
	≥ Ib2	31	20	64.5	
Differentiated degree	High	13	7	53.8	0.047
	Moderate	32	26	81.2	
	Low	19	17	89.5	
Vessel invasion	Yes	45	34	75.6	0.314
	No	19	12	63.2	
Muscular invasion	< 1/2	13	9	69.2	0.026
	≥ 1/2	51	47	92.2	
Lymphatic metastasis	Yes	31	22	71.0	0.041
	No	33	30	90.9	
Pathological pattern	Squamous cell carcinoma	43	39	90.7	0.549
	Adenocarcinoma	21	18	85.7	

### *Association of HDAC3 proteins expression and pathological characteristics of cervical carcinoma*

As shown in **Table 1**, HDAC3 proteins expression are positively correlated with pathology grade, invasive depth of muscular layer and lymphatic metastasis ( $P < 0.05$ ) but there is no significant correlation between HDAC3 proteins expression and ages of patients, invasive depth of vessel and clinical stages ( $P > 0.05$ ).

### *HDAC3 siRNA interference for Hela cells*

Hela cells were transfected with HDAC3 siRNA or vector siRNA, consequently mRNA and proteins expression level was down regulation in Hela cells transfected with HDAC3 siRNA in comparison of vector siRNA group ( $0.887 \pm 0.035$  vs.  $0.093 \pm 0.005$ ,  $P < 0.05$ , **Figure 3**).

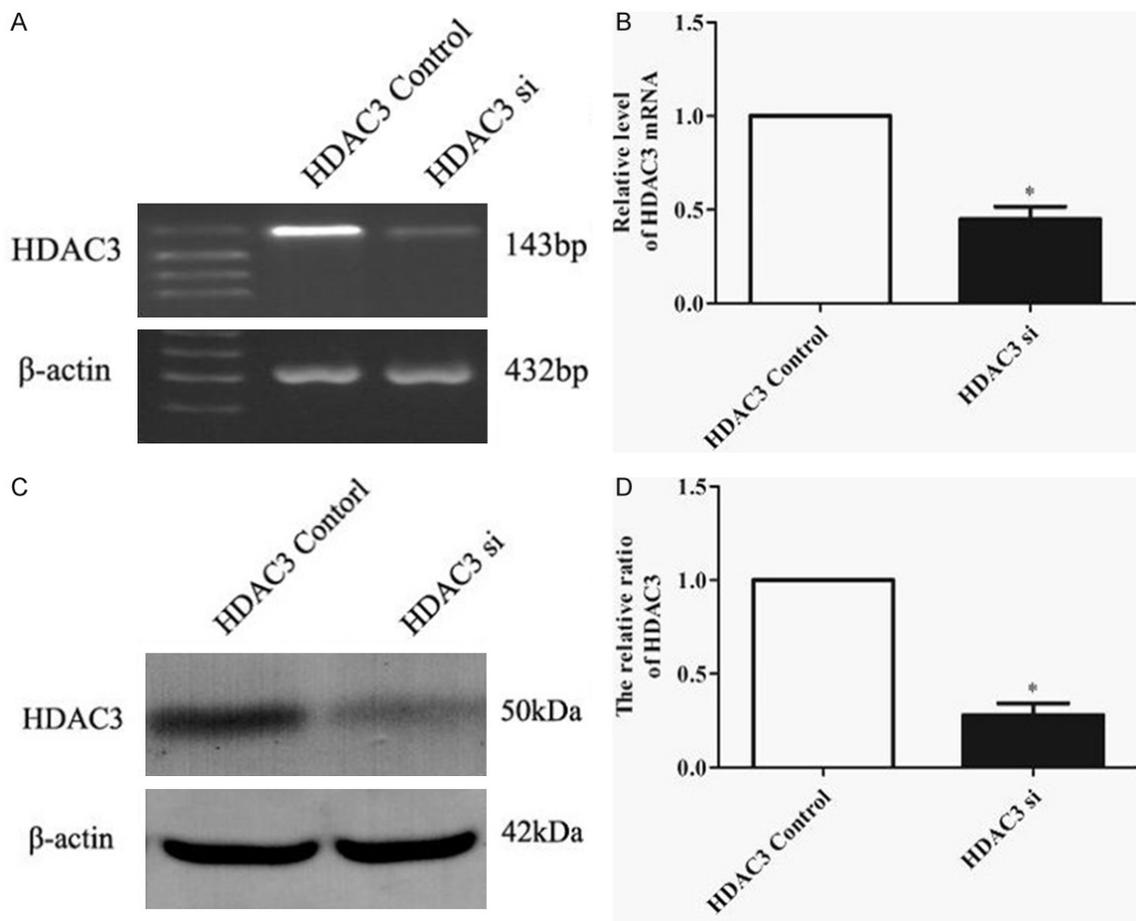
### **Discussion**

Acetylation/deacetylation of histone is crucial in maintaining chromosome stability and regulating genetic transcription [6]. Covalent modification for N-terminal amino acid of histone includes acetylation, methylation and ubiquitination, of which acetylation/deacetylation play the most important role [7]. Interworking of histone acetyltransferase (HAT) and histone deacetylase (HDAC) regulate acetylation of histone [8]. When deacetylation of histone enhancing chromosome turns to be compact, which lead to inhibition of transcription factor bind-

ing to DNA and in some situations induce tumor [9, 10]. HDACs isoforms are known that could be classified into four classes in which HDAC3 belongs to I type. Jian et al reported that HDACs lead to intestinal epithelium paraplasm by inhibiting p21 [11]. Liby et al found expression of HDACs was positively correlated with malignancy grade of tumor [12].

Cervical carcinoma is one of the most widely female genital neoplasms in all across the world. Statistical information indicates 530,000 new cases of cervical carcinoma each year, which occupies 3% of new cancer cases. Furthermore cervical carcinoma possesses high case fatality rate in female genital malignant that is attributed to fourth of all cancer deaths [13]. Thus searching an effective marker to predict cervical carcinoma in the early stage is significant for female health. A slice of molecules have been reported to be related with cervical carcinoma such as FHIT, p53, Rb, c-fos, c-myc, VEGF [14-16], but the association of HDACs and cervical carcinoma elucidated. In this study, we investigated their relation by detecting mRNA, protein expressions of HDAC3 and analyzed association of pathological feature and HDAC3 expression. Finally, we made siRNA interference trial to test the potential of HDAC3 siRNA inhibiting cervical carcinoma. Results demonstrate that HDAC3 expression is positively correlated with cervical carcinoma malignancy on matter in mRNA and protein

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**Figure 3.** HDAC3 siRNA interference for HeLa cells: HeLa cells were transfected with HDAC3 siRNA. 6 h later, the mRNA and protein expression of HDAC3 was detected by semi-quantitative RT-PCR and western blotting respectively. A: mRNA expression of HDAC3; B: Plot representation of relative level of HDAC3 mRNA; C: Protein expression of HDAC3; D: Plot representation of relative ratio of HDAC3. Data were analyzed by Student's t test. \*,  $P < 0.05$ .

expression. Further investigation of pathological feature indicates that HDAC3 proteins expression are positively correlated with pathology grade, invasive depth of muscular layer and lymphatic metastasis but there is no significant correlation between HDAC3 proteins expression and ages of patients, invasive depth of vessel and clinical stages. With siRNA interference HDAC3 expression of cervical carcinoma is significantly blocked.

Histone deacetylase inhibitors have been used in clinical therapy for many tumors and reveal their effectiveness. Borutinskaite et al had present the cell cycle blocking ability of BML-210 against cancer cells [17]. Thus HDACs are considered to be a potential target for many tumors including cervical carcinoma. In order to verify this speculation, we investigated the

interference ability using siRNA which is based on this principle that siRNA combines with single stranded mRNA to induce post-transcriptional gene silencing (PTGS) and following inhibition of gene translation [18, 19] and results confirmed the effectiveness of HDACs siRNA down-regulating HDACs mRNA and protein expression. Interference of siRNA provides potential means to inhibit cervical carcinoma.

In brief, HDAC3 expression is related to cervical carcinoma malignancy and is associated with some pathological feature including pathology grade, invasive depth of muscular layer and lymphatic metastasis. This investigation indicates a possibility of detecting HDAC3 as a marker of cervical carcinoma. Moreover siRNA interference with HDACs suggests an effective means to inhibit cervical carcinoma.

## Disclosure of conflict of interest

None.

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