Abstract

Purpose: The histone deacetylase (HDAC) inhibitor trichostatin A (TSA) has been shown to act as an anti-tumor agent; however, the effect and mechanism of TSA on the invasion of esophageal squamous cell carcinoma (ESCC) remains unknown.

Methods: To determine whether TSA suppresses the invasiveness of ESCC cell via HDAC2, the expression of HDAC2 in ESCC tissues and adjacent non-tumor tissues were compared using Western blot and immunohistochemistry. Cells were transfected with HDAC2 siRNAs and non-targeting control siRNA using Lipofectamine TM 2000. Cell invasion was investigated using a transwell assay. The protein levels of matrix metalloproteinase-2/9 (MMP-2/9) were examined by Western blot analysis.

Results: Expression of HDAC2 was significantly higher in ESCC than in adjacent non-tumor tissues. Additionally, the in vitro invasion assay found that both downregulation of HDAC2 expression and TSA treatment inhibited ESCC cell invasion by approximately 75%. Also, an MMP2/9-specific inhibitor sharply suppressed ESCC cell invasion. Furthermore, both downregulation of HDAC2 and treatment with TSA decreased MMP-2 and MMP-9 protein levels in ESCC cells.

Conclusions: These results suggest that the inhibitory effect of TSA on cancer invasion is mediated through the suppression of HDAC2 expression, and that the reduction of MMP-2 and MMP-9 expression induced by HDAC2 may be involved in the anti-invasive effect of TSA.
Esophageal squamous cell carcinoma (ESCC) is the most common malignant tumor of the digestive system. A high incidence of ESCC is mainly aggregated in northern China (especially in the Henan and Shanxi provinces), South Asia and Africa [1,2]. As one of major causes of cancer mortality in China, despite advances in multimodality therapy, the prognosis for patients with ESCC remains poor with 5-year survival rates generally below 10%. The most important reason for poor survival rate is that patients with early-stage cancers are asymptomatic; most patients are diagnosed at the late stages and tumor invasion and metastasis are the major causes of death [3,4]. The etiology of ESCC has been shown to be multifactorial including environmental and genetic factors [5] and the mechanism of ESCC has not yet been elucidated. To further improve survival rate and prognosis of patients with ESCC, novel diagnostic, therapeutic and molecular prognostic markers must be developed.

Cancer is a pathologic condition related to abnormal genetic and epigenetic events that result in neoplastic transformation [6]. Histone deacetylases (HDACs) are enzymes regulating expression of genes that are associated with various signaling pathways that influence multiple stages of tumorigenesis. By removing the acetylation of histones at active genes and modulating the acetylation of other nuclear proteins, HDACs have emerged as important targets for repressing the cellular processes and reversing the aberrant epigenetic changes associated with cancer [7,8]. As an important type of HDAC, histone deacetylase 2 (HDAC2) has been found to be significantly overexpressed in several tumor types [9,10]. Abundant evidence indicates that HDAC2 plays a crucial role in the control of cell differentiation, survival and migration [7-10]. Furthermore, HDAC2 can indirectly regulate the composition of the extracellular matrix and the levels of signaling mediators [11]. Abnormal expression of HDAC2 correlates with poor prognosis in many cancer cases [10,12,13]. Taken together, these studies indicate that HDAC2 is a promising potential target for tumor diagnosis and therapy.

The histone deacetylase inhibitor, trichostatin A (TSA), is an antifungal antibiotic that noncompetitively inhibits HDAC in cultured mammalian cells, even at low concentrations [14,15]. Recently, TSA has been demonstrated to exert strong anti-neoplastic effects in vitro and in vivo by inhibiting cell invasion and metastasis as well as suppressing tumor angiogenesis [16]. It is proving useful for cancer prevention by reactivating the expression of the epigenetically silenced genes involved in invasion and metastasis [17]. Although TSA effectively inhibit invasion of cells in culture [18], the anti-metastasis efficacy and mechanism of TSA in ESCC cell remain to be elucidated.

In the present study, immunohistochemistry and Western blot analyses were used to compare HDAC2 expression in ESCC and adjacent non-tumor tissues. To further characterize whether anti-invasion effect of TSA is mediated through downregulation of HDAC2 and MMP2/9 expression, invasion assay and Western blot were performed using HDAC siRNA and MMP2/9 inhibitorIII in an ESCC cell line.

Materials and Methods

Tissue Samples

Fresh tissues of esophageal squamous cell carcinoma were obtained from six patients who underwent surgery at The First Affiliated Hospital of Zhengzhou University (Zhengzhou, China). Written informed consent to participate in the study was obtained from each patient before surgery, according to the ethical guidelines of our university. None of patients had received chemotherapy, radiotherapy and immunotherapy before surgery. Both tumor and adjacent non-tumor tissues were sampled, as confirmed by pathological examination, and each specimen was approximate 1 cm³ in size.

Western blot analysis

Western blotting was performed according to the methods described previously [19]. Cell homogenates were prepared, 20-30 µg of proteins were fractionated by SDS-PAGE, and transferred to nitrocellulose membrane, then incubated with specific antibodies. All antibodies were from Santa Cruz Biotech, Inc. (Santa Cruz, CA, USA). Films were digitized and densitometry was performed using an imaging analysis system (Gelpro; Media Cybernetics, Silver Spring, MD, USA).

Immunohistochemistry

All the specimens were fixed in paraformaldehyde and embedded in paraffin. All procedures were performed according to previously described methods [19]. Paraffin-embedded sections (thickness, 4 µm) of tissues were deparaffinized in xylene and rehydrated in a graded series of mixtures of ethyl alcohol and water. Sections were microwaved in citrate buffer to extract antigens. Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide/methanol for 10 min. Sections were incubated with blocking solution and the primary antibody HDAC2 (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. After washing with phosphate-buffered saline, sections were incubated with biotin-labeled secondary
antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 10 min and then incubated in streptavidin peroxidase for 10 min. A diaminobenzidine (DAB) kit (Sigma Diagnostics, St Louis, MO, USA) was used to detect chromogens. The primary antibodies were replaced with PBS for a negative control. Tissue sections were then counterstained with hematoxylin, dehydrated and mounted.

Cell culture and small-interfering RNA

The human ESCC cell line, EC9706, were obtained from the American Type Culture Collection (Manassas, VA, USA). EC9706 cells were grown in RPMI-1640 medium (Gibco, Grand Island, NY, USA). All media were supplemented with 10% fetal bovine serum (PAA, Laboratories, Pasching, Germany). These cells were maintained in a humidified 37°C incubator with 5% CO₂, fed every three days with complete medium, and subcultured when confluence was reached. Both designed siRNAs for HDAC2 and non-targeting control siRNA (Santa Cruz Biotechnology, Santa Cruz, CA) were used for the study. EC9706 cells with 90% confluence were cultured in RPMI-1640 medium without any antibiotics overnight at 37°C in 5% CO₂ and then transfected with HDAC2 siRNA using Lipofectamine TM 2000 (Invitrogen Co., Carlsbad, CA, USA). After 4-6 h, RPMI-1640-containing 10% FBS was added to each well and the cells were cultured for an additional 24-72 h. After 48 h, cells were transfected with or without HDAC2 siRNA.

Cell Invasion Assay

Tumor cell invasion was performed in Matrigel-coated Boyden chamber (BD Biosciences, San Jose, CA, USA). HDACs inhibitor TSA was purchased from Sigma. MMP2/9 inhibitor III was obtained from EMD Biosciences. Briefly, cells transfected with siRNAs (scrambled or HDAC2 siRNA) were trypsinized and seeded onto the upper compartment of an invasion chamber coated with Matrigel. The lower compartment was filled with complete medium. After an incubation period of 16 h at 37°C, the filters were removed. Cells that had invaded onto the bottom of the inserts were fixed, stained and counted under a microscope. The average number of invading cells per microscopic field over the random five fields was counted in each assay from four independent experiments. The same assay was performed to determine the effects of TSA and MMP2/9 inhibitor III on the ESCC cell invasion.

Statistical analysis

Data are expressed as mean ± standard deviation. One-way ANOVA followed by the Dunnett’s multiple comparison post hoc test or the unpaired Student’s t-test were used for statistical analyses using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA).

Results

Upregulation of HDAC2 in ESCC

Western blot was used to detect HDAC expression in six ESCC tissues and adjacent non-tumor tissues. The results showed that protein levels of HDAC2 in ESCC tissues were significantly higher than adjacent tissues (Fig.1). According to the sections detected by immunohistochemistry, positive reddish staining for HDAC2 was observed in ESCC, which is mainly localized in the cytoplasm (Fig.2). Also, weak positive staining was observed in the normal tissue. No staining was observed in the IgG control.
HDAC2 siRNA, TSA and MMP2/9 inhibitor III induce inhibition of ESCC cells invasion

Western blotting showed human ESCC cell lines EC9706 expressed abundant endogenous HDAC2 (Fig. 3). To determine whether HDAC2 is involved in invasion of ESCC cells, HDAC2 siRNA was used to suppress endogenous HDAC2 expression and invasion assay to investigate invasion ability in vitro. Western blot and time-course experiments demonstrated that the expression of HDAC2 in EC9706 cells was inhibited at 24, 48 and 72 h after siRNA transfection (Fig. 3). TSA (500 ng/ml) was significantly inhibited HDAC2 expression after 48 h of treatment. For the invasion assay, cells were cultured in the complete growth media in presence of Scrambled siRNA control, HDAC2 siRNA, HDAC inhibitor TSA (500 ng/ml), MMP2/9 inhibitor III (50 μM) and DMSO control for 48 h (Fig. 4). Results showed that the numbers of invaded cells were markedly decreased in HDAC2 siRNA, TSA and MMP2/9 inhibitor III groups compared with scrambled siRNA and DMSO control groups (p < 0.05) (Fig. 4).

Downregulation of HDAC2 induces reduction of MMP-2 and MMP-9 protein expression in ESCC cells

Cells were treated with HDAC2 siRNA for 0-72 h. Western blot analysis detected that expression of MMP-2 and MMP-9 were both remarkably reduced after 48 and 72 h transfection (Fig. 5). Meanwhile, TSA also decreased MMP-2 and MMP-9 protein levels after 48 h treatment.

Discussion

In this study, HDAC2 was demonstrated to be over-expressed in ESCC; down-regulation of HDAC2 expression prevented cell invasion, possibly by decreasing MMP-2 and MMP-9 expression, indicating that HDAC2 may be involved in ESCC tumorigenesis. HDAC inhibitor TSA was also demonstrated to inhibit ESCC cell invasion. These results have provided an experimental basis for identifying a potential target of ESCC therapy, especially therapy associated with ESCC metastasis.

HDAC2 is a class of cellular proteases belonging to a family of 11 zinc-dependent human HDACs that are ubiquitously expressed. Under normal circumstances, HDAC2 removes acetyl groups from histones, inducing chromatin condensation.
and transcriptional repression [20]. Acetylation and deacetylation of histones emerge as critical aspects of an epigenetic indexing system demarcating transcriptionally active chromatin domains. The balance between acetylation and deacetylation controls several physiological and pathological cellular processes [21]. Overexpression of HDAC leads to enhancement of deacetylase activity and silencing of tumor-suppressor genes [22]. HDAC2 has been found to be significantly overexpressed in several tumor types, such as gastric cancer, prostate cancer and renal cancer, and the overexpressed HDAC2 usually correlated with malignant phenotype and a poor prognosis in human tumors [9,10]. In this study, HDAC2 was shown to be overexpressed in ESCC and mainly localized in the cytoplasm. The alteration of HDAC2 expression may epigenetically mediate gene silencing in ESCC tumorigenesis.

Invasion and metastasis in cancer has been the target of intense research. Invasion is considered to be a prerequisite to metastasis, which is a pathogenic cell migration through tissues and vessels [23]. Abundant evidence indicates that the high invasion ability of ESCC is one of important reasons for poor survival rates and prognosis [7,24]. Invasive cancer results in cell death through the formation of a tumor microenvironment that encourages tumor growth, progression and local immunosuppression [25]. Overexpressed HDAC2 may correlate with

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**FIGURE 4.** Effects of HDAC2 siRNA, TSA and MMP2/9 specific inhibitor on ESCC cell invasion. Cells were transfected with HDAC2 siRNA (scrambled siRNA as negative control) or treated with TSA, MMP2/9 inhibitor III (DMSO as negative control) for 48 h, then cells were subjected to invasion assay. After 16 h, the number of invaded cells were stained and counted using the MetaMorph image analysis program. Quantified data are expressed as means ± SEM (n=3). * p < 0.05, compared with scrambled siRNA or DMSO control, respectively.

**FIGURE 5.** Effects of HDAC2 siRNA and TSA on MMP-2 and MMP-9 protein expression in the ESCC cell line. Cells were treated with HDAC2 siRNA and TSA for various times. Protein levels of MMP-2 and MMP-9 were then subjected to Western blot analysis. GAPDH served as a loading control. Quantified data are expressed as means ± SEM (n=4). * * p < 0.05, MMP-2 and MMP-9 protein levels compared with scrambled siRNA controls.
invasion and poor prognosis in human tumors; for example, cells stably overexpressing HDAC2 retain Hypoxia-Inducible Factor 1 alpha (HIF-1alpha) protein modifications and lead to an increase in cell invasion ability in oral cancer progression [26]. Moreover, knockdown of HDAC2 expression resulted in a decrease in liver cancer cell invasion and progression [27]. Over-expression of HDAC2 decreased P53 expression and enhanced tumor cell invasion in the setting of drug resistance conferred by cancer/testis antigen CAGE [28]. These findings suggest a possible link between the tumor cell invasion potential and the prevalent expression of HDAC2. In this study, downregulation of HDAC2 markedly reduced the invasion of ESCC cells, consistent with previously published studies. These data imply that HDAC2 may be considered as a major regulator of ESCC metastasis.

Degradation or breakdown of the extracellular matrix surrounding tumor cells is a functional prerequisite for invasion and metastasis. The matrix metalloproteinases (MMPs) are a family of structurally related and zinc-dependent enzymes that degrade essentially all components of extracellular matrix [29,30]. MMP-2 and MMP-9, which are associated with the disruption of basement membrane, have an important role in tumor invasion and metastasis. There is considerable evidence to indicate that the expressions of MMP-2 and MMP-9 in ESCC are significantly associated with the tumor invasion depth, tumor-node-metastasis stages, and lymph node metastasis [30-32]. ESCC, with high MMP-9, has a high malignant tendency [31,32]. In this study, HDAC2 downregulation resulted in a decrease in MMP-2 and MMP-9 protein levels. These decreases indicate that HDAC2 may play a key role in ESCC metastasis through MMP-2 and MMP-9 and serve as a novel target for ESCC therapy. This hypothesis was further supported in the current study by the MMP2/9 specific inhibitor-induced effect on ESCC invasion. In addition, MMPs expression started to decline after 48 h of transfection but not at 24 h when HDAC2 expression was at its lowest levels. This implies that complex signaling pathways are involved in the regulation of HDAC2-induced MMP expression and that other molecules may also participate in HDAC2-mediated ESCC cell invasion and metastasis. Previous studies have shown that downregulation of HDAC blocked cancer cell invasion and migration through MMP [32]. Many signaling pathways were involved in MMPs transcription, such as MAPKs and AP-1 [33,34]. Another research group found that knockdown of HDAC2 enhanced p53-dependent NF-κB signaling pathways repression [11]. Those signaling pathways may also be involved in our case.

TSA is a non-specific HDAC inhibitor, and is a potential candidate for anticancer therapy. It has been proposed that TSA’s main mechanism of inhibition is to mimic the substrate and block the catalytic reaction by chelating a zinc ion in the active-site pocket through its hydroxamic acid group [35]. Recently, TSA has been shown to suppress cell invasion in various cancer cells at low concentrations. TSA treatment increased histone acetylation in the promoters and induced the expression of genes that are suppressors of invasion and metastasis, including tissue inhibitors of metalloproteinase and nm23H1/ H2 in gastrointestinal carcinogenesis. TSA suppressed invasion through metalloproteinase such as MMP-2 and MMP-9 [36,37]. Furthermore, TSA reduced PLC-gamma 1 mRNA stability in MCF-7 breast cancer cells and this inhibited malignant cell motility [38]. In the present study, TSA significantly inhibited ESCC cell invasion. Importantly, cell invasion was inhibited by approximately 75% in the TSA treatment group as well as the HDAC2 siRNA group. This observation indicates that the cancer invasive inhibitory effect of TSA is mediated through suppression of HDAC2 expression and activation, and that the reduction of MMP-2 and MMP-9 expression may mediate the invasive inhibitory effect of TSA.

In conclusion, this study demonstrated that HDAC2 is overexpressed in ESCC and that downregulation of HDAC2 leads to the suppression of tumor invasion, indicating that HDAC2 is a key molecule in ESCC invasion. It is conceivable that HDAC2 may serve both as a predictor of the prognosis of the patients with ESCC and as a novel target for ESCC therapy. Since TSA is a HDAC inhibitor and significantly suppresses ESCC cell invasion, TSA could potential be used as a therapeutic against tumor invasion. For this purpose, more detailed mechanistic and functional studies on TSA are crucial.

References
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