RESEARCH ARTICLE

HDAC6 siRNA Inhibits Proliferation and Induces Apoptosis of HeLa Cells and its Related Molecular Mechanism

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Abstract

Objective: To investigate the effects of histone deacetylase 6 (HDAC6) siRNA on cell proliferation and cell apoptosis of the HeLa cervical carcinoma cell line and the molecular mechanisms involved. Methods: Division was into three groups: A, the untreated group; B, the control siRNA group; and C, the HDAC6 siRNA group. Lipofectamine 2000 was used for siRNA transfection, and Western blot analysis was used to determine the protein levels. Cell proliferation and apoptosis were characterized using a CCK-8 assay and flow cytometry, respectively. Results: HDAC6 protein expression in the HDAC6 siRNA-transfection group was significantly lower (P < 0.05) than in the untreated and control siRNA groups. The CCK-8 kit results demonstrated that the proliferation of HeLa cells was clearly inhibited in the HDAC6 siRNA transfection group (P < 0.05). In addition, flow cytometry revealed that the early apoptotic rate (26.0% ± 0.87%) was significantly elevated (P < 0.05) as compared with the untreated group (10.6% ± 1.19%) and control siRNA group (8.61% ± 0.98%). Furthermore, Western blot analysis indicated that bcl-2 protein expression in the HDAC6 siRNA-transfection group was down-regulated, whereas the expression of p21 and bax was up-regulated. Conclusion: HDAC6 plays an essential role in the occurrence and development of cervical carcinoma, and the down-regulation of HDAC6 expression may be useful molecular therapeutic method.

Keywords: Histone deacetylase 6 - RNA interference - cell proliferation - cell apoptosis - molecular target

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Introduction

Cervical cancer is generally the third most common type of tumor in women, with approximately 371,200 new cases worldwide are diagnosed annually. Considering its 51% mortality rate, cervical cancer has become a public health concern (Parkin et al., 1999). However, current cervical cancer treatment methods, which are mainly based on radiotherapy, platinum-based chemotherapy, and surgery, have very limited effects (Pecorelli et al., 2005). Therefore, finding new diagnostic molecular markers and cancer therapeutic targets has become the new goal and direction for improving cancer outcomes.

Histone deacetylase 6 (HDAC6) belongs to the type II HDAC family. Among the 18 members of the HDAC family, HDAC6 has a unique structure with its two tandem deacetylation domains and one zinc finger motif required for its biological functions (Verdel et al., 2000; Zhang et al., 2006). In addition to the unique extraterritorial catalytic structure, HDAC6 contains two nuclear export signals, one in the nuclear signal and the other in a 14-peptide repeat domain, which are closely related to HDAC6 cytoplasmic localization; therefore, HDAC6 is mainly localized in the cytoplasm (Bertos et al., 2004). Some studies have shown that HDAC6 interacts and contains certain nuclear proteins, such as HIF-1α, NF-κB (Zhang and Kone, 2002; Kong et al., 2006), which indicates that it shuttles between the nucleus and cytoplasm, but the specific mechanism is unclear. Previous studies showed that HDAC6 affects cell proliferation in different cancers, such as the Ewing family of tumors. HDAC6 inhibits the activation of p21 expression, thereby accelerating the proliferation of tumor cells (Nakatani et al., 2003). In addition, HDAC overexpression induces cell proliferation (Kouzarides, 1999). Recently, a growing number of studies showed that HDAC6 is closely related to the development of different tumors, including breast cancer (Zhang et al., 2004), ovarian cancer (Bazzaro et al., 2008), oral cancer (Sakuma et al., 2006), acute myeloid leukemia (Xu et al., 2011), neural neuroblastoma (Subramanian et al., 2011), gastric cancer (Park et al., 2011), and colon cancer (Park et al., 2011). However, the relationships between HDAC6 and cervical cancer have not been reported.

Apoptosis is closely associated with tumor occurrence and development. Similarly, bcl-2 and bax protein expression and cell apoptosis are closely related (Sadeghi et al., 2007). HDAC6 may regulate the expression of bcl-2, bax, and other related proteins through the NF-κB signaling pathway (Subramanian et al., 2011).

Objective: To investigate the effects of HDAC6 siRNA on cell proliferation and cell apoptosis of HeLa cervical carcinoma cell line and the molecular mechanisms involved. Methods: Division was into three groups: A, the untreated group; B, the control siRNA group; and C, the HDAC6 siRNA group. Lipofectamine 2000 was used for siRNA transfection, and Western blot analysis was used to determine the protein levels. Cell proliferation and apoptosis were characterized using a CCK-8 assay and flow cytometry, respectively. Results: HDAC6 protein expression in the HDAC6 siRNA-transfection group was significantly lower (P < 0.05) than in the untreated and control siRNA groups. The CCK-8 kit results demonstrated that the proliferation of HeLa cells was clearly inhibited in the HDAC6 siRNA transfection group (P < 0.05). In addition, flow cytometry revealed that the early apoptotic rate (26.0% ± 0.87%) was significantly elevated (P < 0.05) as compared with the untreated group (10.6% ± 1.19%) and control siRNA group (8.61% ± 0.98%). Furthermore, Western blot analysis indicated that bcl-2 protein expression in the HDAC6 siRNA-transfection group was down-regulated, whereas the expression of p21 and bax was up-regulated. Conclusion: HDAC6 plays an essential role in the occurrence and development of cervical carcinoma, and the down-regulation of HDAC6 expression may be useful molecular therapeutic method.

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Hai-Xia Qin et al., 2007; Peng et al., 2009), the ratio of bcl-2/bax in specific parts of cells determines the induction and severity of apoptosis, and the relationships of bcl-2 and bax with cell apoptosis in cervical cancer have not been reported.

Therefore, in the present study, we assayed the cell proliferation and cell apoptosis HeLa cervical carcinoma cells in vitro using RNA interference technology to downregulate HDAC6 expression and determined the levels of P21, bcl2, and bax protein. The underlying molecular mechanism was also explored. This study aims to determine whether HDAC6 plays an important role in the pathogenesis of cervical cancer, and whether HDAC6 can be used in the early diagnosis and treatment of cervical cancer.

Materials and Methods

Cell culture and transfection

The HeLa cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum. When the HeLa cells reached 80% to 90% confluence, the HDAC6 siRNA and control siRNA were transfected into the HeLa cells using Lipofectamine 2000 reagents, according to the manufacturer’s instructions. The cells were assigned into three groups: the untreated group (HeLa cells without any treatment), the control siRNA group (HeLa cells transfected with the control siRNA), and the HDAC6 siRNA-transfected group (HeLa cells transfected with the HDAC6 siRNA).

Western blotting

The HeLa cells were collected 48 h after transfection the total proteins in the cell lysates were extracted. The proteins were isolated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose (NC) membrane. The NC membrane was blocked with TBST containing 5% skim milk and 0.05% Tween solution for 2 h, and incubated overnight on a shaker, with anti-HDAC6, anti-p21, anti-bcl-2, anti-bax and anti-β-actin antibodies (1:200 dilution 4 °C. The resulting mixture was then incubated with the secondary antibodies for 2 h at room temperature. The NC membrane was subjected to enhanced chemiluminescence (ECL) for 1 min to 3 min. The specific protein signals were observed using X-ray exposure, conventional developing and fixation in a darkroom. The gray value of protein expression was analyzed using Gene Tools software, with β-actin as the endogenous reference. The ratio of the gray value of each protein to that of β-actin reflected the relative expression levels of each protein.

Proliferation of HeLa cells

CCK-8 cell proliferation reagent was added to the HeLa cells at a final concentration of 10%, incubated at 37 °C for 3 h, and the absorbance was measured on a microplate reader at 450 nm. Different groups of cells were analyzed at 24 h, 48 h, 72 h, and 96 h after treatment.

Flow cytometry analysis

The HeLa cells were collected 48 h after transfection, washed with ice-cold phosphate-buffered saline, and resuspended to a density of 1 x10⁶ cells/ml. Next, 100 µl cells were added to the flow tube containing 5 µl of Annexin V-FITC (BD Biosciences company) and propidium iodide. After incubating for 15 min in the dark, 1 x10⁴ cells were analyzed by flow cytometry, and CellQuest analyses of apoptosis was conducted on the three groups.

Statistical analysis

SPSS 13.0 statistical software was used for the statistical analysis. The statistical data are presented as mean ± standard deviation. The standard deviations among the three groups were compared using one-way ANOVA and differences with P-values < 0.05 were considered statistically significant.

Results

HDAC6 siRNA reduces HDAC6 protein expression

Western blot analysis showed that the HDAC6 protein expression in the HDAC6 siRNA-transfected group (0.013 ± 0.008) was significantly lower than those in the untreated group (0.902 ± 0.063; P <0.05) and in the control siRNA group (0.905 ± 0.063; P <0.05). However, the difference between the untreated group and the control siRNA group was not statistically significant (P >0.05) (Figure 1).

HDAC6 siRNA inhibited HeLa cell proliferation

As shown in Figure 2, compared with the untreated and the control siRNA group, the cell proliferation rates of the HDAC6 siRNA-transfected HeLa cells were significantly inhibited (P <0.05). No significant difference (P >0.05) in proliferation rate was observed between the control siRNA group and the untreated group. To understand the molecular mechanism of HDAC6 siRNA-mediated
Effects of HDAC6 siRNA on Proliferation and Apoptosis of HeLa Cells

The cell cytometry results indicated that the early apoptosis rate in the HDAC6 siRNA-transfected group (25.96% ± 0.87%) was significantly higher (P < 0.05) than that in the untreated group (10.61% ± 1.19%) and the control siRNA group (8.61% ± 0.98%). However, the differences in bcl-2 expression levels between the untreated group and the control siRNA group was not significant (P > 0.05; Figure 3). The cell cytometry results indicated that the early apoptosis rate in the HDAC6 siRNA-transfected group (10.16% ± 0.44%) was significantly higher (P < 0.05) than that in the untreated group (3.10% ± 0.63%) or the control siRNA group (5.57% ± 0.57%). In addition, the percentage of live cells in the HDAC6 siRNA-transfected group (62.99% ± 1.23%) was significantly lower (P < 0.05) than that in the untreated group (85.19% ± 1.76%) and the control siRNA group (83.49±1.24%), but the difference between the untreated group and the control was not significant (P > 0.05; Figure 4).

**HDAC6 siRNA treatment induced apoptosis**

Gene transcription is both controlled by activators and repressors. Histone deacetylase (HDAC) participates in chromosome relaxation and transcription activation, and it inhibits gene transcription through chromosome condensation. Therefore, as a unique enzyme for transcription, HDAC is involved in epigenetic histone modifications, and it regulates proliferation and differentiation by inhibiting the expression of several key genes. Recently, HDAC was widely recognized and suggested as essential in early tumor development (Knudson, 2001; Lehrmann et al., 2002). Studies indicated that inhibition of HDAC enzyme activity is very promising strategy for cancer treatment (Mehnert and Kelly, 2007; Ropero and Esteller, 2007; Richon et al., 2009). However, the expression of HDAC6 in cervical cancer cells has not been confirmed and its involvement in cervical cancer occurrence and development has not been reported. To investigate the role of HDAC6 in cervical cancer, we inhibited its expression in cervical cancer cells using RNA interference. Our results show that RNA interference effectively reduces HDAC6 protein expression in HeLa cells after transfection for 48 h, unlike in the untreated and the control siRNA groups. Thus, this provides an ideal research platform for further studies on the function of HDAC6 in cervical cancer cells and the molecular mechanism involved.

**Discussion**

As shown by western blot analysis, the bcl-2 expression in the untreated group (0.811 ± 0.057) and that in the control siRNA group (0.795 ± 0.051) were significantly higher than that in the HDAC6 siRNA-transfected group (0.017 ± 0.009). In contrast, the bax protein expression levels in the untreated group (0.034 ± 0.012) and that in the control siRNA group (0.032 ± 0.008) were significantly lower than that in the HDAC6 siRNA-transfected group (0.954 ± 0.081; (P < 0.05). The differences in bcl-2 and bax protein expression between the untreated group and the control siRNA group (P > 0.05) were not significant (Figure 5 and 6).

**Figure 3. HDAC6 siRNA Treatment Increased the Expression of p21 in HeLa Cells, *P < 0.05, compared with the untreated or the control siRNA group. A: untreated group; B: control siRNA group; C: HDAC6 siRNA group**

**Figure 4. Test of the Apoptosis Rate of HeLa Cells by Flow Cytometry.** I: live cell area; II: early apoptotic cells in area; III: late apoptotic or dead cells area; IV: necrotic cell area

**Figure 5. Knockdown of HDAC6 Regulates bcl-2 and Bax Protein Expression. A: untreated group; B: control siRNA group; C: HDAC6 siRNA-transfected group**

**Figure 6. The Relative Expression Levels of bcl-2 and Bax in the Three Groups. P < 0.05, compared with the untreated group and the control siRNA group**
In this study, we transfected the control siRNA and HDAC6 siRNA groups into HeLa cells and compared the rate of cell proliferation through CCK-8 analysis after transfection for 24, 48, 72, and 96 h. The proliferation of HeLa cells in the HDAC6 siRNA group was significantly inhibited. However, the molecular role of HDAC6 siRNA in inhibiting proliferation is still unknown. P21 is the cyclin-dependent kinase inhibitor protein (CDI) that has the most weekly kinase inhibitory activity. P21 can be combined with various cyclin-CDKs to inhibit the kinase activity of cyclin-CDK complexes. P21 cannot phosphorylate the Rb protein, which arrests cells in the G1 phase of the cell cycle (Zhang et al., 1994). The in vitro experiments showed that low p21 concentrations stimulate the phosphorylation of the functional group and promotes cell progression in the G1-S. However, high p21 concentrations negatively regulate CDK function (Waga et al., 1994). P21 inhibits the complexation of proliferating cell nuclear antigen (PCNA) and DNA polymerase, thereby affecting DNA replication (Liu et al., 1996). It is mainly involved in cell cycle regulation, inhibition of abnormal cell proliferation, and cell coordination to complete the repair of DNA damage. Therefore, we determined p21 protein expression through western blot analysis and showed that p21 protein expression was significantly increased in the HDAC6 siRNA-transfected group, which might contribute to the inhibition of HeLa cell proliferation.

Cell apoptosis is strongly related to tumor occurrence and development. Apoptosis induction is mainly regulated by two pathways (Kawiak et al., 1998). One is dependent on bcl-2 and bax, which regulate cytochrome C release, and then activates apoptotic effector proteins caspase-9, caspase-3, and PARP, ultimately inducing apoptotic DNA fragmentation. The other apoptotic pathway is regulated by Fas and Fas-L through the activation of caspase-8, caspase-3, and PPAR. Flow cytometry indicated that the percentage of early apoptotic cells in the HDAC6 siRNA-transfected group was significantly higher than that in the control group (P < 0.05), which suggests that HDAC6 siRNA induces apoptosis in HeLa cells. To determine the initial molecular events involved in apoptosis, we examined the expression of bcl-2 and bax protein, which are closely related with apoptosis. HDAC6 siRNA treatment reduced the bcl-2 expression but increased that of bax. However, the involvement of other molecules requires further exploration.

In conclusion, HDAC6 siRNA significantly reduces HDAC6 protein expression in the HeLa cervical cancer cell line. Meanwhile, HDAC RNA interference inhibits cell proliferation and induces apoptosis, which may be contributed by p21, bcl-2, and bax. Further studies on the role of HDAC6 in cervical cancer cells and the molecular mechanisms involved will provide a theoretical basis for the development of molecular targeted therapy.

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