ORIGINAL ARTICLE

Effect of downregulated histone deacetylase 2 expression on cell proliferation and cell cycle in cervical cancer

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Summary

Purpose: To investigate the effects and molecular mechanism of downregulated histone deacetylase 2 (HDAC2) expression on cell proliferation and cell cycle in cervical cancer Hela cells.

Methods: HDAC2 small interfering (si)RNA and control siRNA were transfected into cervical cancer Hela cells. A cell proliferation assay using a cell counting kit-8 was applied to analyze the change in cell proliferation before and after transfection. Flow cytometry was used to detect the change in cell cycle distribution before and after transfection. Finally, Western blot was used to detect changes in the expression of cell proliferation and cell cycle-related proteins.

Results: HDAC2 siRNA significantly downregulated the expression of HDAC2 proteins in cervical cancer cells, mark-

edly inhibiting their proliferation. In addition, the percentage of Hela cells in the GO/G1 phase in the HDAC2 siRNA group was $63.3\pm2.0\%$, significantly higher than those in the untreated group ($29.3\pm1.7\%$) or the control siRNA group ($29.4\pm1.7\%$) (F=354.181, p=0.000). Furthermore, Western blot analyses demonstrated that downregulated HDAC2 expression inhibited the expression of cyclin D1, cyclin E, and cdk2 proteins but elevated the expression of p21 protein.

Conclusion: The proliferation inhibition and cell cycle arrest mediated by downregulated HDAC2 expression may be tightly associated with the decrease of cyclin D1, cyclin E, and cdk2 proteins expression and the increase in p21 protein expression.

Key words: cell cycle, cell proliferation, cyclins, histone deacetylase 2, uterine cervical neoplasms

Introduction

Cervical cancer is one of the most common gynecologic malignancies. In 2008, 529,800 women were diagnosed with cervical cancer, and 275,100 died of the disease worldwide [1]. Although the treatment of cervical cancer has greatly improved, the prognosis of patients with recurrent or metastatic cervical cancer is still very poor [2]. In recent years, treatment of cancer has gradually extended to the gene or molecular level. Therefore, identifying new genes and achieving better understanding of their relationships with the development of cervical cancer are helpful in the diagnosis and treatment of this disease.

Histone deacetylase (HDAC) can dynamically

regulate protein acetylation in the human body [3-5]. As a member of the HCAC family, HDAC2 has been found to be closely associated with the development of tumors and a high level of expression plays an important role in a variety of tumors [6-8]. However, there have been very few domestic and overseas reports on the relationship between the HDAC2 gene and cervical cancer. Thus, we used HDAC2 siRNA to downregulate the expression of HDAC2 protein in cervical cancer Hela cells, analyzed the effect of downregulated HDAC2 expression on Hela cell proliferation and cell cycle, and explored the molecular mechanism in order to provide a theoretical basis for cervical cancer gene therapy in which HDAC2 is a therapeutic target.

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Methods

Experimental materials

Human cervical cancer Hela cell line was purchased from the American Type Culture Collection; Lipofectamine 2000 was purchased from Invitrogen Co (Carlsbad, Calif, USA); RPMI-1640 medium was purchased from American Sigma Co (St Louis, Missouri, USA); trypsin was purchased from Gibco Co (Calif, USA); fetal calf serum was purchased from Hyclone Co (Logan, USA); cell counting kit-8 reagents were purchased from Chinese Biyuntian Biological Technology Co., Ltd; cell lysis buffer was purchased from Promega Co (Madison, USA); and HDAC2 siRNA and control siR-NA, HDAC2 antibodies, cyclin D1, cyclin E, cdk2, and p21 were purchased from Santa Cruz Biotechnology, USA.

Culture and transfection of Hela cells

Hela cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum. When 80-90% of Hela cells had converged, HDAC2 siRNA and control siRNA were transfected into Hela cells, according to the Lipofectamine 2000 transfection reagent manufacturer's instructions. Then the Hela cells were divided into three groups: untreated, control siRNA (transfected with control siRNA), and HDAC2 siRNA (transfected with HDAC2 siRNA).

Cell proliferation assay

Cell proliferation was analyzed according the cell counting kit-8 manufacturer's instructions. Hela cells from the untreated group and transfected groups were collected at 24, 48, 72, and 96 hrs, respectively, added to 10% cell counting kit-8 reagent, and cultured in a 37 °C incubator for 3 hrs. Finally, the absorbances at 450 nm were measured with a microplate reader.

Cell cycle analysis

Hela cells from three transfected and untransfected groups were collected (each group had about 1×10^6 cells). The cells in each group were rinsed with PBS buffer and fixed in 70% absolute alcohol at 4 °C for 30 min and then rinsed three times with cold PBS. The cells were resuspended in a PBS solution containing 40 µg propidium iodide and 100 µg RNase A and cultured at 37 °C for 30 min. Finally, the DNA contents of samples were analyzed using flow cytometry.

Western blot analysis of expression of cell proliferation and cell cycle-related proteins

Hela cells from transfected and untransfected groups were collected at 48 hrs; the total proteins were extracted from cell lysates and analyzed using Western blot. The total proteins extracted from cells in each group were analyzed using SDS-PAGE electrophoresis. untreated control siRNA HDAC2 siRNA



Figure 1. Expression of HDAC2 proteins in cells of various groups detected by Western blot analysis.

After electrophoresis, the gel was removed and the proteins were electrically transferred to a nitrocellulose membrane. The nitrocellulose membrane was blocked in a TBST solution containing 5% nonfat dry milk for 2 hrs, supplemented with primary antibodies (HDAC2, cyclin D1, cyclin E, cdk2, p21, and β -actin) at a 1:100 ratio, and incubated overnight at 4 °C in a shaker before being incubated at room temperature for 2 hrs. The nitrocellulose membrane was soaked in an enhanced chemiluminesence reaction reagent for 1-3 min and exposed to X-ray film in a darkroom. Conventional methods for developing and fixing films were used to display specific signals for proteins. Gray-scale values for proteins were analyzed using Gene Tools software (Syngene Co, Cambridge, UK). The ratio of target genes to reference genes was taken as the relative protein expression level, and β -actin was taken as an internal reference.

Statistics

The data were analyzed with SPSS 13.0 software (Chicago, IL, USA) and expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) was used for comparisons of groups. p<0.05 was considered as statistically significant.

Results

HDAC2 siRNA may downregulate the expression of HDAC2 proteins in cervical cancer

The change in the expression of HDAC2 proteins in Hela cells was analyzed using Western blot 48 hrs after transfection with HDAC2 siRNA and control siRNA. The results showed that the expression of HDAC2 proteins in Hela cells 48 hrs after transfection with HDAC2 siRNA was significantly lower than those in the untreated group and the control siRNA group (p<0.05; Figure 1). However, HDAC2 protein expression was not significantly different between the untreated group

Downregulated HDAC2 expression inhibited proliferation of cervical cancer cells

Cell counting kit-8 reagents were used to analyze cervical cancer cell proliferation at 24, 48, 72, and 96 hrs after transfection with HDAC2 siRNA and control siRNA. The results showed no differences between the untreated group and the control siRNA group in cervical cancer cell proliferation at various times after transfection (p>0.05; Figure 2). Compared with the untreated group and the control siRNA group, the proliferation of Hela cells at various times after transfection in the HDAC2 siRNA group were inhibited significantly (p<0.05).

Downregulated HDAC2 expressions altered the cell cycle distribution of cervical cancer cells

Cells in each group were collected 48 hrs after transfection with HDAC2 siRNA. Flow cytometry was used to detect changes in cell cycles of various groups (Figures 3 and 4). The results showed that the percentages of cell cycles at the GO/G1 phase in the untreated group and the control siR-NA group were 29.3±1.7% and 29.4 29.3±1.71.7%, respectively (p=0.931). Compared with the untreated group and the control siRNA group, the percentage of G0/G1 phase cells in HDAC2 siR-NA group was significantly higher (63.3±2.0%), (F=354.181, p=0.000). The percentage of Hela cells in the S phase in the HDAC2 siRNA group was $23.5 \pm 1.3\%$, which was significantly lower than those in the untreated group $(29.9 \pm 0.7\%)$ and control siRNA group $(29.8 \pm 0.9\%)$ (F=41.409, p=0.000). There was no significant difference in the percentage of Hela cells in S phase between the untreated group and the control siRNA group (p=0.928). Analysis of the percentage of cells in the G2/M phase showed that the percentage of cells in the G2/M phase in the HDAC2 siRNA group



Figure 2. The proliferation of Hela cells is inhibited by downregulated HDAC2 expression. *p<0.05.

was $13.2\pm 0.8\%$, significantly lower than those in the untreated group (40.8 ± 2.3%) and control siRNA group (40.8 ±1.6%) (F=270.035, p=0.000). These results suggest that downregulated HDAC2 expression can alter the cell cycle distribution of cervical cancer Hela cells.

Effect of downregulated HDAC2 expression on cell proliferation and cell cycle-related protein expression

Cells in each group were collected 48 hrs after transfection with HDAC2 siRNA, and Western blot was used to detect the expression of cell proliferation and proteins closely related to the cell cycle in the Hela cells of each group. The results showed that, compared with the untreated group and the control siRNA group, expression of cyclin D1, cyclin E, and cdk2 proteins in the HDAC2 siRNA group were significantly downregulated, and the expression of p21 protein was significantly upregulated (p<0.05) (Figures 5 and 6). There were no statistically significant differences in the expression of the above-mentioned proteins between the untreated group and the control siRNA group (p>0.05).



Figure 3. Cell cycle distribution in various groups detected using flow cytometry.



Figure 4. Results of statistical analysis of cell cycle in various groups compared with the untreated group and the control siRNA group. *p<0.05.



Figure 5. Expression of cell proliferation and cell cycle-related proteins in Hela cells of various groups detected using Western blot.



Figure 6. Statistical analysis of the relative expression levels of cell proliferation and cell cycle-related proteins in Hela cells of various groups compared with the untreated group and the control siRNA group. *p<0.05.

Discussion

Abnormal changes in gene function and expression patterns are often controlled at epigenetic levels. The epigenetic control of gene expression is achieved mainly through DNA methylation, histone modifications, chromatin remodeling, and noncoding RNA regulation. Histone modifications, particularly acetylation and deacetylation, are the main driving forces of epigenetic gene regulation. HDAC2 is a histone deacetylase closely related to tumor development. Research has shown that HDAC2 was overexpressed in oral tumors and that overexpression of HDAC2 was closely related to the development and poor prognosis of such tumors [8]. Chang et al. [9] detected the expression of HDAC2 in 5 head and neck cancer cell lines (ie, Ca9-22, Cal-27, HSC-3, SAS, and TW2.6), constructed overexpression vectors and small hairpin RNA (shRNA) expression vectors for HDAC2, and studied the effects of upregulation and downregulation of HDAC2 expression on the invasion and metastatic ability of oral tumors. The results showed that knockout of HDAC2 expression in SAS cells resulted in initiation and growth of tumors. Fritzsche et al. [10] found that HDAC2 was highly expressed in renal cancer cells, and its positive expression rate was about 60%. These studies suggested that, as a proto-oncogene, HDAC2 plays an important role in the development of tumors. When we used HDAC2 siRNA to transfect Hela cells, the results showed that HDAC2 siRNA could effectively decrease the expression of HDAC2 proteins in these cells.

To clarify whether downregulated HDAC2 expression can affect the proliferation and cell cycle progression of Hela cells, we used a cell counting kit-8 reagent to study the effect of HDAC2 siR-NA transfection on cell proliferation. The results showed that, compared with the untreated group and the control siRNA group, the proliferation of Hela cells in the HDAC2 siRNA group was inhibited significantly 24-96 hrs after transfection, suggesting that downregulated HDAC2 expression can inhibit the proliferation of Hela cells. HDAC2 is expected to be a molecular target for the treatment of cervical cancer. When we used flow cytometry to analyze changes in cell cycle distribution before and after HDAC2 siRNA transfection, the results showed that downregulated HDAC2 expression could significantly increase the percentage of G0/G1 phase cells in Hela cells, while significantly reducing the percentage of S phase cells. These findings suggest that downregulated HDAC2 expression can arrest the cell cycle at the G0/G1 phase and block DNA synthesis, controlling thus the proliferation of tumor cells.

As we know, uncontrolled cell proliferation and changed cell cycle distribution is a remarkable feature of tumor cells. The molecular mechanism for cell cycle regulation involves interactions between three kinds of molecules, ie cell cycle proteins (cyclins), cyclin-dependent kinases (CDK), and CDK inhibitor proteins; of these, the most important is the expression and regulation of CDK activity. Cyclin D1, cyclin E, cdk2, and p21 proteins play important roles in the cell cycle and thus are closely related to tumor development [11-14]. To explore whether the inhibited Hela cell proliferation and changed cell cycle distribution are closely related to changes of the expression of the previously mentioned cell cycle proteins, we used Western blot to detect the expression of cyclin D1, cyclin E, cdk2, and p21 proteins in Hela cells before and after HDAC2 siRNA transfection. The results showed that HDAC2 siRNA transfection could significantly downregulate the expression of cyclin D1, cyclin E, and cdk2 proteins, while increasing the expression of p21 protein. These results indicated that the inhibition of Hela cell proliferation and cell cycle arrest mediated by downregulated HDAC2 expression may be associated with changes in the expressions of the previously mentioned cell cycle proteins; however, the exact molecular mechanism needs to be further explored.

In conclusion, our study showed that HDAC2 siRNA can effectively decrease the expression of HDAC2 protein in Hela cells. Downregulated HDAC2 protein expression can inhibit the proliferation of Hela cells and arrest the cell cycle at the G0/G1 phase, which may be closely associated with downregulation of cyclin D1, cyclin E, and cdk 2 proteins and upregulation of p21 protein.

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