

## RESEARCH ARTICLE

# Effects of Down-regulation of HDAC6 Expression on Proliferation, Cell Cycling and Migration of Esophageal Squamous Cell Carcinoma Cells and Related Molecular Mechanisms

Ning Li, Xiao-Jing Tie, Pei-Jie Liu, Yan Zhang, Hong-Zheng Ren, Xin Gao, Zhi-Qiao Xu\*

### Abstract

**Objective:** To study the effects of down-regulation of HDAC6 expression on proliferation, cell cycling and migration of esophageal squamous cell carcinoma (ESCC) cells and related molecular mechanisms. **Methods:** ESCC cell line EC9706 cells were randomly divided into untreated (with no transfection), control siRNA (transfected with control siRNA) and HDAC6 siRNA (transfected with HDAC6 small interfering RNA) groups. Effects of HDAC6 siRNA interference on expression of HDAC6 mRNA and protein in EC9706 cells were investigated by semi-quantitative RT-PCR, Western blotting and immunocytochemistry methods. Effects of down-regulation of HDAC6 expression on cell proliferation, cell cycle, and cell migration were studied using a CCK-8 kit, flow cytometry and Boyden chambers, respectively. Changes of mRNA and protein expression levels of cell cycle related factor (p21) and cell migration related factor (E-cadherin) were investigated by semi-quantitative RT-PCR and Western blotting methods. **Results:** After transfection of HDAC6 siRNA, the expression of HDAC6 mRNA and protein in EC9706 cells was significantly downregulated. In the HDAC6 siRNA group, cell proliferation was markedly inhibited, the percentage of cells in G0/G1 phase evidently increased and the percentage of cells in S phase decreased, and the number of migrating cells significantly and obviously decreased. The mRNA and protein expression levels of p21 and E-cadherin in the HDAC6 siRNA group were significantly higher than those in the untreated group and the control siRNA group, respectively. **Conclusions:** HDAC6 siRNA can effectively downregulate the expression of HDAC6 mRNA and protein in EC9706 cells. Down-regulation of HDAC6 expression can obviously inhibit cell proliferation, arrest cell cycling in the G0/G1 phase and reduce cell migration. The latter two functions may be closely related with the elevation of mRNA and protein expression of p21 and E-cadherin.

**Keywords:** Esophageal squamous cell carcinoma - HDAC6 - cell proliferation - cell cycle - cell migration - p21 - E-cadherin

*Asian Pacific J Cancer Prev*, 14 (2), 685-689

### Introduction

Esophageal cancer is a highly aggressive malignancy in digestive tract with high incidence (Parkin et al., 2001-1) and low survival rate (Parkin et al., 2001-2). It is divided into esophageal adenocarcinoma (EA) and esophageal squamous cell carcinoma (ESCC) according to pathological features. More than 90% of esophageal cancers are ESCC (Parkin et al., 2001-1). ESCC mainly appears as chronic inflammation, acute abnormality, carcinoma in situ and invasive carcinoma, with a gradual evolution (Kuwano et al., 1993; Zhang et al., 2009). At present, ESCC in the majority of patients is diagnosed as advanced metastatic cancer of which the 5-year survival rate is less than 10% (Zhang et al., 2004). Therefore, it is of great importance to find metastasis-related genes for diagnosing ESCC.

Histone deacetylase 6 (HDAC6), as an important member in the type-II histone deacetylase family, plays an important role in the occurrence and development of tumor. It has become a useful molecular target for malignancy treatment (Carew et al., 2008). The high expression of HDAC6 has been found in glioma (Wu et al., 2010), breast cancer (Rey et al., 2010), lung cancer (Kakihana et al., 2009), prostate cancer (Ai et al., 2009), multiple myeloma (Hideshima et al., 2005), oral squamous cell carcinoma (Sakuma et al., 2006) and other tumors. Researchs have found that HDAC6 is closely related with the occurrence and development of tumor (Duong et al., 2008; Shen et al., 2008).

There is no report about HDAC6 in ESCC until now. In this paper, the interference of HDAC6 interfering RNA (siRNA) on HDAC6 mRNA and protein expression in EC9706 cells was studied. Effects of down-regulation of

HDAC6 expression on cell proliferation, cell cycle and cell migration were investigated, and the related molecular mechanisms were explored. The objective of this study is to clarify the possible role of HDAC6 in ESCC and provide a theoretical basis for treatment of ESCC with HDAC6 as a target.

## Materials and Methods

### Cell culture and transfection

ESCC cell line EC9706 cells were purchased from the cell bank of Shanghai Institute of Cell Institute, Chinese Academy of Sciences. They were randomly divided into the untreated group, the control siRNA group and the HDAC6 siRNA group. EC9706 cells in the control siRNA group and the HDAC6 siRNA group were transfected with control siRNA and HDAC6 siRNA using Lipofectamine 2000, respectively.

### Detection of HDAC6 mRNA expression by semi-quantitative RT-PCR method

After transfection with HDAC6 siRNA for 24 h, Total RNA of EC9706 cells in three groups was extracted using Trizol. The expression of HDAC6 mRNA was detected by RT-PCR method.

### Detection of HDAC6 protein expression by Western blotting method

The whole procedure was in accordance with the standard procedure of Western Blotting Method.

### Detection of HDAC6 protein expression by immunocytochemistry method

The whole procedure was in accordance with the standard procedure of Immunocytochemistry Method introduced by many researchers (Fan et al., 2006; He et al., 2006; Yuan et al., 2006; Zhang et al., 2008).

### Detection of cell proliferation by CCK-8 kit

EC9706 cells in three groups were collected 24, 48, 72 and 96h after transfection, respectively. The cell counting was conducted using CCK-8 kit to detect the proliferation of EC9706 cells. The targeted cells were collected in 0, 24, 48, 72 and 96 hr to make the concentration of 2000 cells/100 ul in every hole. The rest procedure was in accordance with the standard procedure of cell proliferation method.

### Detection of cell cycle by flow cytometry

EC9706 cells ( $1 \times 10^6$  per group) were collected in 48h and trypsinized with 0.2 % trypsin. After centrifuged 1000 rpm for 5 min, the residue was then washed twice with PBS and solidated with 70% icy ethanol for 30 min and stored in 4 °C for the whole night. The cell cycle was then detected with flow cytometry.

### Detection of cell migration by Boyden chamber

The migration of EC9706 cells was determined by the boyden chamber assay. The sum of cells in 5 fields of high power lens was averaged for the index of Tumorocellular infiltration ability.

### Detection of p21 and E-cadherin mRNA expression by semi-quantitative RT-PCR method

The procedure was in accordance with those of Semi-Quantitative RT-PCR method, while the annealing temperature was set as 62°C for p21 gene and 52°C for E-cadherin gene, respectively.

### Detection of p21 and E-cadherin protein expression by Western blotting method

The procedure was in accordance with those of Western blotting method.

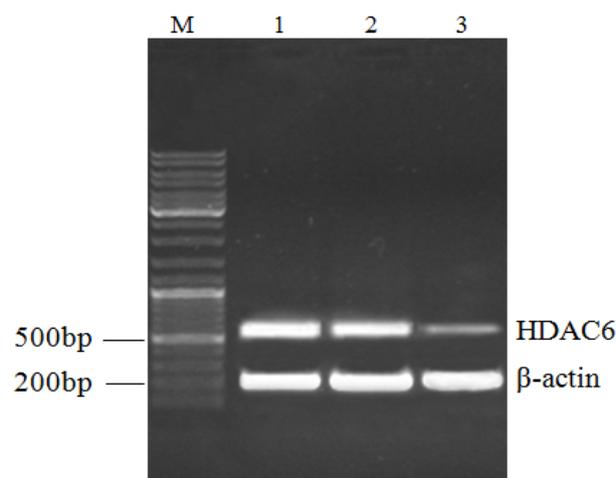
### Statistical analysis

Data were expressed as mean  $\pm$  SD. Statistical analysis was performed using SPSS 13.0 statistical software. A t-test was used to analyze the differences between two groups. A One-way ANOVA was performed to analyze the differences in three groups.  $P < 0.05$  was considered as statistically significant.

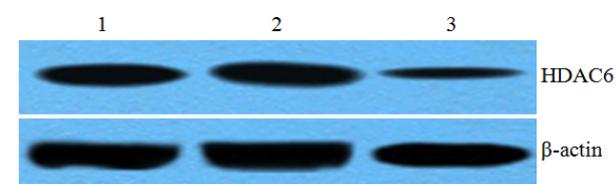
## Results

### Expression of HDAC6 mRNA in EC9706 cells

After transfection, the expression of HDAC6 mRNA in EC9706 cells was detected by semi-quantitative RT-PCR method. Results were shown in Figure 1. The expression of HDAC6 mRNA in the HDAC6 siRNA group (0.044) was significantly reduced compared to the untreated group (0.951) and the control siRNA group (0.947) ( $P < 0.05$ ), respectively. There was no significant difference between the control siRNA group and the untreated group ( $P > 0.05$ ). This indicated that HDAC6 siRNA could effectively downregulate the expression of HDAC6 mRNA in EC9706 cells.



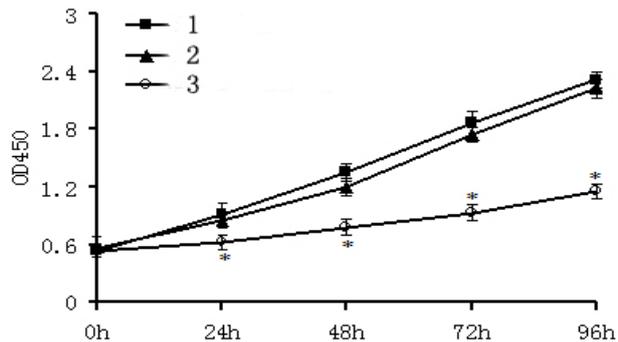
**Figure 1. Expression of HDAC6 mRNA Detected by Semi-quantitative RT-PCR**



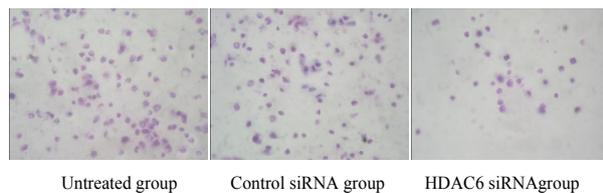
**Figure 2. Expression of HDAC6 Protein Detected by Western Blotting Method**

### Expression of HDAC6 protein in EC9706 cells

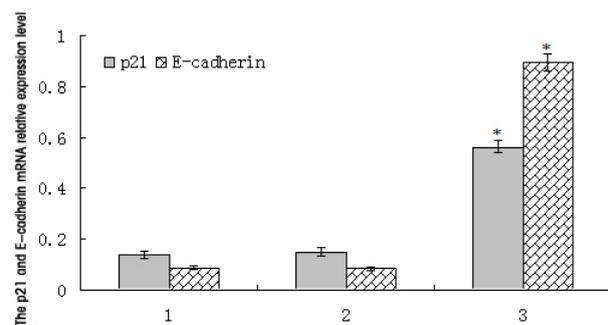
The expression of HDAC6 protein in EC9706 cells was detected by Western blotting and immunocytochemistry methods, respectively. As shown in Figure 2, the expression level of HDAC6 protein in HDAC6 siRNA group (0.114) was significantly lower than that in the untreated group (0.924) and the control siRNA group (0.905) ( $P < 0.05$ ), respectively. There was no significant difference between the later two groups ( $P > 0.05$ ). Results of immunocytochemistry detection showed that the HDAC6 protein was mainly localized in the cytoplasm. There was a strong expression of HDAC6 protein in the



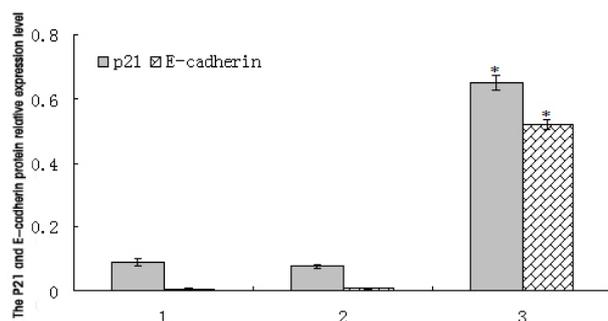
**Figure 3. Effects of Down-regulation of HDAC6 Expression on Proliferation of EC9706 Cells**



**Figure 4. Effects of Down-regulation of HDAC6 Expression on Migration of EC9706 Cells**



**Figure 5. Results of Statistic Analysis on Expression of p21 and E-cadherin mRNA**



**Figure 6. Results of Statistic Analysis on Protein Expression of p21 and E-cadherin**

untreated group and the control siRNA group, and the expression level in the HDAC6 siRNA group was very low. HDAC6 siRNA could also effectively downregulate the expression of HDAC6 protein in EC9706 cells.

### Effects of down-regulation of HDAC6 expression on proliferation of EC9706 cells

The proliferation of EC9706 cells was detected using CCK-8 kit. Results were shown in Figure 3. There was no significant difference of EC9706 cell proliferation between the control siRNA group and the untreated group ( $P > 0.05$ ). The cell proliferation in the HDAC6 siRNA group was significantly reduced compared to the untreated group and the control siRNA group ( $P < 0.05$ ), respectively. This indicated that the down-regulation of HDAC6 expression could effectively inhibit the proliferation of EC9706 cells.

### Effects of down-regulation of HDAC6 expression on cell cycle of EC9706 cells

The cell cycle of EC9706 cells was detected by flow cytometry. The percentage of EC9706 cells in G0/G1 phase in the HDAC6 siRNA group ( $68.55 \pm 2.01\%$ ) was significantly higher than that in the untreated group ( $45.64 \pm 1.26\%$ ) and the control siRNA group ( $46.23 \pm 1.39\%$ ) ( $P < 0.05$ ), respectively. There was no significant difference between the untreated group and the control siRNA group ( $P > 0.05$ ). In addition, the percentage of cells in S phase in the HDAC6 siRNA group ( $25.93 \pm 0.73\%$ ) was significantly lower than that in the untreated group ( $33.13 \pm 1.02\%$ ) and the control siRNA group ( $32.98 \pm 0.91\%$ ) ( $P < 0.05$ ), respectively. Down-regulation of HDAC6 expression could induce the cell cycle of EC9706 cell to arrest in G0/G1 phase and might prevent its DNA synthesis.

### Effects of down-regulation of HDAC6 expression on migration of EC9706 cells

The migration of EC9706 cells was detected by Boyden chamber. Results were shown in Figure 4. The number of migrated EC9706 cells in the HDAC6 siRNA group ( $55.96 \pm 3.98$ ) was significantly less than that in the untreated group ( $134.32 \pm 6.71$ ) and the control siRNA group ( $127.85 \pm 7.12$ ) ( $F = 152.614$ ,  $P > 0.000$ ), respectively. There was no significant difference between the untreated group and the control siRNA group ( $P > 0.05$ ). This indicated that down-regulation of HDAC6 expression can effectively inhibit the migration of EC9706 cells.

### Expression of p21 and E-cadherin mRNA and protein mediated by HDAC6 siRNA

In order to further explore the mechanisms of cell cycle quiescence and migration reduction mediated by HDAC6 siRNA, the mRNA and protein expression of p21 and E-cadherin in EC9706 cells were detected by semi-quantitative RT-PCR and Western blotting methods. Results were shown in Figure 5 and 6. The mRNA and protein expression levels of p21 and E-cadherin in the HDAC6 siRNA group were significantly higher than that in the untreated group and the control siRNA group ( $P < 0.05$ ), respectively. There was no significant difference between the later two groups ( $P > 0.05$ ). This indicated

that the cell cycle quiescence and migration reduction of EC9706 cells mediated by HDAC6 siRNA might be closely related with the elevation of the mRNA and protein expression levels of p21 and E-cadherin RNA.

## Discussion

It is well known that the occurrence and development of tumors were bidirectionally regulated by the genetic modification and the epigenetic modification. The recent rising epigenetics plays a vital role in the occurrence and development of disease. Abnormal epigenetic regulatory mechanisms in body will lead to a variety of diseases including cancer and metabolic, genetic, immune, inflammatory, infectious, cardiovascular diseases. Epigenetics can not only deeply explore the mechanisms of various diseases, but also find the warning molecules and preventive target molecules of disease. It plays a significant role in the treatment and prevention of disease. With the publication of human epigenetics maps and development of epigenetic detection technology, there will be a greater progress of epigenetics in the next 10 years. It has been concluded for a long time that the gene mutation is involved in the tumor formation, but in recent years more and more evidences show that epigenetic modification also plays a very important role in tumor progression (Chen et al., 2011; Unoki et al., 2011). Epigenetic regulation can affect the gene transcription and do not change the DNA sequence. It includes DNA methylation and modifications of chromosomal histone such as methylation, acetylation, phosphorylation, ubiquitination, etc (Banerjee et al., 2009).

HDAC6 is a unique histone deacetylase locating at the cytoplasm. It is combined with the microtubules as a  $\alpha$ -tubulin deacetylase. It is found that a lot of protein can be acetylated, and HDAC6 plays an important role in the physiological and pathological processes such as transcriptional regulation, signal transduction, cell differentiation and apoptosis, growth and development, and occurrence of metabolic diseases and tumors (Barnes et al., 2009; Husain et al., 2009; Carter et al., 2010). Researchs show that HDAC6 can be regarded as the target for cancer treatment (Barnes et al., 2009), and histone deacetylase inhibitor (HDACi) can induce the cell apoptosis in cancer (Jiang et al., 2009; Shen et al., 2011). But so far the research on HDAC6 in ESCC is seldom reported.

The current rising RNA interference is one of the ideal technical means of studying the gene function in vivo and in vitro. It has been used in disease treatment and biological labeling (Baker et al., 2010) with a good prospects. Many studies show that the RNA interference plays an important role in inhibiting cancer gene over-expression (Xu et al., 2011). It may be a new means in cancer treatment. In this study, the interference of HDAC6 siRNA on HDAC6 expression in EC9706 cells is investigated. Results show that HDAC6 siRNA could effectively downregulate the expression of HDAC6 mRNA and protein in EC9706 cells. It has provided a theoretical basis for further studying the function of HDAC6 in ESCC.

Increase of HDAC expression level can accelerate

proliferation of tumor cell, reduce cell apoptosis, alter cell cycle, promote tumor angiogenesis formation and induce cancer gene expression. HDAC is over-expressed in many tumor cells, which results in expression inhibition of many important growth inhibiting genes. This is an important mechanism of HDAC promoting tumor cell proliferation. It is found that the over-expression of HDAC is closely related the decrease of p21 expression (Hrzenjak et al., 2006). Over-expression of HDAC3 in colon cancer cells can lead to the inhibition of p21 expression, and HDAC3 quiescence can promote the expression of p21 (Wilson et al., 2006). In addition, HDAC6 is expressed in the differentiated pre-osteoblasts, and it can interact with the transcription factor Runx2 to regulate the p21 expression (Westendorf et al., 2002). Results of our study show that down-regulation of HDAC6 expression can effectively inhibit the proliferation of EC9706 cells, arrest cell cycle at G0/G1 phase, and prevent the synthesis of DNA. Further study on expression of cell cycle related factor p21 shows that, after transfection with HDAC6 siRNA, the mRNA and protein expression levels of p21 in EC9706 cells significantly increase. This indicated that the cell cycle quiescence and migration reduction of EC9706 cells mediated by HDAC6 siRNA may be closely related with the elevation of p21 expression.

HDAC6 is a kind of multifunctional protein. It can regulate many different biological processes including cell migration, protein folding, misfolding protein degradation, cell stress and immunological synapse formation (Kawaguchi et al., 2003). A lot of studies find that down-regulation of HDAC6 expression can reduce the cell migration. Whether down-regulation of HDAC6 expression can reduce the migration of ESCC it is still unknown. In Our study, the migration of EC9706 cells is detected by Boyden chamber. Results show that down-regulation of HDAC6 expression can effectively inhibit the migration of EC9706 cells. E-cadherin is a glycoprotein with extracellular domain which interacts with neighboring E-cadherin molecular to create connections between epithelial cells. The function reduction and expression loss of E-cadherin may lead to the damage of cell connections which is closely related to the tumor cell invasion and metastasis (Zhang et al., 2012). It is found that inhibition of HDAC6 activity could significantly upregulate the E-cadherin expression (Rey et al., 2010). Our study shows that, after transfected with HDAC6 siRNA, the E-cadherin expression level in EC9706 cells significantly increases. This indicates that the migration reduction of EC9706 cells mediated by down-regulation of HDAC6 expression may be closely related with the rise of E-cadherin expression level, but the specific molecular mechanisms need to be further explored.

In conclusions, HDAC6 siRNA can effectively downregulate the expression of HDAC6 mRNA and protein in EC9706 cells. The down-regulation of HDAC6 expression caould obviously inhibit the cell proliferation of EC9706, inducing the cell cycle quiescence, and reduce the cell migration. These may be closely related with the rising expression levels of p21 and E-cadherin.

## References

- Ai J, Wang Y, Dar JA, et al (2009). HDAC6 regulates androgen receptor hypersensitivity and nuclear localization via modulating Hsp90 acetylation in castration-resistant prostate cancer. *Mol Endocrinol*, **23**, 1963-72.
- Baker M (2010). RNA interference: Homing in on delivery. *Nature*, **464**, 1225-8.
- Banerjee HN, Verma M (2009). Epigenetic mechanisms in cancer. *Biomark Med*, **3**, 397-410.
- Barnes PJ, Adcock IM (2009). Glucocorticoid resistance in inflammatory diseases. *Ancet*, **373**, 1905-17.
- Carew JS, Giles FJ, Nawrocki ST (2008). Histone deacetylase inhibitors: mechanisms of cell death and promise in combination cancer therapy. *Cancer Lett*, **269**, 7-17.
- Carter CL, Lin C, Liu CY, et al (2010). Phosphorylated p68 RNA helicase activates Snail1 transcription by promoting HDAC1 dissociation from the Snail1 promoter. *Oncogene*, **29**, 5427-36.
- Chen J, Zhou H, Li Q, et al (2011). Epigenetic modification of RhoE expression in gastric cancer cells. *Oncol Rep*, **25**, 173-80.
- Duong V, Bret C, Altucci L, et al (2008). Specific activity of class II histone deacetylases in human breast cancer cells. *Mol Cancer Res*, **6**, 1908-19.
- Fan LH, Li LX, Wei YC (2006). The Value of Immunocytochemistry in the Cytological Diagnosis of Serous Ef fusion. *J Mod Lb Med*, **21**, 6-8.
- He QX, Zhu QJ, Chen JO, et al (2006). Application of immunocytochemistry to cytological diagnosis in hydrothorax and ascites. *J Wenzhou Med College*, **36**, 466-8.
- Hideshima T, Bradner JE, Wong J, et al (2005). Small-molecule inhibition of proteasome and aggresome function induces synergistic antitumor activity in multiple myeloma. *Proc Natl Acad Sci USA*, **102**, 8567-72.
- Hrzenjak A, Moirand F, Kremser ML, et al (2005). Valproate inhibition of histone deacetylase 2 affects differentiation and decreases proliferation of endometrial stromal sarcoma cells. *Mol Cancer Ther*, **5**, 2203-10.
- Husain M, Harrod KS (2009). Influenza A virus-induced caspase-3 cleaves the histone deacetylase 6 in infected epithelial cells. *FEBS Lett*, **583**, 2517-20.
- Jiang XX, Zhang QL, Wu WL, et al (2009). Synergistic effects of proteasome inhibitor and Histone Deacetylase Inhibitor on apoptosis and aggresome formation in T Lymphoma cells. *J Experim Hematol*, **17**, 1232-6.
- Kakahana M, Ohira T, Chan D, et al (2009). Induction of E-cadherin in lung cancer and interaction with growth suppression by histone deacetylase inhibition. *J Thorac Oncol*, **4**, 1455-65.
- Kawaguchi Y, Kovacs JJ, McLaurin A, et al (2003). The deacetylase HDAC6 regulates aggresome formation and cell viability in response to misfolded protein stress. *Cell*, **115**, 727-38.
- Kuwano H, Watanabe M, Sadanaga N, et al (1993). Squamous epithelial dysplasia associated with squamous cell carcinoma of the esophagus. *Cancer Lett*, **72**, 141-7.
- Parkin DM (2000). Global cancer statistics in the year 2000. *Lancet Oncol*, **2**, 533-43.
- Parkin DM, Bray FI, Devesa SS (2001). Cancer burden in the year 2000. The global picture. *Eur J Cancer*, **8**, S4-66.
- Rey M, Irondele M, Waharte F, et al (2010). HDAC6 is required for invadopodia activity and invasion by breast tumor cells. *Eur J Cell Biol*, **12**, 25-30.
- Sakuma T, Uzawa K, Onda T, et al (2006). Aberrant expression of histone deacetylase 6 in oral squamous cell carcinoma. *Int J Oncol*, **29**, 117-24.
- Shen J, Wang WQ (2011). Enhancement of sensitivity of oral squamous carcinoma cells to cytochrome c mediated apoptosis by histone deacetylase inhibitors. *J Med Res*, **40**, 62-6.
- Shen R, Chinnaiyan AM, Ghosh D (2008). Pathway analysis reveals functional convergence of gene expression profiles in breast cancer. *BMC Med Genomics*, **1**, 28.
- Unoki M (2011). Current and Potential Anticancer Drugs Targeting Members of the UHRF1 Complex Including Epigenetic Modifiers. *Recent Pat Anticancer Drug Discov*, **6**, 116-30.
- Westendorf JJ, Zaidi SK, Cascino JE, et al (2002). Runx2 (Cbfa1, AML-3) interacts with histone deacetylase 6 and represses the p21(CIP1/WAF1) promoter. *Mol Cell Biol*, **22**, 7982-92.
- Wilson AJ, Byun DS, Popova N, et al (2006). Histone deacetylase 3 (HDAC3) and other class I HDACs regulate colon cell maturation and p21 expression and are deregulated in human colon cancer. *J Biol Chem*, **281**, 13548-58.
- Wu Y, Song SW, Sun J, et al (2010). Iip45 inhibits cell migration through inhibition of HDAC6. *J Biol Chem*, **285**, 3554-60.
- Xu C, Lee SA, Chen X (2011). RNA interference as therapeutics for hepatocellular carcinoma. *Recent Pat Anticancer Drug Discov*, **6**, 105-15.
- Yuan Q, Ye YJ, Fan YS (2006). The application of immunocytochemistry markers in cytological differential diagnosis of effusion smear. **1**, 50-3.
- Zhang H, Chen SH, Li YM (2004). Epidemiological investigation of esophageal carcinoma. *World J Gastroenterol*, **10**, 1834-5.
- Zhang J, He Y, Wand SK, et al (2012). Expression of E-cadherin in the tumor tissue and serum of patients with esophageal squamous cell carcinoma. *J Central South Univ (Medical Science)*, **3**, 228-32.
- Zhang XG, Li Z, Wang JF, et al (2009). Evaluation of risk factors and prevention strategies of esophageal cancer. *World Chinese J Digestol*, **17**, 677-80.
- Zhang ZH, Zhao LL, Guo HQ, et al (2008). Application of thin-layer cytology smears and immunocytochemistry in identifying the cell types of malignant tumors. *JOURNAL???* **35**, 332-5.