RESEARCH ARTICLE

MicroRNA-101 Inhibits Cell Proliferation, Invasion, and Promotes Apoptosis by Regulating Cyclooxygenase-2 in Hela Cervical Carcinoma Cells

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Abstract

Aim: Although aberrant miRNA expression has been documented, altered miR-101 expression in cervical cancer and its carcinogenic effects and mechanisms remain unexplored. The aim of our study was to investigate the role of miR-101 alteration in cervical carcinogenesis. Methods: Expression of miR-101 was examined by quantitative real-time reverse transcriptase PCR (qRT-PCR) in Hela cells. After modulating miR-101 expression using miR-101 mimics, cell growth, apoptosis and proliferation, and migration were tested separately by MTT or flow cytometry and cell wound healing assay and protein expression was detected by qRT-PCR. The expression of COX-2 in Hela cell was also examined by immunohistochemical staining and the correlation with miR-101 expression was analysed. Results: The miR-101 demonstrated significantly low expression in Hela cell. When we transfected miR-101 mimics into Hela cells, the modulation of miR-101 expression remarkably influenced cell proliferation, cycling and apoptosis: 1) The expression of microRNA-101 tended to increase after transfection; 2) Overexpression of miR-101 was able to promote cell apoptosis, the apoptosis rate being markedly higher (97.6%) than that seen pre-transfection (12.2%) ($P<0.05$); 3) The miR-101 negatively regulates cell migration and invasion, scratch results being lower (42.7um±2um) than that observed pre-transfection (181.4 um±2 um); 4) miRNA-101 inhibits the proliferation of Hela cells as well as the level of COX-2 protein, which was negatively correlated with miR-101 expression. Conclusions: Overexpression of miR-101 has obvious inhibitory effects on cell proliferation, migration and invasion. Thus reduced miR-101 expression could participate in the development of cervical cancer at least partly through loss of inhibition of target gene COX-2, which probably occurs in a relative late phase of carcinogenesis. Our data suggest an important role of miR-101 in the molecular etiology of cancer and indicate potential application of miR-101 in cancer therapy.

Keywords: MicroRNA - cervical cancer - proliferation - transfection - COX-2

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Introduction

Cervical cancer is malignant with second highest morbidity and mortality in women worldwide (Arbyn et al., 2011). In Xinjiang, especially in the southern border region, cervical cancer is one of the leading cause of death in minority women (Lalaisuzuke et al., 2006). The incidence is higher in developing countries than that of developed countries. Chronic inflammation has been revealed to contribute to the development of malignant tumors (Li et al., 2013). Most patients with cervical cancer are diagnosed when the disease has already been in a severe status. It is appealing to investigate new endogenous and therapeutic targets for prevention and treatment of cervical cancer.

Although great progresses have been seen in using surgical technique, diagnostic method and new chemotherapy regimens for cervical carcinoma, effective targeting therapy is rarely available, and the exact molecular mechanism (s) (of cervical cancer) has not yet been fully elucidated. Thus, further study and understanding the potential molecular mechanism of cervical carcinoma is still a must.

One of the latest and progressive approaches for molecular characterization of various tumors today is based on micoRNA (miRNA) expression profiles. MicroRNAs (miRNAs) are a group of endogenously expressed, non-coding small RNAs (20-25 nucleotides in length) known to negatively regulate gene expression by suppressing translation or decreasing the stability of mRNAs by directly binding to the 3'-untranslated region (3'-UTRs) of target mRNAs (Dykxhoorn, 2010; Cui et al., 2011). Functional studies indicate that miRNAs are important to several fundamental biological processes, including proliferation, apoptosis, development, and cellular differentiation (Bumgarner et al., 2009; Yu et al.,

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2010). Computational and experimental outcomes predict that miRNAs regulate at least 30% of protein-coding genes (Ivey and Srivastava, 2010) and approximately 50% of miRNAs genes are located in cancer-related genomic regions (Kim et al., 2010). miRNAs can through these genes to regulate cancer development as tumor suppressors or oncogenes (Krol et al., 2010). For example,Some miRNAs can function as oncogenes or tumor suppressors by regulating the expression of target genes, such as miR-7 (Kefas et al., 2008), miR-128 (Godlewski et al., 2008) have all been shown to be down-regulated in tumor specimens and function as tumor suppressors; miR-17 (WU et al., 2013) and miR-21 (Kumar et al., 2013) have been shown to be up-regulated in tumor specimens and function as oncogenes. These studies indicate that the dysregulation of miRNAs is frequently involved in carcinogenesis and cancer progression and, moreover, they suggest that the pathogenesis of cervical carcinoma may be attributed to miRNAs. In addition, miRNA expression profiling can also be used to predict the clinical outcome of cancer patients (Jiang et al., 2008; Xie et al., 2012).

MiR-101 belongs to a family of miRNAs that are involved in a series of cellular activities, e.g. cell proliferation, invasion, and angiogenesis (Semaan et al., 2011). MiR-101-1 has been found in the genomic fragile regions that are associated with abnormal deletion or amplification in cancer (Buechner et al., 2011). In recent years, several published studies have shown that miR-101 is decreased expressed in several cancer types including breast, lung, prostate, ovarian, colon, and liver cancers, and emerging evidence suggests a tumour suppressive role for this miRNA (Zhang et al., 2012; Schwarzenbacher et al., 2013; Zhang et al., 2013). However, miR-101 as a significantly down-regulated miRNA, was further studied in greater detail because the signal pathway (s) regulated by miR-101 and the role of miR-101 in tumorigenesis have not yet been elucidated. In cervical cancer cell lines, the biological functions and regulatory mechanisms of miR-101 is unclear.

Cyclooxygenase enzymes, COX-1 and COX-2, perform the rate-limiting step in the conversion of free arachidonic acid into prostaglandins and have distinct roles in physiologic and pathologic conditions (Wang and Dubois, 2010). COX-2 expression depends on the interplay between different cellular pathways involving both transcriptional and post-transcriptional regulation (Agra Andrieu et al., 2012). Normally absent in most cells, COX-2 (PTGS2) expression is rapidly induced by proinflammatory and growth-associated stimuli. Substantial evidence has shown unregulated COX-2 expression to be a contributing factor in many chronic diseases and cancer (Menter et al., 2010). Many studies have confirmed, COX-2 gene has a closely relation with variety of human tumors in the occurrence and development , such as gastric cancer (He et al., 2012), breast cancer (Hsia et al., 2013), colorectal neoplasia (Dixon et al., 2013), etc. Furthermore ,The many current researchs also showed that between COX-2 and microRNAs exist certain relationship, or even co-regulated to the tumor incidence. However, it is still uncertain whether COX-2 could be regulated by miRNAs or RNA-binding proteins in human cervical carcinoma cell lines and whether COX-2 levels in human cervical carcinoma cell lines correlate with an altered expression of microRNA-101.

In this study, we aim to identify the mechanism and function of miR-101 when regulated. Further investigation to revealed that a frequently up-regulated miRNA, the miR-101, could sensitize to apoptosis and impaired the ability of cancer cells. Moreover, COX-2 , was characterized as a direct target of miR-101. Our findings will help to elucidate the functions of miRNAs and their roles in tumorigenesis. And to explore the therapeutic potential of miR-101 in cervical cancer.

Materials and Methods

Cell lines and culture

The cervical carcinoma cell (HeLa cells) were obtained from the Cell Bank of Shanghai. Cells were grown in DMEM medium (Gibco) and with 10% fetal bovine serum, 50 U/mL of penicillin, and 50 mg/mL of streptomycin. At 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Transfection

The human miR-101 duplex mimic (miR-101) and negative control oligonucleotide duplex mimic (miR-NC) were designed and provided by Invitrogen. RNA oligonucleotides were transfected by using Lipofectamine™2000. After 24 h transfection, cells were used for subsequent experiments including proliferation, apoptosis, migration, and invasion assays.

Real-time PCR

TotalRNA from the frozen tissue specimens and cultured cells was isolated using the TRIzol kit (Invitrogen) following to the manufacturer's instructions and then using the Thermo Scientific RevertAid First Strand cDNA Synthesis kit (Thermo) to reverse transcribed and amplifed the RNA, Real-time PCR was performed using the QuantiFast SYBR Green PCR mixture (Invitrogen). The U6 small nuclear RNA was amplifed as a loading control. The primers for this U6 internal control were purchased from Invitrogen company. Data were shown as fold change ($2^{-\Delta\Delta Ct}$) and analyzed initially using GraphPad Prism 5.0 software. MiR-101 specific primers used for PCR amplification were as follows: 5'-TACAGTACTGTGA TAACTGAA-3'for hsa-miR-101 ,5'-GTCGTATCCAGT GCAGGGTCCGAGGTATTCGCACTGGATACGACTT CAGT -3' for hsa-miR-101-RT. They were synthesized by Invitrogencompany. The experiment was repeated three times.

MTT

The MTT method was used to estimate cell viability. The cells were plated at an initial density of 10^4 cells per well in flat-bottom, 96-well cell culture plate and allowed to grow for 48 hours. MTT (BIO-BOX) was added to each well followed by a 4-hour incubation at 37°C. After removing the media, dimethyl sulfoxide was added to each well for solubilizing the formazan formed. After 30

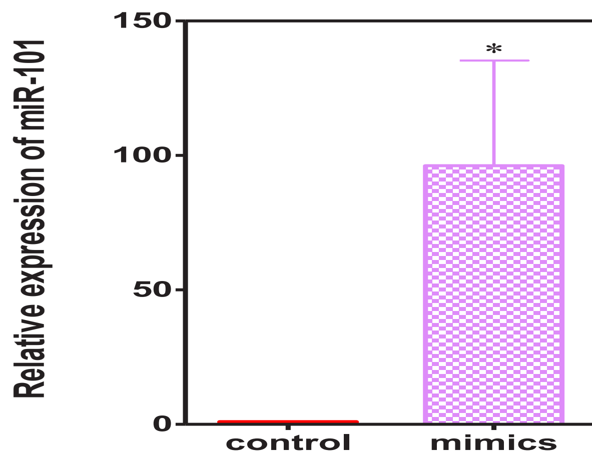

Figure 1. Mature miR-101was Extremely Low Expressed in Normal Hela Cell, after Transfected with miR-101 Mimics While the Expression Level of miR-101was Significantly Increased. The posttransfection expression level of miR-101 is ninety eight times than normal time. The difference was statistically significant

minutes at room temperature, the plates were scanned spectrophotometrically with a microplate reader set at 490 nm for measuring the absorbance.

Detection of apoptosis

Apoptosis and cell death were assessed using the Annexin V-EGFP Apoptosis Detection Kit, Briefly, the cells were seeded at 1.25×10^5 cell/ml in a 6-well plate and incubated for 72 h.The cell were then washed twice with 1×PBS and harvested, resupended in 1×Annexin V-binding buffer at a concentration of 1×10^6 cells/ml. One hundred microlitres of the cell suspension was incubated with 5 ul of Annexin V-FITC and 5 ul of propidiun iodide in the dark for 15 min at room temperature. The samples were then analysed on the FACSCalibur™ flow cytometer after the addition of 400ul of 1×binding buffer at the end of incubation. The results were expressed as the percentage of apoptotic cells and non-viable cells amongst the total cells counted.

Cell wound healing assay

Hela cell transfected by miR-101 mimics and miR-101 NC were seeded in a 6 well plate and cultured for 48 h to obtain 80% monolayer confluency. A wound was created by scraping the cells using a plastic pipette tip n the bottom of the culture flasks, and the medium was replaced with fresh medium. Images were captured immediately (day 0) and every day for 3 days. Take photos for three consecutive days Cell migration was qualitatively assessed by the size of the wounds at the end of the experiment.

Immunohistochemistry

Immunhistochemistry was performed according to the method described previously. Briefly, cell washed twice with 10×PBS, 4% paraformaldehyde for 15 minutes, washed twice with 10×PBS, 3% H_2O_2 for 15 minutes, washed twice with 10×PBS. Serum closed, serum in blocking solution in the kit, 37°C, 20 minutes. Remove the serum closed, Add 1:150 primary antibody, 4°C 12 h. Negative control antibody was replaced with PBS. Washed twice with 10×PBS, Adding secondary antibody, 37°C, 20 minutes. DAB colorimetric method to color for 10 minutes. PBS to terminate and take photos. The immunohistochemical staining of each sample was scored based on staining intensity and percentage of the cells stained. In detail, the staining intensity was assigned a rating from0 to 3: 0 means negative in cells; 1 weakly positive; 2 moderately positive; and 3 strongly positive. The percentage of positive cells was quantitated in four randomly selected fields and graded using following five categories: 0 = 0-9% of cells positively stained; 1 = 10-24% of cells positively stained; 2 = 25-49% of cells positively stained; 3 = 50-74% of cells positively stained and 4 = 75-100% of cells positively stained. Finally, the intensity score and percentage score were multiplied to yield an overall score. According to overall score, immunohistochemical reactivity for EZH2 expression in each sample was classified into 4 groups: 0 - was defined as 'negative'; 4 - defined as 'weak'; 6 - defined as 'moderate' and 8 - defined as 'strong'.

Statistical analysis

All mapping were using GraphPad Prism 5.0 software. All statistical analyses were carried out using SPSS version 17.0 statistical software (SPSS Inc, Chicago, IL, USA). P values < 0.05 were considered statistically signifi cant and all of them are two-sided. Quantitative data were expressed as the mean ± SD. Student's t test and ANOVA was used to determine statistical significance. Differences were considered significant at $P < 0.01$.

Results

Expression of miR-101 is frequently reduced in human Hela cell

Previously, it has been reported that miRNA-101 is down-regulated in human bladder cancer tissue versus normal adjacent tissue. However, The expression of miR-101 in Hela cell lines has not been well documented. To test whether miR-101 was correlated with cervical carcinoma cell development, We measured the expression of miR-101 in Hela cell transfected with or not, miR-NC, miR-101 mimic using quantitative RT-PCR. The quantitative real-time PCR analysis displayed that in Hela cell (miR-NC), miRNA-101 exhibited apparently low level expression, whereas the expression of miRNA-101 was significantly (P<0.01) up-regulated in Hela cell which transfected into miR-101 mimic. In addition, miR-101 mimics group level of expression was ninety eight times rising than normal group (Figure 1). This finding indicated that the overexpression of miR-101 is remarkable up-regulated.

Overexpression of miR-101 can promotes Cell Apoptosis

We also analyzed the effect of miRNA-101 on apoptosis in Hela cells by conducting Annexin V and PI double staining. The Annexin V-positive early-phase apoptotic cells were significantly increased in cells transfected with miRNA-101 mimics oligo -nucleotide when compared with miR-NC or non-transfected cells.

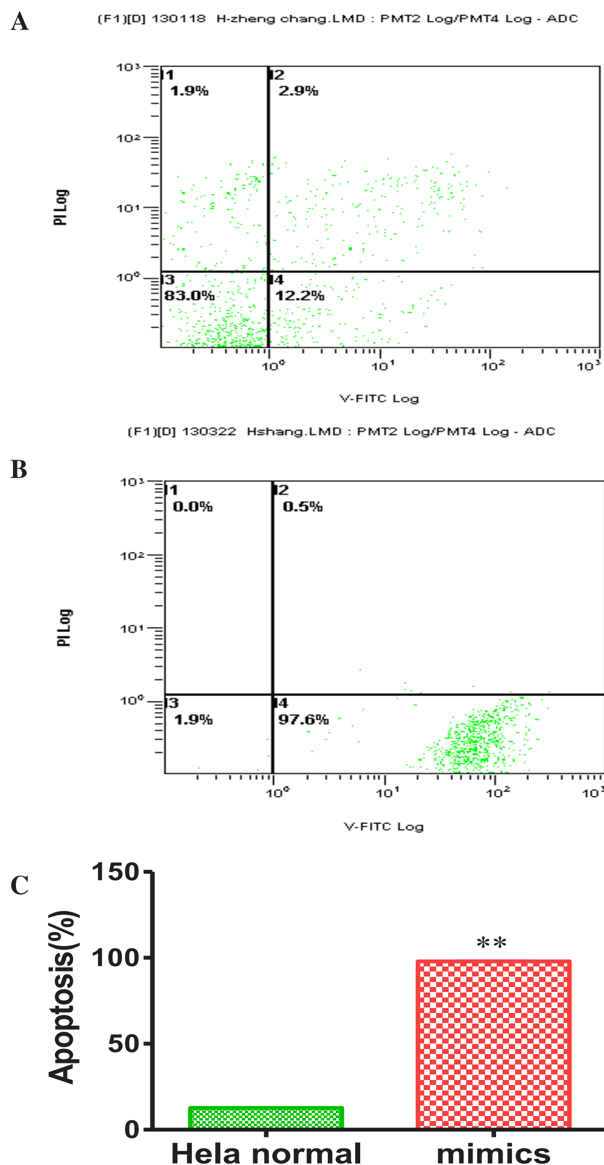

Figure 2. Up-regulated miR-101 Significantly Encouraged Cell Apoptosis in Hela Cells by Flow Cytometry. (A) The apoptosis rate when normal Hela cell. (B) The apoptotic rate when imported mimics of miR-101 into the cell. (C) The picture of both Apoptpsis (%). After transfection, apoptosis rate was eight times than normal, The difference was statistically significant

Percentages of apoptotic cells are shown in the histogram (Figure 2A, B). The apoptosis rate were higher (97.6%) than pre-trasfection (12.2%) (Figure 2C). Annexin-V-FITC/PI double staining assay showed that miRNA-101 induced apoptosis of Hela cells. Taken together, the results suggest that miR-101 could enhanced Hela cells to apoptosis by transfect into mimics.

miR-101 negatively Regulating Cell Migration and Invasion

Invasive growth is an important biological characteristic of malignant cancer cells. To evaluate the impact of miRNA-101 on invasive ability of Hela cells, we employed cell wound healing assay. In Hela cell, cells transfected with or not, miR-NC, miR-101 mimic, were applied to Cell wound healing assay. The result of scratch detected were lower (42.65 um±2um) than pre-

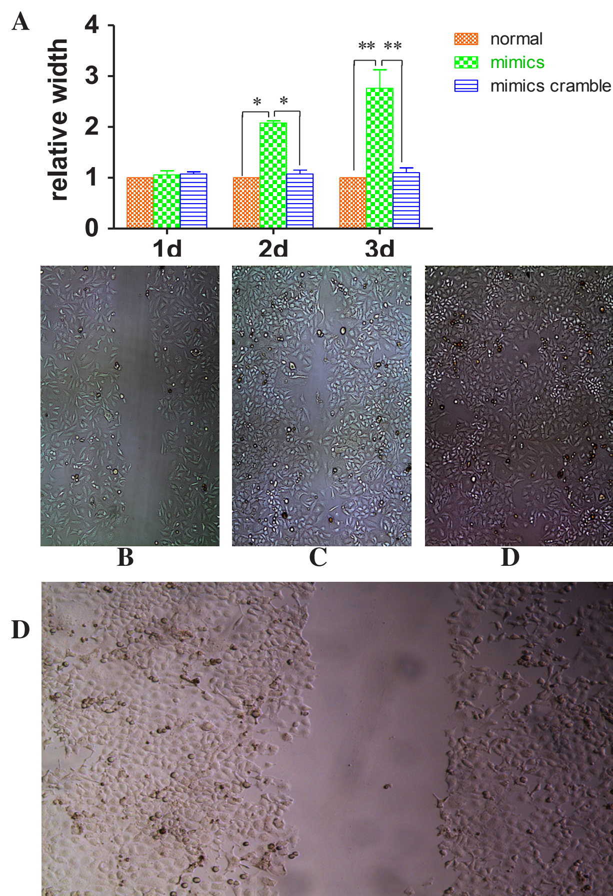

Figure 3. Up-regulated miR-101 Significantly Inhibited Cell Migration and Invasion in Hela Cells by Cell Wound Healing Assay. (A) Normal Hela cell by Cell wound healing assay. The Relative Width at the time of 1day,2day, and 3 day posttransfection of miR-101 mimics, the result of scratch detected were lower (42.65um±2um) than pre-transfection (181.38um±2 um). The migration inhibition rates were 0%,50%,33% ,respectively. (B, C, D) The picture of normal Hela cell three days scratches. FigureC5,6,7: The picture of posttransfection three days scratches

transfection (181.38 um±2 um) (Figure 3). These results suggested that miR-101 function can inhibit migration and invasion in Hela cells.

miRNA-101 inhibits the proliferation of Hela cells

The growth ability of Hela cell was determined by MTT assay. At 0-72 h time point were shown that the inhibition rates were 86%, 27%, 19%and13% ,respectively (Figure 3). These results imply that miRNA-101might function as a tumor suppressor in Hela cell.

miR-101 and COX-2 are inversely expressed in Hela cell

The binding of miR-101 to COX-2 mRNA was analyzed by Immunohistochemistry analysis. Observed under a microscope at 10x, Hela cells in the normal group, there are 960 cells is positive, 40 cells emerge negative (Provisions of its total number of observations is 1000 cells), while after transfected into miR-101 mimics, 670 cells were positive, 330 cells appear negative. The immunoprecipita- COX-2 exhibited increased expression in the miR-101 inhibtor of Hela cell compared to the miR-101 mimic (Figure 3).

00.0
75.0
50.0
25.0
0
6.3
56.3
31.3
Newly diagnosed without treatment

Discussion

Cervical cancer is the second women worldwide common malignancy, rank only second to breast cancer. In developing countries, ranks No. 2 after breast cancer is the most common malignancy of the female reproductive tract. In 2008, there are 529,800 new cases of cervical cancer to estimated, 25.51 million deaths, 85% of which were new cases in the developing countries (Shen et al., 2013). The most important etiologic agent in the pathogenesisis human papilloma virus (HPV), However, not all women infected with high-risk HPV develop cervical carcinoma, there are also related to some changes in genetic abnormalities, such as the aberrantion of miRNAs (Forman et al., 2012; Peralta-Zaragoza et al., 2013).

Increasing evidence suggests that miRNAs play a critical role in carcinogenesis and cancer progression. Altered miRNA expression levels have been implicated in the initiation and development of tumors; modulation of miRNAs that function as a negative regulator of oncogenes, or tumor suppressors in promotion of cancer cell proliferation and growth (Chen et al., 2013). Previous studies have shown that the role of miR-101 in the progression of various types of tumors is controversial. The expression pattern and targets of miR-101 vary in different types of tumors. miR-101 expression is down-regulated in some tumors, functioning as a tumor suppressor and oncogene by targeting oncogenes in some cancers. In recent years, relevant article said that MicroRNA 101 could suppresses bladder cancer cells (Hu et al., 2013), human hepatocellular carcinoma (Su et al., 2009), vascular endothelial (Chen et al., 2012), lung cancer (Cho et al., 2011) and some other cancers, caused by some presentations on cancer cell proliferation, apoptosis. It is proved that microRNA-101 have a close relationship with tumor occurrence and development.

In this study, We show that miR-101 is frequently down-regulated in Hela cell. We also reveals that in miR-101-overexpressing cells, Hela, which we observed, significant effects on cell proliferation, migration and invasion, and the result of which can decrease cell growth, and increase cellular apoptosis. These effects of cell proliferation and migration are similar with those observed here in other human cancer cells that have been reported. We further characterize COX-2 as the functional target of miR-101. It can reduce expression of miR-101 in Hela cell. Collectively, these findings emphasize a fundamental role of miR-101 in tumorigenesis, especially when up-regulating miR-101, it may inhibit the initiation and progression of cervical carcinoma.

Apoptosis is a major barrier that must be circumvented during malignant transformation. Cancer cells evolve to evade apoptosis so that they can escape from being cleared away by the surveillance system and can survive in the crucial tumor microenvironment (Palumbo et al., 2013). In this study, we show that up-regulated miR-101 can significantly promote apoptosis of Hela cell. This performance shows that Hela cell may be particularly sensitive to miR-101. Growing numbers of miRNAs have been implicated in the regulation of apoptotic cell death and in the development of cancers. For instance, miR-15a and miR-16-1, which are down-regulated in the majority of chronic lymphocytic leukemia patients, induce apoptosis by down-regulating Bcl-2 (Ding et al., 2013).

To date, miR-101 significantly inhibits cellular proliferation, migration and invasion of gastric cancer cellsby targeting EZH2, Cox-2, Mcl-1 and Fos (Wang et al., 2010). Cyclooxygenase-2 gene is located on chromosome NO.1 1q25.2-q25.3, length 8.3 kb, consists of 10 exons and 9 introns, encoding 604 amino acid residues from the polypeptide consisting of base. Regulation Carcinogenic mechanism of COX-2, is now more accepted view is that COX-2 can promote cell proliferation, inhibition of apoptosis, promote angiogenesis, suppression of the immune function and other mechanisms involved in tumor development and progression. As an aspect of the relationship with the microRNA-101, the prediction based on PicTar and TargetScan DNA analysis software suggest that COX-2 could be one direct target for miR-101 because miR-101 has a seed region, which is able to bind to the COX-2 mRNA 3-UTR. Such as miR-101 and COX-2 can control the development of mammary gland (Tanaka et al., 2009), colon cancer (Strillacci et al 2009) and prostate cancer (Hao et al., 2011). In recent years, studies have found that some cancer cells ,which have a high-expression of COX-2, their ability of the proliferation and metastasis have also been highly focused. In this research, we find that over-expressed the miR-101 can make the expression of COX-2 tend to fall, which suppressed the ability of proliferation and migration and, thus promote apoptosis of the Hela cells. In other words , in cervical cancer cells , We found that there is a significant correlation between expression levels of miR-101 and protein levels of COX-2 through Immunhistochemical means. The COX-2 protein in the positive group , the expression levels of miR-101 is significantly lower than that in the negative group. It prompts that miR-101 in Hela cells may regulate the expression of COX-2 protein, when COX-2 shows itself as a high expression in the Hela cell ,while miR-101 is has a low trend. We speculate that it may be involved in some of the tumor suppressor gene promoter methylation status, leading to tumor suppressor gene silencing and, thereby, contributing to the incidence and development of cervical cancer .

In conclusion, our study demonstrates that miR-101 is significantly down-regulated in Hela, and miR-101 is a kind of miRNAs which as a potential novel cancer suppressor in Hela, which might play a role in receding cell proliferation, migration and invasion of Hela cell. These results indicate that promotion of miR-101may be a potential therapeutic strategy for treatment of cervical carcinoma. It is worthwhile to fully study and understand its molecular mechanism by which miR-101 playing a role as cancer suppressor gene in the development and progression of cervical carcinoma.

On the other hand, we find that Lipofectamine™2000 has toxic effects on cells. It more or less has a certain influence on cell proliferation and apoptosis, and affect the results of the experiment. In the next experiment we will improve and review the ways of transfection. For example, we can use the technology of gene gun or Phage infection technique as an alternative and effective way to try and see the results.

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