Aberrant Expression of miR-20a and miR-203 in Cervical Cancer

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Abstract

MicroRNAs (miRNAs) are small, non-coding RNAs that are critical regulators of various diseases. MicroRNA-20a (miR-20a) and microRNA-203 (miR-203) have previously shown significant alteration in a range of cancers. In this study, the expression levels of miR-20a and miR-203 in 100 cervical cancer tissues were detected by qRT–PCR and compared to patient matched-nontumor cervical tissues. Correlations between expression level and clinicopathologic characteristics of cervical cancer were also analyzed. Finally, we studied the effect of miR-20a and miR-203 on cell proliferation in cervical cancer cell lines by MTT. We found that the expression level of miR-20a ($P<0.001$) was significantly higher in cervical cancer patients than in healthy controls, while that of miR-203 ($P<0.001$) was lower. Aberrant expression of miR-20a was correlated with lymph node metastasis (LNM), histological grade and tumor diameter, but down-regulated miR-203 was correlated with LNM only. Furthermore, we found that over-expression of miR-203 decreased cell proliferation, while reduction of miR-20a also prevented tumor progression. Our results support the involvement of miR-20a and miR-203 in cervical tumorigenesis. We propose that miRNAs might be used as therapeutic agents for cervical cancer.

Keywords: miR-20a - miR-203 - cervical cancer - therapeutic agents

Introduction

Cervical cancer is the third most common type of cancer in women all over the world (Jemal et al., 2011), which is a leading cause of cancer death, resulting in about 300,000 deaths each year. Most cervical cancer patients receive standard radiotherapy and chemotherapy. However, clinical outcomes vary significantly. So many researchers devote themselves to find pathogenesis and more effective tumor therapy.

Many genetic events are required for cancer development. Recently aberrant expression of miRNAs is reported in various types of cancers. miRNAs are small non-coding RNAs of approximately 22 nucleotides (nt) and act as post-transcriptional regulators of gene expression. These small molecules have been found to regulate genes involved in diverse biological processes such as cell proliferation, development, differentiation, apoptosis and others (Lagos-Quintana et al., 2001; Lee et al., 2001). Numerous of studies have shown that alterations in miRNAs synthesis in human cancers are often related to tumor development, progression and metastasis. There is a hypothesis that deregulated synthesis of miRNAs, which in turn regulate protein synthesis, is one of the most important factors contributing to cancer development (Lin et al., 2012; Ma et al., 2012; Liang et al., 2013). Altered miRNA expression profiles have also been reported in cervical cancer as compared with normal cervix (Lee et al., 2008; Hu et al., 2010). miRNAs are different in diverse tumors, research on miRNAs expression profile will contribute to the classification of tumors. To inhibit the oncogene-like miRNAs or to over-express the anti-tumor miRNAs will be a novel method on tumor therapy.

In this study we investigated the expression profiles of mir-20a and mir-203 in cervical cancer tissues and cervical cancer cell line. Our primary aim was to determine whether there were significant correlations between miRNAs expression and histological characteristics. Then we change their expression in cell line to detect the anti-tumor efficacy, which could be a promising starting point for developing future miRNA-based cervical cancer therapy.

Materials and Methods

All cervical tissue samples were collected at the department of gynecologic oncology, Guangxi Tumor Hospital between 2010 and 2011. Eighty cervical cancer samples of International Federation of Gynecology and Obstetrics (FIGO) stage I-II were obtained from patients who underwent surgical treatment. Twenty samples of stage IIB-IV were got from cervical biopsy. All samples were squamous cell carcinoma. No previous local or systemic treatment had been conducted on these patients before the operation or biopsy. LNM was confirmed in patients of stage I-II which we used operation for the first treatment. Normal cervical epithelium samples were collected from twenty patients who had hysterectomy for benign disease. The median age for patients was 49

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years with a range from 25 to 69 years. The mean age for control subjects was 45 years, ranging from 33 to 57 years. The tissues were frozen in liquid nitrogen immediately after surgical removal and stored at -80°C until use. All protocols were approved by the Ethics Committee of the Guangxi Medical University. The human cervical cancer cell SiHa was kept by our laboratory. All cells were cultured in RPMI 1640 medium containing 10 % fetal bovine serum (FBS) in a humidified 37°C incubator with 5% CO₂.

RNA was extracted from frozen fresh cervical cancer tissues, normal cervical epithelium tissues and cervical cancer cells using the miRcut miRNA isolation kit(Tiangen, China) according to the manufacturer’s instructions. The reverse-transcription reactions were carried out using an MiraMasTM Kit (Bioo scientific, USA), which contains poly (A) polymerase used for polyadenylation of miRNA. qRT-PCR was performed using a standard SYBR Green PCR kit (takara, Japan). The primers were synthesized (Shanghai GenePharma, China) as follows: miR-20a forwards primer: TAC GAT AAA GTG CTT ATA GTG CAG GTA G. miR-203 forwards primer: TAC GAG TGA AAT GTT TAG GAC CAC TAG. U6 forwards primer: ATT GGA ACG ATA CAG AGA AGA TT. Universal reverse primer: GTC CTT GGT GCC CGA GTG. The 20 μl mixture of PCR consisted of 12.5 μl SYBR Green supermix, 3.5 μl RNase-free water, 1 μl forward primers, 1 μl reverse primers, and 2 μl reverse transcribed product. The reactive condition was 40 amplification cycles of 95°C for 3 min, 95°C for 12 s, and 62°C for 50 s using a BIO-chromo4 (Bio-Rad, USA) quantitative Real-Time PCR System. U6 was used as references for miRNAs. Each sample was analyzed in triplicate. Comparative threshold cycle (CT) method-fold change (2⁻ΔΔCT) was used to analyze relative changes.

Cell transfection and MTT assay: miR-20a inhibitor, miR-203 mimics, miRNA negative control (NC) were chemically synthesized by GenePharma (Shanghai GenePharma, China) as follows: miR-20a inhibitor: CUA CCU GUA CUU AUA GCA CUU UA. miR-203 mimics: 5'-GUG AAA UGU UUA GGA CCA CUA G-3'; 3'-AGU GCC CUU AAA CAU UUC ACU U-5'. Negative control: 5'-UUC UCC GAA CGU –GUC ACG UTT-3'; 3'-ACG UGA CAC GUU CGG AGA ATT-5'. We transiently transfected the miR-20a inhibitor (200 nM), miR-203 mimics (100 nM) and NC (100 nM) in cultured cells at 30-50% confluence using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer’s instructions. The capacity for cellular proliferation was measured with the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Twenty-four hours after transfection, 1×10⁴ cells were seeded into 96-well microtiter plate for 24, 48, 72, and 96 h, respectively. Then, the cells were incubated with 20 μl of MTT (5 mg/ml, PH=7.4) for 4 h at 37°C and 150 μl of dimethyl sulfoxide was added to solubilize the crystals for 20 min at room temperature. Optical density (OD) was measured at a wavelength of 490 nm. All experiments were performed three times and were calculated using average results, which we used to draw the growth curves. Growth inhibition rate was calculated as following: (AC−AT)/AC×100% (AC = absorbance value of the NC and AT = absorbance value of the experimental group) (Luan et al., 2010).

All data were processed using PASW Statistics 16. Since the results did not display normal distribution, we chose to analyze the data with non-parametric methods. (Mann-Whitney U test between two groups and Kruskal-Wallis H test for three or more groups). P-value<0.05 was considered significant.

**Results**

In order to assess the role of miRNAs in cervical cancer development, we measured miR-20a and miR-203 expression levels in normal cervical tissues and cervical cancer tissues by qRT-PCR. In our study, the data reported in Figure 1A shows miR-20a is definitely up-regulated in
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Table 1. Association Between the Expression of miR-20a and miR-203 with Clinicopathological Features in Patients with Cervical Cancer

|                                | N       | Fold change of miR-20a (median*) | Fold change of miR-203 (median*) |
|--------------------------------|---------|---------------------------------|---------------------------------|
| Age <50                        | 60      | 6.94(1.71-55.8)                 | 0.23(0.029-0.927)               |
| ≥50                           | 40      | 6.92(1.7-27.786)                | 0.12(0.058-1.874)               |
| p                              | 0.602   | 0.814                           |                                 |
| Diameter of tumor              |         |                                 |                                 |
| <4cm                           | 83      | 3.56(1.7-27.8)                  | 0.23(0.029-1.888)               |
| ≥4cm                           | 17      | 55.8(6.9-224.9)                 | 0.12(0.058-0.347)               |
| p                              | 0.013   | 0.283                           |                                 |
| FIGO stage                     |         |                                 |                                 |
| I                              | 51      | 1.74(0.9-27.3)                  | 0.46(0.015-3.683)               |
| II                             | 35      | 6.97(1.73-28.28)                | 0.12(0.029-0.911)               |
| III                            | 9       | 27.83(17.1-83.6)                | 0.23(0.086-0.232)               |
| IV                             | 5       | 226(113-666)                    | 0.06(0.037-0.118)               |
| p                              | 0.004   | 0.611                           |                                 |
| Histologic grade               |         |                                 |                                 |
| Well                           | 61      | 3.51(1.3-27.6)                  | 0.12(0.015-1.887)               |
| Moderate                       | 18      | 10.39(0.4-34.6)                 | 0.18(0.058-0.913)               |
| Poorly                         | 21      | 28.01(4.3-110.6)                | 0.12(0.059-0.464)               |
| p                              | 0.027   | 0.952                           |                                 |
| LNM stage                      |         |                                 |                                 |
| negative                       | 28      | 1.72(0.54-6.05)                 | 1.87(0.203-6.497)               |
| positive                       | 23      | 13.75(0.9-111.5)                | 0.058(0.004-0.232)              |
| p                              | 0.001   | 0                               |                                 |
| stageIIA                       |         |                                 |                                 |
| negative                       | 12      | 1.74(0.55-6.08)                 | 0.704(0.073-3.728)              |
| positive                       | 17      | 27.5(1.3-55.8)                  | 0.029(0.007-0.347)              |
| p                              | 0.001   | 0                               |                                 |

*Median of relative expression with 25th-75th percentile is recorded.

Discussion

Currently, hundreds of human miRNAs have been identified. It is also known that a single miRNA can influence on the expression of several thousands of genes, thus controlling one third of the human genome (Lewis et al., 2005). miRNAs are able to regulate various biological processes such as cell growth, their differentiation and death (Flynt et al., 2008), neuroprocesses and immune response (Lodish et al., 2008; Stadler et al., 2008). The high specificity of miRNAs compared with mRNAs indicates that these small molecules can serve as highly informative cancer biomarkers (Jiang et al., 2005; Lu et al.,...
miR-20a have relationship with LNM, increased tumor tissue with a median of 6.92, up-regulated expression of miR-203 was down-regulated in sample group in comparison to non-tumor counterparts (Viticchiè et al., 2011; Takeshita et al., 2011). A Series of works demonstrated the direct correlation between miRNAs and epigenetic mechanisms, including hypermethylation of CpG islands located next to gene promoter regions, causing the silencing of tumor suppressor genes. Moreover, histone post-translational modifications, such as deacetylation and methylation, are also common. These modifications have important roles in tumor initiation and maintenance. Treatment of lymphoma B cells with demethylating agents led to increased miR-203 expression, which was reported by Craig et al. (2011). miR-20a could also be affected by DNA methylation (Lee et al., 2012). Botezatu found the results for the high risk-HPV precursor lesions and tumors indicate a possible involvement of the high risk HPV genotype in the miRNA methylation process (Botezatu et al., 2011). A Series of works demonstrated the direct correlation between miRNAs and epigenetic mechanisms in cancer which could be an important mechanism for the transcriptional regulation of miRNAs.

In conclusion, our results suggest that miR-20a and miR-203 expression may be related with malignant process of cervical cancer, especially invasion and metastasis. Large-scale and long-term follow-up studies are needed to confirm the significance of miR-20a and miR-203 in cervical cancer. miRNAs may be an attractive target for therapeutic intervention.

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