RESEARCH ARTICLE

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

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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) stageI-IIA were obtained from patients who underwent surgical treatment. Twenty samples of stageIIB-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-IIA 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% CO_2.

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 synthetize (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 ($2^{-\Delta\Delta CT}$) 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 CUA UAA GCA CUU UA. miR-203 mimics: 5'-GUG AAA UGU UUA GGA CCA CUA G-3'; 3'-AGU GGU CCU 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 transected 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).

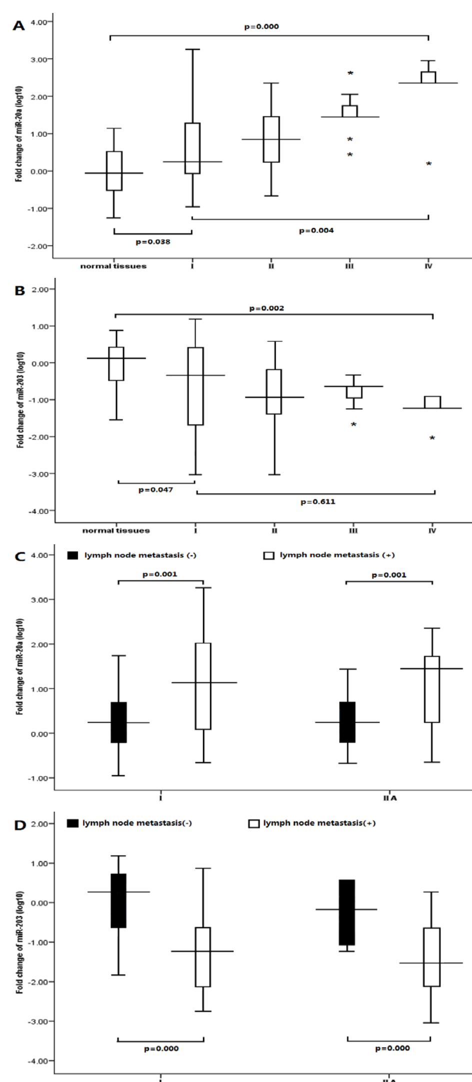

Figure 1. Expression of miR-20a and miR-203 in 100 Patients with Cervical Cancer. A, B: miR-20a and miR-203 were measured by qRT-PCR. Data were presented as log10 of fold-change. The Mann–Whitney test was performed to examine the difference between normal controls and cervical cancer patients, Kruskal–Wallis H test was used to define the difference among stages I–IV of cervical cancer patients. C, D: we compared the expression between patients which had lymp node metastasis or not in the same stage. *P*-value<0.05 was considered significant

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

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(0.029-1.88)
≥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			
stageI			
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

the impaired tissue with a median of 6.92. On the other hand, miR-203 was down-regulated in sample group in comparison to control group, by a median of 0.12. Figure 1B showed the distribution of miR-203 expression levels. Patients with up-regulated expression of miR-20a tended to have LNM (P=0.001) as in Figure 1C, increased tumor sizes (P=0.013, Mann-Whitney U test), advanced stage (P=0.004, Kruskal-Wallis H test), and advanced histological grade (P=0.027, Mann-Whitney U test) of cervical cancer (Table 1). While a low expression of miR-203 in cervical cancer was associated with LNM as in Figure 1D, There was no significant difference between low expression of miR-203 and other clinicopathological characteristics such as age, tumor diameter, histological grade, FIGO stage.

We used SiHa cell line to investigate the effect of miR-20a and miR-203 on cell proliferation using cells transfected with miR-20a inhibitor and miR-203 mimics (or control miRNA). As shown in Figure 2B, mature miR-203 was highly expressed in cells transfected with miR-203 mimics while the expression level of miR-203 was still very low in cells transfected with negative control 48 h posttransfection. After we transfected miR-20a inhibitor, the expression was down-regulated, as in Figure 2A. In addition, we found that the cells which transfected with miR-203 mimics and miR-20a inhibitor in SiHa cells had an obviously growth inhibition compared to matched

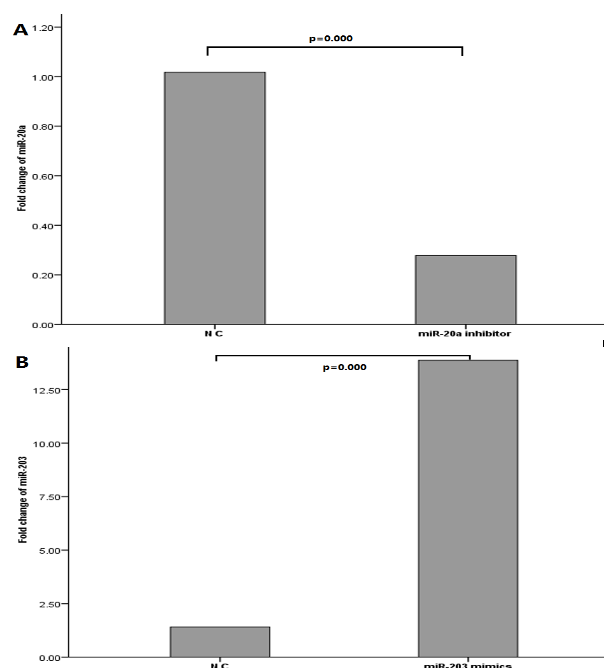

Figure 2. A: Mature miR-20a was lower expressed in cells transfected with miR-20a inhibitor while the expression level of miR-20a was still very high in cells transfected with negative control 48 h posttransfection. B: After we transfected miR-203 mimics, the expression was up-regulated

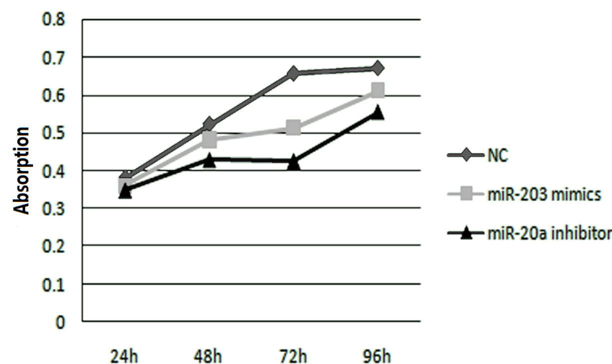

Figure 3. Up-regulated miR-203 or Down-regulated miR-20a Significantly Inhibited Cell Proliferation in SiHa Cells by MTT Assay

NC by MTT assay (Figure 3). At the time point of 24, 48, 72, and 96 h posttransfection of miR-20a inhibitor, the inhibition rates were 8.4%, 18%, 35.2% and 17.4%, respectively. After we transfected cells with miR-203 mimics, there was also a growth inhibition, 10.5%, 15.3%, 21.9% and 11.9%, respectively.

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.,

2005). It is possible to extract high-quality miRNA from a wide range of cell and tissue sources, The perspective of using miRNA in clinical practice is not restricted by only early detection of the disease. It is also possible to use them in cancer therapy. If miRNA acts as tumor suppressor, it is necessary to increase the level of its expression by either increasing copying or deleting repression of the encoded gene or guiding the mature miRNA into the tumor cell. If miRNA acts as oncogene, it is necessary to inhibit its expression. Understanding of the role of miRNAs is allowing new insights on the molecular basis of cancers, and new biomarkers for cancer diagnosis, prognosis and therapy.

In this study we investigated the expression levels of two key miRNAs identified in the literature in cervical tissue stratified by histological diagnosis in order to determine their impact in cervical cancer malignant progression and LNM. In the recent study, miR-203 has significantly low expression in cancer tissues compared to non-tumor counterparts (Viticchiè et al., 2011; Takeshita et al., 2012; Gu et al., 2013), while there were up-regulation of miR-203 was observed in pancreatic adenocarcinomas and breast, cancers (Naoki et al., 2010; Madhavan et al., 2012), we think the differential expression of miRNAs may be the result of tissue-specific differences. Just as Baffa et al suggested (Baffa et al., 2009). miR-203 was proved to be down-regulated in squamous cell carcinoma (Yuan et al., 2011; Boldrup et al., 2012; Takeshita et al., 2012). Expression profiling using quantitative real-time RT-PCR of 202 miRNAs was performed on micro-dissected high-grade CIN (CIN 2/3) tissues and compared to normal cervical epithelium, then Cheung TH found miR-203 could distinguished the high-grade CIN specimens from normal cervical epithelium (Cheung et al., 2012). So we chose cervical squamous cell carcinoma to verify relationship between aberrant expression of miR-203 and cervical tumorigenesis. Our investigation showed miR-203 was down-regulated in sample group in comparison to control group that low expression of miR-203 was correlated with LNM, this is exactly the same result as Hu proved in their study (Hu et al., 2010). miRNA-20a was known to belong to the miR-17-92 cluster, which is the most extensively studied cluster that has an oncogenic function. It comprises seven miRNAs, which reside in intron3 of the C13orf25 gene at 13q31.3 (Ota et al., 2004). This cluster is widely expressed in healthy tissues and is important for the regulation of the immune and hematopoietic systems and lung development (Lu et al., 2007; Ventura et al., 2008; Xiao et al., 2008). However, it is situated in a region that is commonly amplified and, therefore, is usually over-expressed in many kinds of tumor types, such as lymphomas and cancers of the lung, breast, follicular lymphoma, chronic myeloid leukemia, osteosarcoma, and also cervical cancer (Hayashita et al., 2005; Venturini et al., 2007; Huang et al., 2012; Kang et al., 2012; Li et al., 2012; Wang et al., 2012). Our study showed miR-20a is definitely up-regulated in the impaired tissue with a median of 6.92, up-regulated expression of miR-20a have relationship with LNM, increased tumor sizes, advanced stage, and advanced histological grade of cervical cancer.

Above all, we consider that miR-20a and miR-203 may be related with tumorigenesis of cervix. Then we used experiment in vitro to verify the function. Results of MTT showed miR-20a inhibitor and miR-203 mimics can significantly inhibit the cell proliferation in SiHa cells. At the time point of 24, 48, 72 and 96 h posttransfection of miR-20a inhibitor, the inhibition rates were 8.4%, 18%, 35.2% and 17.4%, respectively. After we transfected cells with miR-203 mimics, there was also a growth inhibition, 10.5%, 15.3%, 21.9% and 11.9%, respectively. So we suppose that miR-20a and miR-203 may play an important role in malignant process of cervical cancer, especially invasion and metastasis, which could lead poor prognosis.

miRNAs are involved in the development of such processes and could be prognostic markers and therapeutic targets in metastatic tumors. To be the tumor suppressor miRNAs, restrain the expression of miR-203 could up-regulate some kinds of oncogene which had been confirmed to be the target genes of miR-203. On the other hand, oncomiRs are miRNAs that generally modulate the expression of tumor suppressor genes and are usually over-expressed or amplified in tumor cells, contributing to tumor development, as previously reported for miR-20a. This may be the main mechanism during tumorigenesis caused by miRNAs. There were also other reasons that make miRNAs involved in cancer progress. Aberrant epigenomic patterns in tumor cells are frequently seen, 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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