Original Article
Role and clinical significance of miRNA-204 in human cervical carcinoma

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Abstract: Objective: This study is to investigate the role and mechanism of miRNA-204 in human cervical carcinoma. Methods: 73 cases of cervical carcinoma patients diagnosed and operated in our hospital from February 2014 to August 2014 were enrolled. And, 85 aged-matched healthy volunteers were set as controls. Tumor tissues, the corresponding tumor-adjacent tissues and blood specimens were collected. qRT-PCR was applied for detecting expression levels of Bcl-2 mRNA and miR-204, Western Blot and ELISA were applied for detecting protein level of Bcl-2 in tissues, SiHa cells and serum, respectively. Luciferase reporter assay was applied to investigate whether miR-204 could regulate the expression of Bcl-2 by direct binding to the 3’UTR. Flow cytometry was applied to evaluate the cell apoptosis rate of miR-204 mimics and siRNA transfected SiHa cells. Results: The expressions of miRNA-204 were significantly down-regulated in tumor tissues and blood in cervical carcinoma patients, while Bcl-2 mRNA and protein were significantly up-regulated accordingly. Overexpression of miRNA-204 resulted in significantly down-regulated expression of Bcl-2, and increased apoptosis rate of SiHa cells. Luciferase reporter assay showed that miRNA-204 regulated the expression of Bcl-2 by binding to the 3’-UTR. Conclusion: The expression of Bcl-2 was significantly up-regulated in tumor tissues and blood in cervical cancer patients, and this increase may be related to the down-regulated expression of miRNA-204. Our data suggests that miRNA-204 may regulate cell apoptosis and expression of related proteins in cervical carcinoma cells through Bcl-2.

Keywords: Cervical carcinoma, miR-204, Bcl-2

Introduction
Cervical carcinoma is a common prevalent female malignancy, and the incidence rate ranks as the fourth leading cause of cancer mortality in women worldwide, with an estimated about 530,000 new cases and 175,000 deaths each year [1]. As there is a marked tendency of this disease occurs in individuals at younger ages [2], and the 5-year survival rate is still less than 40% for stage III and above patients [3]. Although cervical carcinoma has its own characteristic clinical features and distinctive pathogenesis, the detailed underlying molecular pathogenesis of cervical carcinoma is far from been fully elucidated, many demographic, environmental, life style-associated and genetic factors are suggested to relate with the pathogenesis and development of cervical carcinoma [4, 5]. Previous studies have verified some evidences of genetic components to cervical carcinoma, especially microRNAs (miRNA) are reported to involve in the occurrence and development of cervical carcinoma [6-9].

As a class of endogenous and highly conserved non-coding small RNAs with the length 18 to 24 nucleotides, miRNAs play important roles in a variety of basic physiological processes, such as cell growth, differentiation, proliferation and apoptosis [10-13]. The basic function of miRNA is binding to the specific pairing bases of target mRNA (i.e. miRNA specific binding to the 3’UTR of target mRNA), causing degradation or translational repression of the target mRNA, and resulting in post-transcriptional gene silencing [14]. As a recently identified miRNA, miRNA-204 was verified to play important role in the pathogenesis and development of many can-
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cers [15], such as human non-small cell lung cancer [16], endometrial carcinoma [17], breast cancer [18], bladder and prostate cancer [19] and gastric cancer [20]. As many miRNAs are reported to involve in pathogenesis or development of cervical carcinoma [6, 7, 21], suggesting it is very likely that miRNA-204 is related to the pathogenesis or development of cervical carcinoma.

Bcl-2 is a well-known key oncoprotein to inhibit cell apoptosis, which is perceived as an anti-apoptotic regulator in the pathogenesis and development of many cancers, such as breast cancer [18], bladder and prostate cancer [19], gastric cancer [20], oral squamous cell carcinoma [22], and cervical carcinoma [23]. Some previous studies showed Bcl-2 worked as a targets for miR-204 in breast cancer [18], bladder and prostate cancer [19] and gastric cancer [20], suggesting miRNA-204 may be related to the pathogenesis or development of cervical carcinoma through Bcl-2.

In this study, qRT-PCR, Western Blot, ELISA, cell transfection, luciferase reporter assay, gene bioinformatics prediction, and flow cytometry analysis were applied. The expression of Bcl-2 mRNA and protein, miRNA-204 in cervical carcinoma tumor tissues, the corresponding tumor-adjacent tissues and blood samples was detected. The relationship among miRNA-204, Bcl-2 and cell apoptosis was analyzed. Our findings may further elaborate the regulation mechanism of miRNA-204 in the pathogenesis and development of cervical carcinoma, and can provide a new theoretical basis for the treatment of human cervical carcinoma.

Materials and methods

Subjects and samples

Totally 73 cases of cervical carcinoma patients diagnosed and operated in our hospital from February 2014 to August 2014 were enrolled. And, 85 age-matched healthy volunteers were set as controls. Tumor tissues and the corresponding tumor-adjacent tissues were collected from cervical carcinoma patients, and blood specimens were collected from both cervical carcinoma patients and healthy controls. The sampled tissues and blood specimens were immediately snap-frozen in liquid nitrogen and stored at -80°C. Prior written and informed consent was obtained from every patient and healthy control. The study was approved by the ethics review board of the Affiliated Cancer Hospital of Xiangya School of Medicine, Central South University.

qRT-PCR

The total RNA from tissues, blood specimens and cell lines were extracted by Trizol (Invitrogen, Carlsbad, USA) according to the manufacturer’s instructions. RNA was reverse transcribed into cDNA. The levels of Bcl-2 and miR-204 were quantified by quantitative Real-Time PCR (qRT-PCR) miRNA Detection Kit (Ambion, Austin, USA) combined with SYBR Green Super Real PreMix (Tiangen, Beijing, China) on the iQ5 real-time PCR detection systems (Bio-Rad, Hercules, USA). β-Actin and U6 were set as internal controls for Bcl-2 and miRNA-204, respectively. The primer sequences were as follows: 5'-CTTTTGTGTAACTGTACC-3' (forward) and 5'-CTTTGGCAGTAAATAGCTGATCGAC-3' (reverse) for Bcl-2, and 5'-CACCGGGCGTGATGTT-3' (forward) and 5'-CTCCAAACATGATCGGTGTC-3' (reverse) for U6. Quantitative PCR for Bcl-2 and β-actin were performed with the following procedure: 95°C for 30 s, followed by 40 cycles of 95°C for 10 s and 55°C for 10 s and 72°C for 30 s. And quantitative PCR for miRNA-204 and U6 were performed with the following procedure: 95°C for 30 s, followed by 40 cycles of 95°C for 10 s, 60°C for 10 s and 72°C for 30 s. The relative expression was calculated by 2^(-ΔΔCT) method.

Western blot

Total protein was extracted from tissues or cells, and protein concentration was determined by BCA protein assay kit (Pierce, Rockford, USA). 20 µg samples were subjected to 10% SDS-PAGE for Western Blot analysis. The primary antibodies, including anti-Bcl-2 antibody (Abcam, Cambridge, UK, and diluted 1:1000) and anti-β-actin antibody (Abcam, Cambridge, UK, and diluted 1:5000) were added and incubated overnight at 4°C. Then, secondary goat anti-rabbit antibodies (Abcam, Cambridge, UK, and diluted 1:3000) were added and incubated at room temperature for 1 h. Membrane was placed in ECL solution for color development. And, image was obtained by gel imaging system and analyzed by Image
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Lab3.0 software (Bio-Rad, Hercules, USA). The relative content of Bcl-2 protein was calculated as the ratio of Bcl-2 gray value to β-actin gray value.

**ELISA assay**

Serum was separated from blood sample by centrifugation at 3000 rpm for 10 min. Then, 50 µg samples (diluted 1:4) or standard reference solutions were added to the corresponding well. 100 µL HRP-conjugated detection antibodies (Abcam, Cambridge, UK) was added to each well, sealed and incubated in constant temperature incubator for 1 h. After washing for 5 times, 50 µL substrate A and B was added to each well. After incubation at 37°C for 15 min, 50 µL termination solutions were added, and OD value of each well at 450 nm wavelength were determined within 15 minutes.

**Bioinformatics prediction**

The miRanda, TargetSean, PieTar, MiRanda, BibiServ and other target gene prediction software were applied for predicting Bcl-2 upstream regulatory miRNAs and miRNA-204 targeting sites in genome wide, and the possible regulatory sites were also predicted.

**Luciferase reporter assay**

Wild type (5'-ACUGGAAAAGGAAAUA-3') and mutated type (5'-ACUGGACACCGAAAUA-3') of the predicted miRNA-204 binding site in the 3'-UTR of Bcl-2 gene were chemically synthesized in vitro, with adding Sac-1 and Hind III cleavage sites, then DNA fragments were cloned into pMIR-REPORT luciferase reporter plasmid (Ambion, Woodlands, USA), and the recombinant plasmids with 3'-UTR or mutated 3'-UTR were transfected into 293 T cells by liposome method, finally transfected with agomiR-204. After cultured at 37°C for 24 hours, the fluorescence values were measured by GloMax 20/20 luminometer (Promega, Madison, USA). Renilla fluorescent activity was set as internal control, and all the procedures were strictly performed according to producer’s instructions.

**SiHa cells transfection**

1.5-2×10⁵ logarithmic growth phase SiHa cells were plated into each well of 24-well plates, cultured in antibiotic-free 10% FBS DMEM media 24 hours before transfection. When cell reached about 80-90% confluency, transfection was performed by use of Lipofectamine 2000 (Invitrogen, Carlsbad, USA) in accordance with the manufacturer’s protocol. For each dish, 3-5 µg purified control plasmid, siRNA or agomiR mixed with 8-10 µL Lipofectamine in 2 ml serum-free DMEM was used. After co-cultured for 6 h, the medium was replaced by fresh growth medium. The cells were further incubated in 5% CO₂ at 37°C for 48 hour, and then harvested for detection the expression levels of targeted Bcl-2 mRNA and protein.

**Flow cytometry analysis**

Cell apoptosis rate was evaluated by flow cytometry (FCM) with reference to according to the described methods by Ni Sima et al [9]. In brief, after 48 hours post-transfection with control plasmid, siRNA or agomiR, cells were harvested with 0.2% trypsin, washed in cold PBS, fixed in 70% ice-cold ethanol, stained with propidium iodide (PI) overnight at -20°C, then were run on a FACSsort flow cytometer (Becton-Dickinson, Franklin Lake, USA), and the apoptosis rates were evaluated by the CELLQuest software system (BD Biosciences, San Jose, USA).

**Statistical analysis**

All statistical analyses were performed on the software package SPSS 18.0 (SPSS Inc, Chicago, USA). Data were presented as mean ± standard deviation. All data were analyzed with normality test. One-way ANOVA was applied for multiple sets of measurement data analysis. LSD and SNK method were applied when there was homogeneity of variance, and Tamhane’s T2 or Dunnett’s T3 method was applied when there was not homogeneity of variance. A P value <0.05 was considered statistically significant. All experiments were repeated at least thrice.

**Results**

**Changes of Bcl-2 mRNA expression in cervical carcinoma tumor tissues and blood samples**

qRT-PCR was applied to detect the expression of Bcl-2 mRNA in cervical carcinoma tumor tissues, the corresponding tumor-adjacent tissues and blood samples. As shown in Figure 1A, compared with that in the corresponding tumor-adjacent tissues, expression levels of
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Bcl-2 mRNA were significantly increased in cervical carcinoma tumor tissues (P<0.05). As shown in Figure 1B, compared with that in healthy controls, expression levels of Bcl-2 mRNA in blood samples were significantly increased in cervical carcinoma patients (P<0.05). This result indicates that Bcl-2 may play a certain role in the regulation of cervical carcinoma.

Western Blot and ELISA were applied to detect the expression of Bcl-2 protein in cervical carcinoma tumor tissues, the corresponding tumor-adjacent tissues and blood samples. As shown in Figure 2A, compared with that in the corresponding tumor-adjacent tissues, expression levels of Bcl-2 protein were significantly increased in cervical carcinoma tumor tissues (P<0.05). Similarly, as shown in Figure 2B, Bcl-2 protein expressions in blood samples of cervical carcinoma patients were significant-

**Figure 1.** Relative expression levels of Bcl-2 mRNA in cervical carcinoma tumor tissues and tumor-adjacent tissues (A) and in blood (B). Compared with control group (tumor-adjacent tissues or healthy controls), **Represents P<0.01.

**Figure 2.** Relative expression levels of Bcl-2 protein in cervical carcinoma tumor tissues and tumor-adjacent tissues (A) and in blood (B). Compared with control group (tumor-adjacent tissues or healthy controls), *Represents P<0.05.

**Figure 3.** The predicted specific regulatory binding sequences of miRNA-204 to 3’UTR of Bcl-2 mRNA.
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**Figure 4.** Relative expression levels of miR-204 in cervical carcinoma tumor tissues and tumor-adjacent tissues (A) and in blood (B). Compared with control group (tumor-adjacent tissues or healthy controls), *Represents P<0.05.

**Figure 5.** Comaprison of fluorescence values in luciferase reporter assay. Compared with control (negative control mimic), **Represents P<0.01.

...higher than those in healthy controls (P<0.05). In consistent with the trend of mRNA, this result indicates that expression levels of Bcl-2 protein were also significantly up-regulated in cervical carcinoma tumor tissues and blood samples in cervical carcinoma patients.

*Bcl-2 is one of the target genes of miRNA-204*

The Bcl-2 upstream regulatory miRNAs and miRNA targeting sites in genome wide were predicted with target gene prediction software. Results showed that there may be targeted regulatory relationship between miRNA-204 and Bcl-2, and the specific regulatory binding sequences as shown in Figure 3.

**Changes of miRNA-204 expression in cervical carcinoma tumor tissues and blood samples**

qRT-PCR was applied to detect the expression of miRNA-204 in cervical carcinoma tumor tissues, the corresponding tumor-adjacent tissues and blood samples. As shown in Figure 4A, compared with that in corresponding tumor-adjacent tissues, the expression levels of miRNA-204 were significantly decreased in cervical carcinoma tumor tissues (P<0.05). The expression levels of miRNA-204 in blood samples were significantly lower in cervical carcinoma patients than in healthy controls (P<0.05) (Figure 4B). These results indicate that miRNA-204 may play a certain role in the regulation of cervical carcinoma, and this regulation function may be related to the transcriptional regulation of Bcl-2.

**Luciferase reporter assay**

Luciferase reporter assay was applied to verify the relationship between miRNA-204 and Bcl-2. As shown in Figure 5, the fluorescence value was significantly decreased in wild type Bcl-2 and agomiR-204 co-transfecting 293T cells (P<0.05), while had no statistical difference in the mutant Bcl-2 and agomiR-204 co-transfecting 293T cells (P>0.05). This result indicates that miRNA-204 may regulate the expression of Bcl-2 through binding to the 3’-UTR of Bcl-2.

**Effect of agomiR-204 and siRNA-204 transfection on SiHa cells**

qRT-PCR, Western Blot and flow cytometry analysis were applied to detect the expression of miRNA-204 and Bcl-2 mRNA, the expression of Bcl-2 protein and cell apoptosis rate in agomiR-204 and siRNA-204 transfected SiHa cells, respectively. As shown in Figure 6, com-
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pared with those in control plasmid transfected SiHa cells, expression levels of miRNA-204 were significantly increased and decreased in agomiR-204 and siRNA-204 transfected SiHa cells (Figure 6A), expression levels of Bcl-2 mRNA and protein were significantly decreased and increased in agomiR-204 and siRNA-204 transfected SiHa cells (Figure 6B and 6C), and cell apoptosis rates were significantly increased and decreased in agomiR-204 and siRNA-204 transfected SiHa cells (Figure 6D). This result indicates that up-regulated expression of miRNA-204 may promote cell apoptosis through regulating Bcl-2 expression in SiHa cells.

Discussion

Although the advances in diagnosis and appropriately systemic therapy contribute to the prognosis of cervical carcinoma, the incidence and mortality have declined over the past few decades in some developed countries, cervical carcinoma remains as one of the most common cause of cancer death in females worldwide, especially in the developing countries [5]. For cervical carcinoma, beside precise clinical stage judgment and appropriate treatment system and formulas, it is badly in need to develop more useful prognostic biomarkers [24, 25]. As a class of highly conserved non-coding small RNAs, miRNAs play regulatory role in post-transcriptional gene silencing and are involved in many important biological processes, including human cancer [10, 12, 13]. Previous studied showed that the recently identified miRNA-204 can regulate the expression of an key anti-apoptotic regulator gene Bcl-2, Bcl-2 acted an anti-apoptotic effect in many
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human cancers, such as breast cancer [18], bladder and prostate cancer [19], gastric cancer [20], oral squamous cell carcinoma [22], and cervical carcinoma [23], we speculated that miRNA-204 may play an important role in the development and progression of human cervical carcinoma through Bcl-2.

In this study, we detected the expression levels of Bcl-2 mRNA and protein and miRNA-204 in cervical carcinoma tumor tissues, the corresponding tumor-adjacent tissues and blood samples, preliminary explored the biological functions of miRNA-204 and Bcl-2, and studied the molecular mechanisms of miRNA-204 on cervical carcinoma. A significant increase of Bcl-2 mRNA and protein expression was observed in cervical carcinoma tumor tissues and blood in cervical carcinoma patients, which is consistent with previous studies that Bcl-2 acted an anti-apoptotic effect in many human cancers, and indicating that the changes in expression levels of Bcl-2 is related to the development and progression of cervical carcinoma, and suggesting the abnormal expression of Bcl-2 may be a key factor to the pathogenesis and development of human, and the expression levels of Bcl-2 mRNA and protein in tissue or blood may indirectly reflect the malignant degree and metastasis of human cervical carcinoma cells. Through genome wide Bcl-2 upstream regulatory miRNAs and miRNA-204 targeting sites prediction and analysis, we found that miRNA-204 may be an upstream regulation miRNA of Bcl-2. Luciferase reporter assay was applied to verify the directly binding and the specific binding sites of miRNA-204 to 3′UTR of Bcl-2 mRNA, results showed that miRNA-204 can directly bind to the targeted seed region in 3′-UTR of Bcl-2 mRNA to inhibit the expression of Bcl-2. As many endogenous, small, non-encoding miRNAs may splice and inhibit the translation of Bcl-2 mRNA, and play important roles in the development of cancers [26, 27], miR-204 targets Bcl-2 expression and enhances responsiveness of gastric cancer [20], miRNA-204 promotes cell apoptosis by targeting to Bcl-2 in bladder and prostate cancer cells [19], microRNA-204 targets JAK2 in breast cancer and induces cell apoptosis through the STAT3/Bcl-2/survivin pathway [18], and so on. The expression levels of miRNA-204 were significantly decreased in cervical carcinoma tissues and blood in cervical carcinoma patients. In considered that the expression levels of Bcl-2 were abnormally increased in cervical carcinoma tissues and blood and miRNA-204 can inhibit the expression of Bcl-2 by directly binding to the 3′UTR of Bcl-2 mRNA, we hypothesize that down-regulated expression of miRNA-204 results in up-regulated expression of Bcl-2, which may further affect cell apoptosis in cervical carcinoma cells. To further study the possible underlying molecular mechanism, effect of agomiR-204 and siRNA-204 transfection on SiHa cells was investigated. Results showed that up-regulated expression of miRNA-204 indeed resulted in the decreased expression level of Bcl-2 and increased cell apoptosis rates of SiHa cells. The results further confirmed that miRNA-204 can regulate cell apoptosis rate through inhibit the expression of Bcl-2 by directly binding to the targeted seed region in 3′-UTR of Bcl-2.

In summary, our findings show that miRNA-204 may inhibit the expression of Bcl-2, result in expression changes of associated proteins, then promote cell apoptosis, and play its biological functions in the pathogenesis and development of cervical carcinoma, suggesting miRNA-204 may be used as a prognostic biomarker for cervical carcinoma diagnose and treatment.

Disclosure of conflict of interest

None.

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References

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