ORIGINAL ARTICLE ___

miR-99a inhibits proliferation and migration of cervical cancer cells by targeting IGF1R

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Summary

Purpose: To explore the effects of miR-99a on the proliferation and migration of cervical cancer cells (CCCs) by targeting IGF1R.

Methods: miR-99a and IGF1R expression in C-33 A and C-4 II cells was interfered. Their effects on the proliferation, apoptosis, and migration of CCCs were analyzed by MTT assay, flow cytometry, and Transwell assay. The mechanism of action of miR-99a was analyzed by a rescue experiment and a dual luciferase reporter gene assay (DLRGA). Differences in miR-99a and IGF1R expression were detected in cervical cancer and adjacent tissues (n=30 each), and the correlation of the expression with clinicopathological characteristics of patients with cervical cancer was analyzed

Results: miR-99a expression was lower but IGF1R expression was higher in C-33 A and C-4 II cells than that in normal cervical epithelial cells. The results showed that both the promotion and the inhibition significantly decreased the proliferation and migration of the two CCCs, but increased their apoptosis. To further verify the correlation of miR-99a with IGF1R in cervical cancer, we co-transfected miR-99a and IGF1R overexpression vectors into the cells and found that compared with CCCs transfected with miR-99a overexpression vectors alone, the expression of IGF1R in the co-transfection group increased, while the expression of miR-99a did not change significantly. Additionally, the proliferation and migration of the cells in the co-transfection group increased, while their apoptotic rate decreased. DLRGA showed the targeted inhibition of miR-99a on IGF1R expression.

Conclusions: miR-99a can specifically inhibit IGF1R expression and thus inhibit the proliferation and migration of CCCs.

Key words: miR-99a, IGF1R, cervical cancer, proliferation, migration

Introduction

As a major cause of cancer-related deaths all over the world, cervical cancer has the highest incidence and mortality rates among all gynecologic tumors, with 528,000 new cases and 266,000 deaths each year [1,2]. Currently, surgery, radiotherapy, and chemotherapy are commonly used to treat this disease, but conventional chemotherapy has a limited therapeutic effect and causes serious systemic toxicity. Moreover, the tolerance of tumor cells to radiotherapy and chemotherapy gradually

[3,4]. Therefore, it is necessary to find new targets for the treatment of cervical cancer.

We have a new understanding of tumor pathogenesis due to the discovery of microRNAs (miR-NAs/miRs), which has also provided an important research direction for finding new therapeutic targets. miRNAs are generally about 22 nucleotides in length and regulate target gene expression through the targeted complementary pairing with the 3'-untranslated region (UTR) of mRNAs [5,6]. enhances with the extension of treatment time Located at human chromosome 21q21.1, miR-99a

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inhibits cervical cancer cells (CCCs) to proliferate and invade via targeting mTOR signaling pathway [7]. Later research has found that the target of miR-99a, insulin-like growth factor 1 receptor (IGF1R) inhibits breast cancer cells to proliferate, migrate and invade [8] and also esophageal cancer cells [9] through inhibiting IGF1R. This receptor promotes glycolysis, and glycolysis disorders are an important feature of cancer cells, so its activation significantly enhances the growth, survival, and movement of tumor cells [10,11]. IGF1R is also reported to regulate the biological behavior of CCCs such as proliferation and invasion [12,13]. However, the role of miR-99a regulating IGF1R on CCCs has been rarely studied.

In this study, the influence of miR-99a targeting IGF1R on CCC proliferation and migration were explored to provide more experimental basis for clinical search for therapeutic targets of cervical cancer.

Methods

Cell sources

Human primary cervical epithelial cells (PC-480-011), C-33 A cells (HTB-31), C-4 II (CRL-1595), and their culture media were purchased from ATCC. The medium for the cervical epithelial cells was a Cervical Epithelial Cell Basal Medium, and the culture medium for C-33 A cells was Eagle's Minimum Essential Medium + 10% fetal bovine serum (FBS), and the culture medium for C-4 II cells was Walmouth's MB 752/1 medium + 10% FBS. The cells were cultured at 37°C and with 95% air + 5% carbon dioxide, and the culture medium was changed 2-3 times weekly.

Cell passage

After recovery, the cells were adherently cultured, and then rinsed with 0.25% trypsin + 0.03% ethylenediaminetetraacetic acid (EDTA) solution after the old culture medium was sucked out. After the liquid was removed, the cells were added with trypsin-EDTA solution (1-2 mL), which was removed after the cells were separated. After the fresh culture medium was added, the cells were suspended, and then separately cultured for 2-3 generations.

Construction and transfection of expression vectors

All primers and expression vectors were designed and synthesized by TaKaRa (Tokyo, Japan), including a miR-99a interference vector (inhibitor), a miR-99a overexpression vector (mimic), a blank vector miR-NC, an IGF1R interference vector (si-IGF1R), an IGF1R overexpression vector (sh-IGF1R), and blank vectors (sh-NC, PGL3-wt-IGF1R, and PGL3-mt-IGF1R). Trypsin was used to digest the cells 24 h before transfection, and the expression vectors were transfected into the cells when they were fused to about 80%, with specific operating steps referring to the kit instruction. After that, the cells were cultured in an incubator for 48 h (37°C, 5% CO_2), with the culture medium changed every 6 h. qRT-PCR and Western blotting were used to measure the transfection results. LipofectamineTM2000 (11668019) was purchased from ThermoScientific.

Cell grouping and intervention

The cells were divided into the control, miR-NC, miR-99a mimic, si-NC, si-IGF1R, and co-transfection groups. Those in the first group were not treated, while those in other groups were transfected with miR-NC, miR-99a mimic, si-NC, si-IGF1R, and miR-99a mimic+sh-IGF1R, respectively.

Tissue sources

The cervical cancer tissues and their corresponding adjacent tissues (n=30 each) were selected, preserved from August 2013 to May 2014 and confirmed by pathology. The patients had complete clinical medical records and follow-up data. This study was approved by the Medical Ethics Committee of our hospital. The patients consented through telephone or text message and signed the informed consent form.

qRT-PCR

qRT-PCR was performed to detect miR-99a expression in cells/tissues, from which total RNA was drawn using the TRIzol reagent. An UV spectrophotometer and agarose gel electrophoresis were applied to detect its purity, concentration, and integrity. Experimental requirements were met with A260/A280 value between 1.8 and 2.1. Based on the kit instruction, the first strand cDNA was synthesized, and then PCR amplification was performed. The system was upstream and downstream primers (0.4 μL each), 2×TransTaq® Tip Green qPCR SuperMix (10 µL), Passive Reference Dye (50X) (0.4 μ L), and ddH₂O finally added to make up to 20 μ L. The conditions were pre-degeneration (94°C, 30 s), degeneration (94°C, 5 s), annealing and extension (60°C, 30 s), for a total of 40 cycles. With U6 used as a reaction internal reference, its upstream and downstream sequences were 5'-GCGCGTCGTGAAGCGTTC-3' and 5'-GTGCAGGGTCCGAGGT-3'. miR-99a sequences were 5'-AGTTACTTAAGCTCGGGCCC-3' and 5'-TTGTTCTCG-GTGGGCTTGGC-3'. The primer sequences were designed by Thermo, and the TransStart® Tip Green qPCR SuperMix (AQ142-21) was purchased from TransGen Biotech Co., Ltd, Beijing.

Western blotting

The repeated freeze-thaw method was used to extract protein from the cells/tissues, and its concentration was detected using the BCA method. The protein was separated with 12% polyacrylamide gel electrophoresis (PAGE) after the concentration was adjusted to 4 μ g/ μ L, with the voltage initially being 90 V and then increasing to 120V to move the samples to the appropriate position of the separation gel. Then, the protein was transferred to the membrane (100V constant voltage, 100 min) and sealed (37°C, 60 min). Next, the transferred membrane

was sealed in 5% skimmed milk for immune reaction. It was incubated with primary antibody (1: 1000) (all night, 4°C), warmly cleaned with PBS (5 min) for 3 times on the next day, and then incubated with secondary antibody (1: 1000) (room temperature, 1 hour). Finally, it was developed and fixed with an ECL reagent. Protein bands scanned by the film were statistically analyzed by Quantity One. The relative expression of the protein = gray values of protein bands / gray values of internal reference. The BCA protein kit (23250), ECL kit (35055), and trypsin (90058) were all purchased from Thermo Scientific[™]. Rabbit anti-IGF1R (monoclonal antibody, ab182408) and goat anti-rabbit IgG (secondary antibody, ab6721) were all purchased from Abcam, USA.

MTT assay for cell proliferation

The cells transfected for 24 h were collected, and conventionally digested. After their density was adjusted to 3×10^4 cells/well, they were inoculated on 96-well plates, incubated (37°C, 48 h), then added with 20 µL of MTT solution (5 mg/mL), and finally cultured (37°C, 4 hours). 150 µL of dimethyl sulfoxide was added to each well. Subsequently, optical density (OD) values in each group were measured at 490 nm using a microplate reader (Shanghai Flash Spectrum Biological Technology Co., Ltd.). The MTT assay kit (V13154) was purchased from Thermo Fisher (China).

Flow cytometry for cell apoptosis

The cells were digested with 0.25% trypsin, cleaned twice with PBS, then added with binding buffer (100 μ L), and finally prepared into a suspension (1×10⁶ cells/mL). After sequentially added with Annexin V-FITC and PI, the cells were incubated in the dark (room temperature, 5 min). A FACSCalibur flow cytometer (BD Biosciences, CA, USA) was used for the detection, and the experiment was repeatedly conducted for 3 times to obtain the average value. The Annexin V-FITC/PI apoptosis detection kit (V35113) was purchased from Invitrogen, USA.

Detection of cell migration and invasion

The upper chamber of Transwell was inoculated with 5×10^4 cells, and the L-15 medium (10% FBS) was added into the lower one. After the Transwell was cultured for 24 h, the cells under the membrane were fixed with 75% methanol, and stained with crystal violet, so as to count the number of transmembrane cells in 5 visual fields under 40*10 field of view. Three parallel experiments were conducted. The Transwell chamber and its related reagents were purchased from Corning, New York, USA.

Dual luciferase reporter gene assay (DLRGA)

Human embryonic kidney cells 293T (BNCC100530) were purchased from BeNa Culture Collection. After cultured to logarithmic growth phase, the cells were transfected with PGL3-wt-IGF1R or PGL3-mt-IGF1R, miR-99 mimic, miR-99 inhibitor, and miR-NC for 48 h. After that, the fluorescence intensity was detected by the dual luciferase reporter assay system (CytoFLEX S flow cytometer, Beckman, USA).

Statistics

SPSS 19.0 (Asia Analytics Formerly SPSS China) was used for statistical analyses. Measurement data were expressed as mean±SD, and their comparison between two groups was analyzed by independent samples t-test, while their comparison between multiple groups was analyzed by one-way analysis of variance (ANOVA). *Post hoc* test was used for LSD test, and Pearson test was used to analyze the correlation. P values were bilateral, and the difference was statistically significant when p<0.05.

Results

miR-99a expression and IGF1R in cervical cancer cell lines

Compared with normal cervical epithelial cells, C-33 A and C-4 II cells had remarkably lower miR-99a expression (p<0.05), but remarkably higher IGF1R expression (p<0.05) (Figure 1).

Effects of miR-99a on proliferation and migration of CCCs

miR-99a was lowly expressed in CCCs, so we transfected miR-99a mimic vectors into the cells. After the transfection, miR-99a expression in the CCCs remarkably rose (p<0.05); the cells' proliferation and migration in the mimic group were remarkably lower than those of the un-transfected cells (p<0.05), but their apoptosis was remarkably higher in the mimic group (p<0.05) (Figure 2).

Effects of IGF1R on proliferation and migration of CCCs

Similarly, we transfected si-IGF1R vectors. After the transfection, IGF1R expression in CCCs remarkably rose (p<0.05); the cells' proliferation and migration in the si-IGF1R group were remarkably lower than those of the un-transfected cells (p<0.05), but their apoptosis was remarkably higher in the si-IGF1R group (p<0.05) (Figure 3).



Figure 1. Expression of miR-99a and IGF1R in cervical cancer cell lines. **A:** miR-99a. **B:** IGF1R. *p<0.05.

Effects of co-transfection with miR-99a mimic+sh-IG- tent (p<0.05), and their apoptotic rate also reduced F1R on proliferation and migration of CCCs

In order to verify the effects of IGF1R on tumor inhibition by miR-99a, we co-transfected miR-99amamic+sh-IGF1R vectors. According to the results, IGF1R expression in the co-transfection group was remarkably higher than that of the cells transfected with miR-99a mimic vectors alone (p<0.05), but miR-99a expression was not different between the two cells (p>0.05). According to the comparison of the biological behavior, the proliferation and migration of the cells in the co-transfection group were restored to a certain ex(p<0.05). (Figure 4).

DLRGA

The binding site between miR-99a and IGF1R was predicted through targetscan7.2, so as to design the wild and mutant types of IGF1R. According to the DL-RGA, the up-regulation of miR-99a expression could significantly inhibit the fluorescence intensity of PGL3wt-IGF1R (p<0.05), and the down-regulation of miR-99a could increase the fluorescence intensity (p<0.05), but the two interference vectors had no effect on the fluorescence intensity of PGL3-mt-IGF1R (Figure 5).



Figure 2. Effects of miR-99a on proliferation and migration of CCCs. A: Results of transfection with miR-99a mimic. B: Changes in the proliferation of CCCs. C: Changes in the apoptosis of CCCs. D: Changes in the migration of CCCs. *p<0.05.



Figure 3. Effects of IGF1R on proliferation and migration of CCCs. A: Results of transfection with si-IGF1R. B: Changes in the proliferation of CCCs. C: Changes in the apoptosis of CCCs. D: Changes in the migration of CCCs. *p<0.05.



Figure 4. Effects of co-transfection with miR-99a mimic+sh-IGF1R on proliferation and migration of CCCs. **A:** Results of transfection with miR-99amimic+sh-IGF1R. **B:** Changes in the proliferation of CCCs. **C:** Changes in the apoptosis of CCCs. **D:** Changes in the migration of CCCs. *****p<0.05.



Figure 5. DLRGA. **A:** Results of miR-99a intervention. **B:** Effects of miR-99a on fluorescence intensity. **a** indicates p<0.05 compared with that in the blank group. **b** indicates p<0.05 compared with that in the miR-NC group. **c** indicates p<0.05 compared with that in the miR-99a mimic group.

miR-99a and IGF1R expression in cervical cancer tissues

miR-99a expression reduced (p<0.05), while IGF1R expression rose in cervical cancer tissues. Their expression was associated with the grade of differentiation, FIGO staging, and 5-year survival rate (p<0.05). (Table 1).

Discussion

As one of the most destructive gynecologic tumors, cervical cancer affects women's health and quality of life. Advanced cervical cancer has a 5-year survival rate of only 40%, so new and effective therapeutic methods for treating this disease are urgently needed [14,15]. In this study, the mechanism of action of miR-99a on affecting CCC proliferation and migration was analyzed, so as to find potential targets for its treatment.

Previous studies have reported the anti-cancer effect of miR-99a. In a study by Wang et al, miR-99a has a negative correlation with lymph node metastasis, so its overexpression inhibits CCC growth and invasion through the targeted inhibition of mTOR signaling pathway [7]. According to Li et al, the targeted inhibition of miR-99a on this pathway also inhibits endometrial cancer cells to proliferate and invade, and the regulatory effect of miR-99a on cell cycles blocks the cells' G1/S phase transition and induce their apoptosis [16]. At present, no research has reported the regulatory relationship between miR-99a and IGF1R, but the promotion of IGF1R in tumor occurrence and development (including cervical cancer) can be confirmed. Wu et al have found that miRNA-375 may inhibit the growth of CCCs by inhibiting IGF1R signaling pathway [17]. According to an earlier report, knocking out IGF1R can inhibit the

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	п	miR-99a	x^2/t	р	IGF1R	x^2/t	р
Cancer tissues	30	1.235±0.106	23.244	<0.001	1.121±0.121	14.984	<0.001
Adjacent tissues	30	1.845±0.137			0.742±0.067		
Age, years			1.865	0.073		0.698	0.491
<50	10	1.927±0.117			0.754±0.077		
≥50	30	1.839±0.124			0.736±.062		
Tumor diameter			0.652	0.520		0.594	0.598
<4cm	13	1.851±0.117			0.733±0.058		
≥4cm	17	1.882±0.136			0.748±0.073		
Degree of differentiation			3.590	0.001		3.674	0.001
Medium-high	17	1.930±0.108			0.709±0.533		
Low	13	1.788±0.105			0.784±0.058		
FIGO staging			4.211	< 0.001		4.722	< 0.001
I and II	16	1.941±0.097			0.701±0.046		
III and IV	14	1.785±0.106			0.788±0.056		
5-year survival rate			3.108	0.004		2.841	0.008
Surviving	23	1.983±0.057			0.685±0.040		

Table 1. Correlations of miR-99a and IGF1R with pathological characteristics

Dead

studies have also found that this receptor can be used as a predictor of efficacy on cervical cancer, and its high expression is associated with the formation of chemoradiotherapy resistance in patients with the disease, resulting in the reduction of long-term local control of the patients [19-21]. Additionally, patients with its high expression have significantly reducing 5-year recurrencefree survival rate and overall survival rate [22]. It is clear that miR-99a has a targeted inhibitory and regulatory effect on IGF1R, which is similar to reports on bladder cancer and gastric cancer [23]. Accordingly, miR-99a regulates the growth of tumor cells through the targeted inhibition of IGF1R. This study supplements the role of miR-99a on the growth of CCCs through the targeted inhibition.

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1.834±0.122

We purchased PC-480-011, C-33 A, and C-4 II cells from ATCC. The detection results showed that miR-99a expression was lower but IGF1R expression was higher in the two CCCs than that in the normal cervical epithelial cells. Therefore, we promoted miR-99a expression and inhibited IG-F1R expression, and then analyzed the cells' biological behavior after the interference. The results showed that both the promotion and the inhibition significantly decreased the proliferation and migration of the two CCCs, but increased their apoptosis. To further verify the correlation of miR-99a in normal people are unknown, and there are few

cells' growth and colony formation [18]. Many with IGF1R in cervical cancer, we co-transfected miR-99a and IGF1R overexpression vectors into the cells. During this process, we found that compared with CCCs transfected with miR-99a overexpression vectors alone, the expression of IGF1R in the co-transfection group increased, while the expression of miR-99a did not change significantly. Additionally, the cell proliferation and migration in the co-transfection group increased, while their apoptotic rate decreased. The DLRGA showed targeted inhibition of miR-99a on IGF1R expression. Therefore, from the cell experiment level, we have preliminarily verified that miR-99a can specifically inhibit IGF1R expression and thus inhibit the proliferation and migration of CCCs. We also analyzed the correlation of miR-99a and IGF1R expression in cervical cancer tissues with clinicopathological characteristics of the patients. miR-99a and IGF1R were correlated with the grade of differentiation, FIGO staging, and 5-year survival rate, which supports the conclusions of our cell experiments to some extent.

0.759±0.064

However, there are still deficiencies in this study. Animal experiments were not conducted for further verification. In vitro experiments cannot simulate the microenvironment in vivo that has a great influence on gene action, so in vivo experiments are needed to further verify our conclusions. Moreover, miR-99a and IGF1R expression cancer, so the clinical significance of their expression in the disease requires more research.

In summary, miR-99a is lowly expressed but IGF1R is highly expressed in CCCs, both of which are associated with the grade of differentiation, FIGO staging, and 5-year survival rate of patients

clinical reports on miR-99a and IGF1R in cervical with the disease. miR-99a can specifically inhibit IGF1R expression and thus inhibit the proliferation and migration of CCCs.

Conflict of interests

The authors declare no conflict of interests.

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