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Original Research

MiRNA-214 promotes the pyroptosis and inhibits the proliferation of cervical cancer cells via regulating the expression of NLRP3

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Abstract: Inflammasome mediates the maturation of interleukin-1β (IL-1β) and IL-18, triggers the pyroptosis and associates with multiple autoimmune diseases. In light of this, we hope to investigate the regulatory role of miRNA-214 in the inflammasome of cervical cancer. With the samples collected from 50 cervical cancer patients and 50 age-matched healthy subjects, real-time PCR and Western blotting were employed to detect the mRNA and/or protein expression profiles of the NOD-like receptor protein family, including NLRP1, NLRP3, NLRC4, Caspase-1, IL-1β, IL-18 and miR-214. Corresponding plasmids were used to transfect the Hela, HCC94, Siha or HUCEL normal cell lines to upregulate or downregulate the expression of targeted genes and to construct the cervical cancer models on rats. In addition, RT-PCR and Western blot were also considered to detect the expression of miR-214 and pyroptosis-related genes, while the pyroptosis of cells was evaluated by using the caspase-1 activity detection kit. Downregulation of miR-214 was found in the cervical cancer patients and the cervical cancer cell lines (** P <0.01), while overexpression of miR-214 could induce the pyroptosis of cervical cancer cell by targeting NLRP3. In cervical cancer patients, miR-214 and NLRP3 are downregulated, while upregulation of miR-214, by enhancing the expression of NLRP3, can advance the pyroptosis of cervical cancer cells. In addition, we, for the first time, clarify the correlation of cervical cancer with the miR-214 and NLRP3.

Key words: Inflammasome; Cell pyroptosis; Cervical cancer; miR-214; NLRP3.

Introduction

Cervical cancer is a type of cancer that begins in the cervix. The disease is caused by abnormal cell growth, and these cells can spread to other parts of the body or attack them. At the onset of the disease, there are usually no symptoms. Subsequent symptoms include vaginal bleeding, pelvic pain, or pain. Cervical cancer, as the most common gynecological disease worldwide, has been giving rise to 529,800 new cases every year, and 275,100 corresponding death tolls as per the estimate of the prevalence of cervical cancer (1-4). An increasing body of evidence has suggested the key role of an inflammatory mechanism in the development and progression of cervical cancer (3, 5-8).

NLRP3 inflammasome is a kind of intracellular multiprotein complex consisting of NLRP3, ASC and procaspase 1 (9-11). Activation of NLRP3 promotes the release of interleukin-1βand IL-18(12, 13), thereby inducing the inflammatory responses. It can also mediate the pyroptosis of cells which is a pro-inflammatory form of programmed cell death (10, 14, 15).

In this study, we explored the potential mechanism of miR-214 in cervical cancer, and as a result, confirmed that miR-214 is downregulated in the cervical cancer models, while overexpression of miR-214 and NLRP3 can advance the pyroptosis of cervical cancer cells. Detailed information on this study is reported in the following text.

Materials and Methods

Samples

Cervical cancer samples and healthy cervical samples were respectively collected from 50 cervical cancer patients and 50 healthy subjects in Yidu central hospital of Weifangafter they signed the written informed consents. Before the implementation, the protocol of this study had been approved by the Ethical Board ofYidu central hospital of Weifang

Construction of cervical cancer models

Wistar rats weighing between 180 and 220 g were housed at 22°C, 55% of relative humidity, with free access to water and food. Following one-week of adaption, rats were prepared to construct the cervical cancer models by injecting the human cervical cancer cell suspension (Hela cells) into the left axillary, and those models were further treated as per the treatment for models in cervical cancer (CC) group, pcDNA3.1- miRNA-214 group (CC+pcDNA3.1-miR-214 group) and pc DNA 3.1 NLRP3 group (CC+ pc DNA 3.1 NLRP3 group).

Cell culture and transfection

Cervical cancer cell lines (Hela, HCC94 and Siha)

and normal cervical epithelial cell line (HUCEC) were provided by the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences. All cells were maintained in the DMEM (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C, 5% CO_2. As per the differences in treatment, those cells were divided into the following groups: HUCEC group, CC group, miR-214 mimics group, p-miR-214 group and p-NLRP3 group.

RT-PCR

The extraction of total RNA from the cervical tissue and cell was conducted by using the extraction kit provided by Life Technologies (US) (16). RT-PCR was then carried out to detect the expression of miR-214 and the pyroptosis-related genes in the tissues or cells. Briefly, reverse transcription was carried out to prepare the cDNA by using the total RNA in a 10 μL system consisting of 2 μL 10 mMdNTP, 1μL primer, 1 μLRevertAid RT, 1 μL ribonuclease inhibitor and 1 μL RNase-free water for a reaction at 42°C for 60 min and 70°C for 5 min. Following RT-PCR was conducted on Roche LightCycler 480 (Roche, Switzerland) under the following conditions: 95°C for 30 s and 45 cycles of 95°C for 5 s and 60°C for 30 s. The resulting melting curve was used for analysis to ascertain the magnification of the product. Primer sequences are list as follows:

miRNA-214 Forward:5'-ATCCAGTGCGTGTCGTG-3'
miRNA-214 Reverse: 5'-TGCTACAGCAGGCACAGAC-3'
ASC Forward:5'- AACCCAAGCAAGATGCGGAAG-3'
ASC Reverse:5'- TTAGGGCCTGGAGGAGCAAG -3'
NLRP1 Forward: 5'-CCACAACCCTCTGTCTACATTAC-3'
NLRP1 Reverse: 5'-GCCCCATCTAACCCATGCTTC-3'
NLRC4 Forward: 5'-CCAGTCCCCTCACCATAGAAG-3'
NLRC4 Reverse: 5'-ACCCAAGCTGTCAGTCAGACC-3'
NLRP3 Forward: 5'-GTGGAGATCCTAGGTTTCTCTG-3'
NLRP3 Reverse: 5'-CAGGATCTCATTCTCTTGGATC-3'
caspase-1 Forward: 5'-AAGGTCCTGAGGGCAAAGAG-3'
caspase-1 Reverse: 5'-GTGTTGCAGATAATGAGGGC-3'
IL-1β Forward: 5'-CCCTGCAGCTGGAGAGTGTGG-3'
IL-1β Reverse: 5'-TGTGCTCTGCTTGAGAGGTGCT-3'
IL-18 Forward: 5'-ACAACCGCAGTAATACGGAGCA-3'
IL-18 Reverse: 5'- TGTGCTCTGCTTGAGAGGTGCT-3'
GAPDH Forward: 5'-CAGTGCCAGCCTCGTCTCAT-3'
GAPDH Reverse: 5'-AGGGGCCATCCACAGTCTTC-3'

Western blot

Protein samples collected from the tissue samples were loaded into the SDS-PAGE (25 μg/lane) for electrophoresis and then transferred onto the PVDF membrane. Thereafter, the unoccupied sites on the PVDF membrane were blocked in 5% non-fat milk for 1 h at room temperature. Proteins on the membrane were incubated with the corresponding antibodies at 4°C overnight, and the unattached antibodies were eluted by washing in TBST three times. The immunoblots were further incubated with the horseradish peroxidase-conjugated secondary antibodies (1:5000, Jackson ImmunoResearch) at room temperature. The final immunoblots were developed by incubating with the ECL reagent (Amersham Biosciences) to analyze the intensity of bands, with the intensity of β-actin as an endogenous control.

Detection of cell pyroptosis

Caspase-1-positive (caspase-1+) cells and PI-positive (PI+) cells were labeled by using the caspase-1 activity detection kit and PI staining kit, respectively, from which 5×10^4 labeled cells were screened by using the Bio-Rad flow cytometer to calculate the caspase-1+/PI+ cells. This step was repeated four times.

Statistical analysis

SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA) was utilized to perform the statistical analysis. All data were presented with the mean ± standard deviation. The difference between or among groups was testified by using the unpaired student *t*-test or One-way ANOVA. Statistical plots were prepared by using the GraphPad Prism 8. $P<0.05$ suggested that the difference had statistical significance.

Results

MiR-214 and pyroptosis-related genes are downregulated in the cervical cancer tissues and cells

We found that in the cervical cancer tissues, miR-214 expression was significantly suppressed in comparison with the healthy controls (Figure 1A, $P<0.01$). In addition, we also detected the expression of pyroptosis-related genes, including NLRP1, NLRP3, NLRC4, caspase-1, IL-18 and IL-1β, and found that expression of genes above was also suppressed in the cervical cancer tissues (Figure 1B-G, $P<0.01$). Results of Western blotting also revealed that in comparison with the healthy controls, NLRP3 expression in the cervical cancer group was downregulated sharply (Figure 1H). Meanwhile, similar results were also seen in the cervical cancer cell lines, including Hela, HCC94 and Siha cells (Figure 1I-J, $P<0.01$). Thus, miR-214 was downregulated in cervical cancer patients, while the pyroptosis pathway in the cervical cancer cell was suppressed.

Upregulation of miR-214 promotes the pyroptosis of cervical cancer cells

To further investigate the role of miR-214 in pyroptosis of cervical cancer cells, we transfected the Hela cells with pcDNA3.1-miR-214 plasmid to upregulate the expression of miR-214 and detect the pyroptosis of Hela cells. As a result, we found that transfection

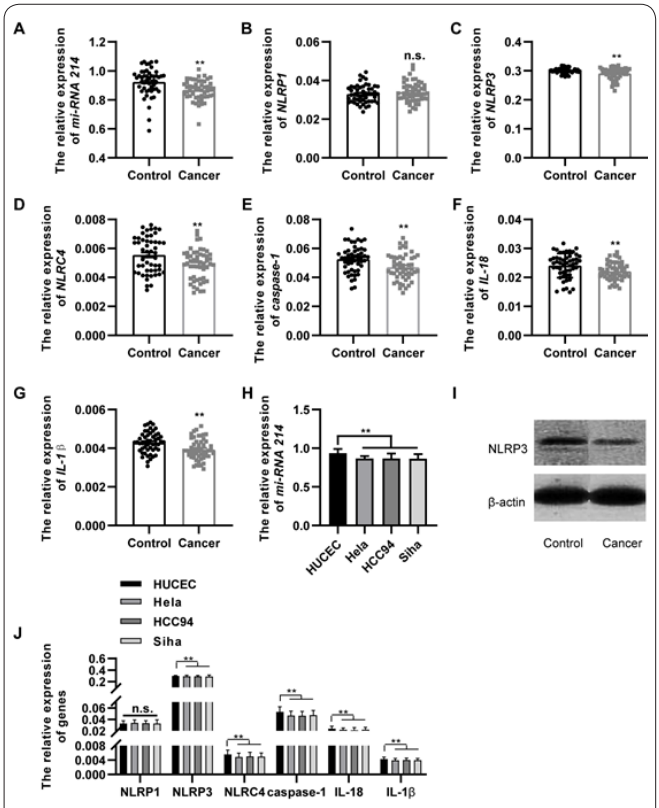

Figure 1. miR-214, NLRP3 and pyroptosis-related genes are suppressed in the cervical cancer tissues and cells. A-G, miR-214, NLRP1, NLRP3, NLRC4, caspase-1, IL-18 and IL-1β are downregulated in cervical cancer tissues; H, miR-214 is downregulated in the cervical cancer cells; I, NLRP3 protein expression is suppressed in the cervical cancer tissues; miR-214, NLRP1, NLRP3, NLRC4, caspase-1, IL-18and IL-1β are downregulated in cervical cancer cells.

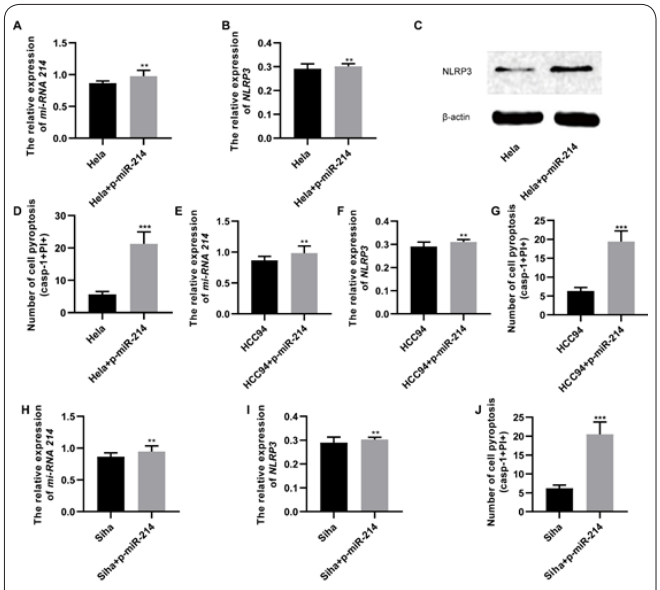

Figure 2. Transfection of pcDNA3.1-miR-214 plasmid enhances the expression of the pyroptosis-related pathway in cervical cancer cells. A: Transection of pcDNA3.1-miR-214 plasmid enhances the expression of miR-214 in Hela cells; B-C: Transection of pcDNA3.1-miR-214 plasmid enhances the mRNA and protein expression of NLRP3 in Hela cells; D: Transection of pcDNA3.1-miR-214 plasmid increases the proportion of Hela cells in pyroptosis; E-J: Transection of pcDNA3.1-miR-214 plasmid enhances the mRNA expression of miR-214 and NLRP3 in HCC94 and Siha cells, with increases in the proportion of cells in pyroptosis.

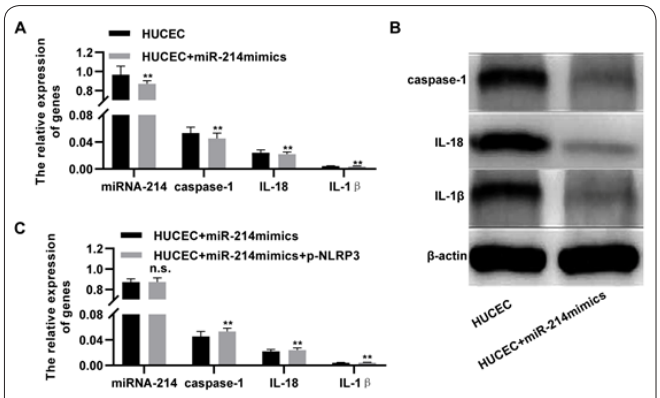

Figure 3. miR-214 regulates the pyroptosis of cervical cancer cells by targeting NLRP3. A: Transfection of miR-214-mimics reduces the gene expression of miR-214 and pyroptosis-related genes in normal cells; B: Transfection of miR-214-mimics reduces the protein expression of pyroptosis-related genes in normal cells; C: Transfection of pcDNA3.1-NLRP3 upregulates the gene expression of pyroptosis-related genes, without any alteration of miR-214 expression.

of pcDNA3.1-miR-214 could successfully upregulate miR-214 (Figure 2A, P <0.01), with the significant upregulation of NLRP3 in mRNA and protein expression (Figure 2B-C, P <0.01), and also enhanced the pyroptosis of Hela cells (Figure 2D). Similar results were also witnessed in the HCC94 cells and Siha cells (Figure 2E-H). Together, the results above revealed the obvious correlation between miR-214 and pyroptosis of cervical cancer cells and suggested that in cervical cancer tissues and cells, NLRP3 was downregulated, which could be reversed by the upregulation of miR-214.

MiR-214 regulates the pyroptosis of cervical cancer cells by targeting NLRP3

To validate whether miR-214 regulates the pyroptosis of cervical cancer cells by targeting NLRP3, we detected the changes in the activity of the NLRP3 pathway by transfecting the normal cells and cervical cancer cells with miR-214 mimics and pcDNA3.1-NLRP3. Results of RT-PCR showed that after the HUCEC cells were transfected by miR-214 mimics, miR-214, caspase-1, IL-1β and IL-18 were downregulated evidently (Figure 3A, P <0.01), and similar changes were also seen in the Western blotting (Figure 3B). Subsequent transfection of pcDNA3.1-NLRP3 abolished the miR-214 mimics-induced downregulation of NLRP3 but showed no significant effect on miR-214 (Figure 3C, P <0.01).

NLRP3 inhibitor reduces the pyroptosis of cervical cancer cells induced by pcDNA3.1-miR-214 but does not alter the expression of miR-214

The results above showed that the upregulation of miR-214 can enhance the expression of NLRP3. Subsequently, we validated the existence of a negative-feedback network in the regulation of miR-214 expression in cervical cancer cells. First, cervical cancer cell lines (Hela, HCC94 and Siha cells) were selected for transfection by using the pcDNA3.1-miR-214, followed by the treatment of NLRP3 inhibitor at a certain concentration that could not alter the activity of cells or the miR-214 expression, but inhibit the expression of NLRP3 (Figure 4A-C; P <0.01). Thus, miR-214 could regulate

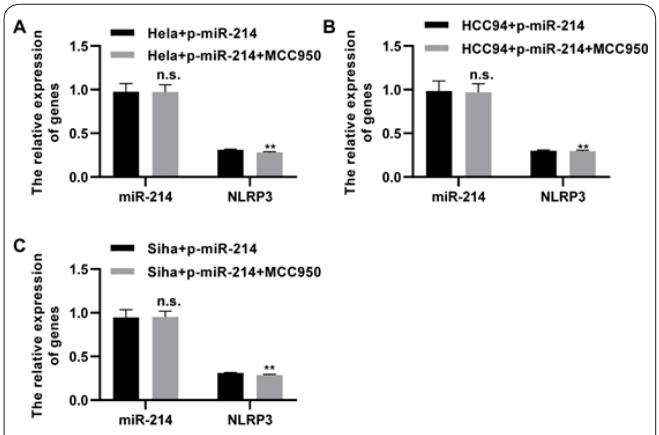

Figure 4. NLRP3 inhibitor reduces the pyroptosis of cervical cancer cell-induced by transfection of pcDNA3.1-miR-214, but with no influence on the expression of miR-214. A-C: NLRP3 inhibitor reduces the expression of NLRP3 in cervical cancer cells, but with no effect on the expression of miR-214.

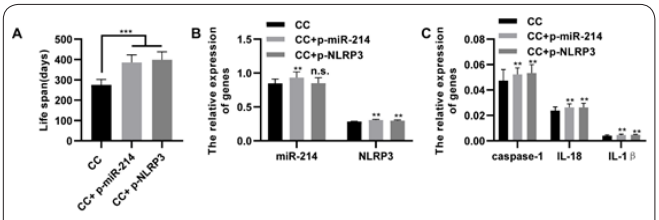

Figure 5. The upregulation of miR-214 and NLRP3 can prolong the survival of cervical cancer rats. A: Injection of pcDNA3.1-miR-214plasmidand pcDNA3.1-NLRP3 plasmid can prolong the survival of cervical cancer rats; B: Injection of pcDNA3.1-NLRP3 plasmid does not alter the expression of miR-214 in cervical cancer rats; C: Injection of pcDNA3.1-miR-214plasmidand pcDNA3.1-NLRP3 plasmid enhances the expression of pyroptosis-related genes.

the expression of NLRP3, not in a negative-feedback pattern.

Upregulation of miR-214 and NLRP3 prolongs the survival of cervical cancer rats

To further validate the role of miR-214, we constructed the cervical cancer models on Wistar rats to detect the effect of pcDNA3.1-miR-214 on rats in the CC + p-miR-214 group. In addition, cervical cancer rats were also treated by the pcDNA3.1-NLRP3 to verify its effect on the pyroptosis of cells. As a result, transfection of pcDNA3.1-miR-214 or pcDNA3.1-NLRP3 could prolong the survival of cervical cancer rats (Figure 5A, $P < 0.01$). Results of RT-PCR also indicated that following the injection of pcDNA3.1-miR-214, cervical cancer rats manifested the significant upregulation of miR-214, while the injection of pcDNA3.1-NLRP3 resulted in no significant change in expression of miR-214 (Figure 6B). However, both of the injection of pcDNA3.1-miR-214 and pcDNA3.1-NLRP3 upregulated the expression of NLRP3 (Figure 6B). Moreover, cervical cancer rats showed obvious downregulation of the NLRP3 pathway, which was reversed after the injection of pcDNA3.1-miR-214 or pcDNA3.1-NLRP3 (Figure 3C). Thus, miR-214 could regulate the pyroptosis of cervical cancer cells by targeting miR-214/NLRP3 axis *in vivo.*

Discussion

MiRNAs are proved to be critical to the development and progression of a variant of gynecological diseases, including pelvic inflammation and uterine fibroid, while the specific mechanisms of miRNAs regarding the pyroptosis remain unknown (17-21). In this study, we confirmed that miR-214 is downregulated in the cervical cancer tissues and cells, while overexpression of miR-214, by targeting NLRP3, can induce the pyroptosis of cervical cells to inhibit the development and progression of cervical cancer.

Previous studies have shown the involvement of miRNAs in the development and progression of cervical cancer (21). For instance, miR-137 overexpression, via binding to GREM1, can inhibit the activity of the TGF-β/Smad pathway, thereby inhibiting the invasion, migration and epithelium-mesenchyme transformation of cervical cancer cells (21). In addition, miR-575-5p can also inhibit the growth of cervical cancer cells and enhance their sensitivity to chemotherapeutics by targeting QKI, a kind of RNA-binding protein. Furthermore, an increasing body of evidence suggested that miR-214 plays key roles in various diseases (22-26), which, nevertheless, has not yet covered the correlation between miR-214 and cell pyroptosis. In our study, miR-214 is downregulated obviously in cervical cancer, while the upregulation of miR-214 can decrease the pyroptosis of cervical cancer cell lines.

Dysregulation or dysfunction of miRNAs may participate in the progression of cancer, manifesting the intimate correlations with the development, progression and prognosis of cervical cancer. At present, enormous studies are investigating the relationship between cervical cancer and miRNAs, including miR-137, miR-574-5p and miR-21, and involvement of these miRNAs in the development and progression of cervical cancer (3, 21, 27). But there remains no evidence suggesting the molecular mechanism of miRNA in the development and progression of cervical cancer. MiR-214, according to the previous evidence, experiences the most obvious downregulation in cervical cancer patients (24).

Cell pyroptosis is a kind of inflammation-related programmed cell death that is involved in the development and progression of cervical cancer. Initiation of cell pyroptosis requires the formation of the inflammasome, which can further induce the generation of caspase-1 that accelerates the release of IL-1β和IL-18(28). This process can induce the oxidative stress of mitochondria to promote the apoptosis of cells and affect the abnormal metabolism of cells (28). More and more evidence has shown that enhancing the inflammation can promote the death of cancer cells and delay the development and progression of cancer (29, 30). Extensive research has been conducted on the expression of genes in various organisms (31-36). Control to reduce expression or increase gene expression can lead to good traits (37-40). Genome editing can be very effective in this regard (42). In our study, the upregulation of miR-214 can advance the pyroptosis of cancer cells by targeting NLRP3, which we believed to be associated with the inflammation. In future work, more efforts should be focused on the role of miR-214 in the regulation of other pathological processes of cervical cancer.

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