Research Paper

Exploration of miR-22 inhibiting cervical cancer progression through suppressing TGF-β/Smad3 pathway by targeting TGFBR1

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ABSTRACT

Background: The TGF-β/Smad3 pathway is frequently active in cervical cancer, preventing tumor cell apoptosis and fostering tumor cell survival and growth. According to reports, miR-22 inhibits the activation of the TGF-β/Smad3 pathway by targeting the TGF-β receptor TGFBR1. The therapeutic potential of miR-22 in cervical cancer has also been reported. However, the relationship between the two has not been fully studied.

Methods: Through clinicopathological tissue examination and *in vitro* tests, this study will investigate the link between miR-22 and TGF-β/Smad3 pathway expression. Collect cervical cancer tissues and para-cancerous tissues from patients, and compare the expression of miR-22 and TGF-β/Smad3 pathway. Hela cell lines were used as cell models for *in vitro* experiments. Cell lines overexpressing/inhibiting miR-22 (miR-22 mimic/inhibitor) and TGFBR1 overexpressing (oe-TGFBR1) were cultivated by cell transfection. Cell proliferation was detected by colony formation. Cell migration was discovered using the cell scratch test. Cell apoptosis was discovered using flow cytometry. Dual luciferase reporter assay was used to confirm the binding of miR-22 and TGFBR1. TGFBR1, TGF-β1, and Smad3 mRNA expression levels were identified using RT-PCR. TGFBR1, TGF-β1, and Smad3 protein expression was found using Western blot.

Results: Significantly less miR-22 was expressed in cervical carcinoma tissue. Hela cell growth was suppressed and cell apoptosis was aided by miR-22 overexpression. The TGF-β/Smad3 pathway was blocked by miR-22 overexpression. TGFBR1 was the binding target of miR-22. Overexpression of TGFBR1 reversed the effect of overexpression of miR-22 on Hela cells. Overexpression of TGFBR1 reversed the inhibition of TGF-β/Smad3 pathway by overexpression of miR-22.

Conclusion: In this work, thorough clinical and *in vitro* cell studies were performed to show that miR-22 suppressed the expression of the TGF-β/Smad3 pathway by targeting TGFBR1, consequently decreasing cervical cancer cell growth and increasing death.

INTRODUCTION

Cervical cancer is cancer that occurs in the cervix. Cervical cancer is usually asymptomatic in its early stages, but abnormal vaginal bleeding and pelvic pain may occur in advanced stages [1]. Sexually transmitted HPV viruses are the primary cause of more than 95% of cervical malignancies [2]. However, the majority of HPV virus-infected individuals will not develop cancer [3]. The importance of HPV infection outweighs that of other risk factors, which include smoking, immunological dysfunction, frequent use of contraception, unsafe sex, many partners, etc., [4]. Typical cervical cancer develops from precancerous lesions that develop

over a period of 10 to 20 years. Cervical cancer can be divided into several types, 90% are squamous cell carcinoma, 10% are adenocarcinoma, and other types account for only a few [5]. Typically, a Pap smear test is used to screen for cervical cancer, which is then followed by a biopsy. To determine if cancer has spread, doctors employ medical imaging [6]. Cervical cancer is the fourth most common type of cancer in the world and the fourth most common cause of cancer-related death in women [7].

An essential protein route in the onset and progression of cancer is the TGF-β/Smad3 signaling pathway [8]. TGF-β/Smad3 signaling has been linked to the development of cancer by fostering cell proliferation, neovascularization, and immunosuppression, according to studies [9]. The TGF-β/Smad3 pathway is typically active in cervical cancer cells, which prevents tumor cell apoptosis (programmed cell death) and encourages tumor cell survival and development [10, 11].

A short RNA molecule known as miR-22 is used in molecular biology. MicroRNAs are a common type of molecule with an average length of 22 nucleotides. By attaching to the 3′UTR of mRNAs produced in cells, microRNAs can post-transcriptionally control gene expression [12]. The immortal cell line HeLa, which is generated from cervical cancer cells, was where miR-22 was first discovered, although it was later discovered to be widely expressed in many tissues. It has previously been discovered that miR-22 plays a role in erythrocyte maturation. Numerous miRNAs have been found to be dysregulated, which has been linked to oncogenesis [13]. miR-22 was shown to be up-regulated in breast cancer, cholangiocarcinoma, multiple myeloma, and hepatocellular carcinoma but down-regulated in prostate cancer. Several datasets on breast cancer showed a correlation between miR-22 expression and survival [14].

Growing and evolving element the 53 kDa Beta receptor I, also known as activin A receptor type II-like kinase, is a membrane-bound member of the TGF-beta receptor family and the TGF beta superfamily of signaling molecules. The human gene for it is TGFBR1 [15]. When the type II TGF-β receptors and the protein produced by this gene connect to TGF-β, a heteromeric complex is created that transmits the signal from the cell surface to the cytoplasm. A serine/threonine protein kinase is the protein that is encoded. Loeys-Dietz aortic aneurysm syndrome, also known as LDAS and LDS, has been linked to mutations in this gene [16–18].

miR-22 has been reported to target TGF-β receptor TGFBR1, thereby inhibiting the activation of TGF-β/Smad3 pathway [19]. miR-22 has also been reported for its therapeutic potential in cervical cancer [20, 21]. However, the relationship between the two has not been fully studied in cervical cancer. Therefore, this study intends to explore the relationship between miR-22 and TGF-β/Smad3 pathway expression through clinicopathological tissue analysis and *in vitro* experiments, so as to find clinical treatment methods for cervical cancer.

MATERIALS AND METHODS

Experiment design

Clinical experiment

Collect cervical cancer tissues and para-cancerous tissues from patients, and compare the expression of miR-22 and TGF-β/Smad3 pathway.

Cell experiments

Hela cell lines were used as cell models for *in vitro* experiments. Cell lines overexpressing/inhibiting miR-22 (miR-22 mimic/inhibitor) and TGFBR1 overexpressing (oe-TGFBR1) were cultivated by cell transfection. Cell proliferation was detected by colony formation. The cell scratch test was used to identify cell migration. Using flow cytometry, cell apoptosis was identified. Using a dual luciferase reporter experiment, it was determined that miR-22 and TGFBR1 were really bound. RT-PCR was used to determine the mRNA expression levels of TGFBR1, TGF-β1, and Smad3. Western blot was used to detect the protein expression of TGFBR1, TGF-β1, and Smad3.

RT-PCR analysis

The TRIzol reagent was used to extract total RNA from cervical cancer tissue cells, para-cancerous tissue cells, HaCaT cells, and cervical cancer cells. The Revert Aid TW first Strand cDNA Synthesis Kit was used to produce first strand cDNA. The QuantiNova SyBr Green PCR Kit was used for the PCR analysis. Pre-denaturation at 95°C for one minute, denaturation at 95°C for thirty seconds, annealing at 60°C for thirty seconds, and elongation at 72°C for thirty seconds were the reaction conditions.

Western blot analysis

Each six-well plate has to have 200 μl of cell lysate added to it once each group's cells have been collected. The cells were sonicated and then put on ice for an hour to produce lysing. The lysed cell sample was centrifuged for 15 minutes at 4°C and 12,500 rpm. The supernatant should then be transferred from the centrifuge tube into a fresh one. We measured the amount of protein using a kit for measuring β-actin protein. The temperature of the protein samples under examination was −80°C. For Western blot electro-

phoresis, 50 μg of protein loading was used per well. The membrane was then transferred and blocked following SDS-PAGE electrophoresis. The proteins TGFBR1, TGF-β1, and Smad3 were diluted using a primary antibody (1: 500, anti-human, Thermo-Fisher, USA) in order to use concentration. All through the night, the samples oscillated at 4°C in an incubator. The samples were rinsed with PBS before being incubated with the secondary antibody (1: 1000, anti-human, Thermo-Fisher, USA) for 30 minutes at room temperature in the dark. The developer was then captured on camera and developed.

Cell cloning experiment

Each logarithmic growth phase cell collection must be extracted using a pipette, digested with 0.25% trypsin, and suspended for subsequent use in a culture medium containing 10% fetal bovine serum. Before being injected at a gradient density of 50, 100, and 200 cells per dish into a plate containing 10 ml of pre-warmed culture material at 37°C, each batch of cells was gently spun to ensure equal distribution. After that, a gradient was used to dilute the cell solution many times. For two to three weeks, placed it in a cell incubator that is adjusted at 37°C, 5% CO_2, and saturated humidity. In general, the culture should be stopped when there are obvious clones in the petri dish. Before being thrown away, the supernatant was carefully cleaned with PBS three times. For 15 min, 5 mL of 4% paraformaldehyde is used to fix the cells. The dye solution is then applied to the area to be colored for 10 to 30 min before being gradually rinsed off with running water. Next, the fixative solution is removed, the required amount of GIMSA is added, and the dye solution is applied.

Cell scratch test

Eight 1 × 10^5 cells/mL from each batch were split across 35 mm^2 growth dishes after the cells in each batch had been digested and counted. Aspirate the culture medium, mark the dish's bottom with a marker, and then mark the cells in the dish perpendicular to the marker using a 10 μL pipette tip. Add serum-free culture media to the indicated cells after washing them with PBS to remove them from the culture. Choose the junction of the marker line and the cell scratch as the observation point and keep an eye on a fixed place while capturing pictures after 24 h.

Flow cytometry analysis

Each group's cells were put to a 2 mL centrifuge tube, which was then spun at 1500 rpm for 5 min before collecting the supernatant. Use 0.1% Triton X-100 to fix at room temperature for 10 min after fixing with 4% paraformaldehyde (PFA) at 4°C for 30 min. 200 μL of the primary antibody should be added, diluted with PBA, and then incubated at 4°C for two hours. After that, spin the mixture to separate the supernatant, and then PBS-wash it. 200 μL of a secondary antibody that has been fluorescein-labeled and diluted with PBA should be incubated for 30 min at 4°C in the dark. Following that, 500 μL of PBS was added to the flow tube with the cells, and a flow cytometer was used to identify the cells.

Dual luciferase reporter analysis

Prior to transfecting cells with target plasmids in 96-well plates, it is advised to wait until the cell density is between 50–70%. 10 mL of DMEM should include 0.16 mg of lncRNA (circRNA/3′UTR)/Mut target plasmid and 5 pmol of miR/Negative. Carolina's regulations in a neutral setting, repeat (solution A). After that, mix 10 mL of DMEM with 0.3 mL of transfection agent (solution B) and let sit for 5 minutes at room temperature. Allow the solution A/B combination to remain at room temperature for 20 min. New medium was first added to the cells, then the transfection solution, and finally they were merged. At 37°C and 5% CO_2, cells were developed. Cells were collected and new medium was added six hours after transfection.

The experiment's detection kit was the Promega Dual-Luciferase System. A 96-well plate was filled with 100 μL of 1′PLB that had been diluted with 5′PLB's distilled water. Pipette the cell lysate into a 1.5 mL centrifuge tube after giving the cells 15 min of moderate shaking at room temperature to disperse the cells. The supernatant was transferred to a fresh tube after the first tube had been centrifuged at a speed of 13200 g for 10 minutes at 4°C. A 96-well plate has to have 100 μL of the Luciferase Assay Reagent II (LAR II) working solution (Luciferase Assay Reagent, Promega). After 20 μL of cell lysate have been added and well mixed by pipetting 2–3 times, the Firefly luciferase value should be determined and recorded as the internal reference value. The Firefly luciferase reporter gene fluorescence value must be measured and recorded. You should add 100 μl of the Stop&Glo® Reagent (Luciferase Assay Reagent, Promega).

Statistical analysis

The statistical method used in this assignment was the paired *T*-test. The experimental data's mean and standard deviation are shown. For the statistical analysis, SPSS 23.0 was utilized as the program. The figures were produced using Origin 2022b and Adobe Illustrator 2021 software.

RESULTS AND DISCUSSION

miR-22 expression decreased significantly in cervical cancer tissue

Figure 1 shows the RNA expression levels of miR-22, TGFBR1, TGF-β1, and Smad3, as well as the protein levels of TGFBR1, TGF-β1, and Smad3 in cervical cancer and surrounding tissue. The figure shows that the RNA expression of miR-22 in cervical cancer tissues was much lower than in neighboring tissues, but TGFBR1, TGF-β1, and Smad3 were significantly higher. TGFBR1, TGF-β1, and Smad3 protein expression levels were very consistent with the RNA expression pattern. There was a significantly statistical difference. This indicated that the expression of

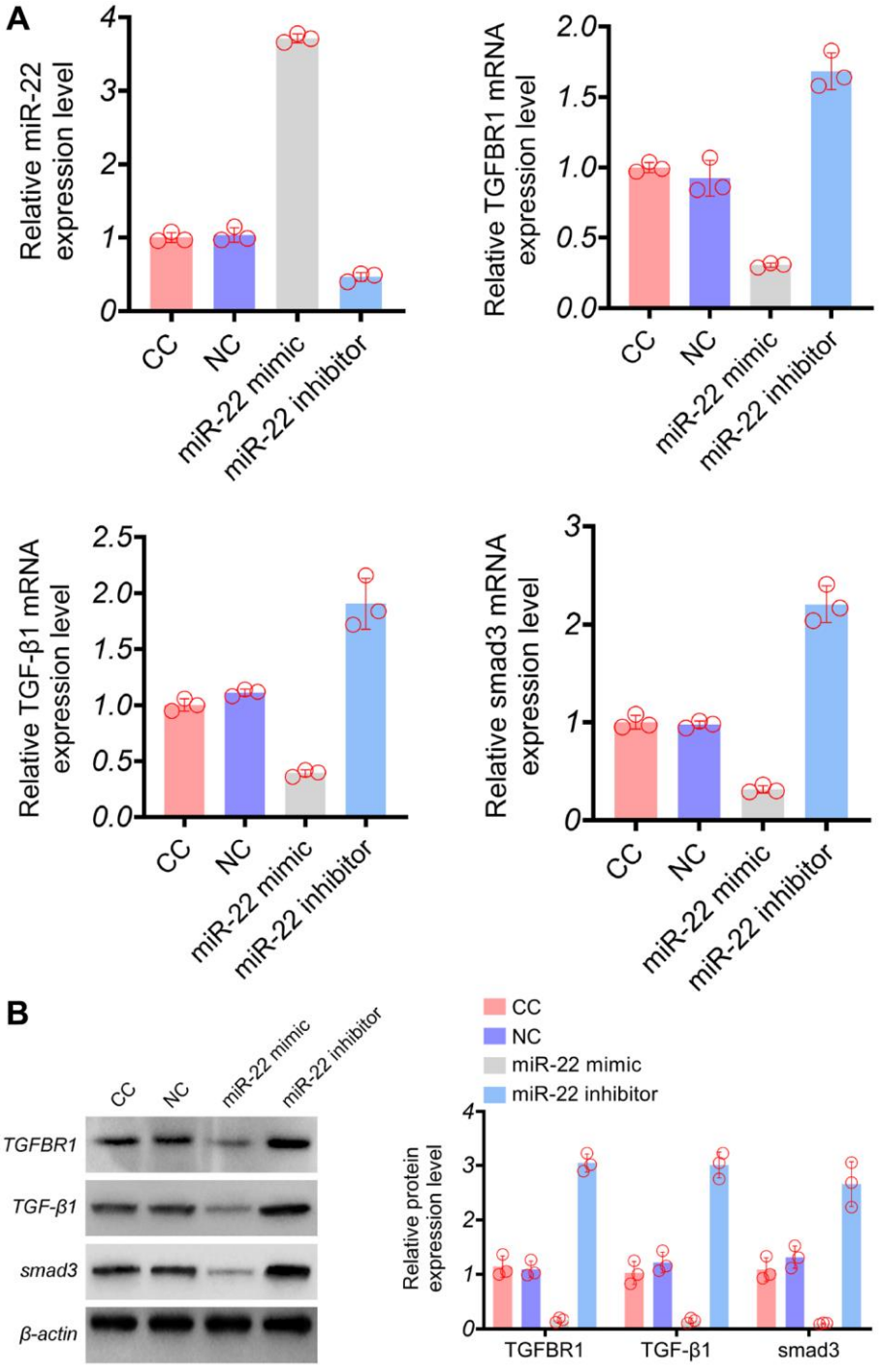

Figure 1. (**A**) The results of RNA expression level of miR-22, TGFBR1, TGF-β1 in cervical cancer tissue and adjacent tissue. (**B**) The results of protein expression level of TGFBR1, TGF-β1 and Smad3 in cervical cancer tissue and adjacent tissue.

miR-22 was inhibited in cervical cancer tissues, while TGF-β/Smad3 pathway was activated. The activation of the TGF-β/Smad3 pathway promotes cancer growth, which is consistent with existing research results. Further validation is needed to determine whether the inhibition of miR-22 expression is associated with the activation of the TGF-β/Smad3 pathway.

miR-22 overexpression decreased Hela cell growth and accelerated cell death

The effects of overexpression of miR-22 and inhibition of miR-22 expression on the proliferation and apoptosis of Hela cells were verified by cell cloning, cell scratch, and flow cytometry, as shown in Figure 2. An essential technical technique for identifying cell proliferation, invasiveness, and susceptibility to lethal agents is the cell cloning experiment. The two crucial characteristics of cell population dependency and proliferation capacity are reflected in the clone creation rate. The most common technique for determining a tumor cell's capacity for invasion is cell scratching. After 24 hours, the scratch's potential to allow cells to invade is stronger the smaller it is. Flow cytometry can visually display the proportion of cell apoptosis. As shown in the figure, overexpression of miR-22 significantly inhibited the proliferation and migration of Hela cells, while promoting their apoptosis. Inhibiting the expression of miR-22 had the opposite effect. This fully demonstrated the potential of miR-22 in treating cervical cancer.

Overexpression of miR-22 inhibited the TGF-β/Smad3 pathway

Figure 3 shows how miR-22 expression may be raised or lowered to alter TGFBR1, TGF-β1, and Smad3 RNA and protein expression. When miR-22 is overexpressed, it can significantly reduce the synthesis of TGFBR1, TGF-β1, and Smad3 in both RNA and protein. Inhibiting miR-22 expression would have the opposite effect. TGFBR1, the TGF-β1 receptor, was the target of miR-22, which inhibited the TGF-β/Smad3 pathway. This was consistent with the findings of other investigations.

TGFBR1 was the target of miR-22

The results of the dual luciferase reporter detection of miR-22 binding to TGFBR1 are shown in Figure 4. When TGFBR1-wt and miR-22 mimic were co-transfected into cells, the activity of the luciferase decreased ($p < 0.05$) in comparison to the NC group,

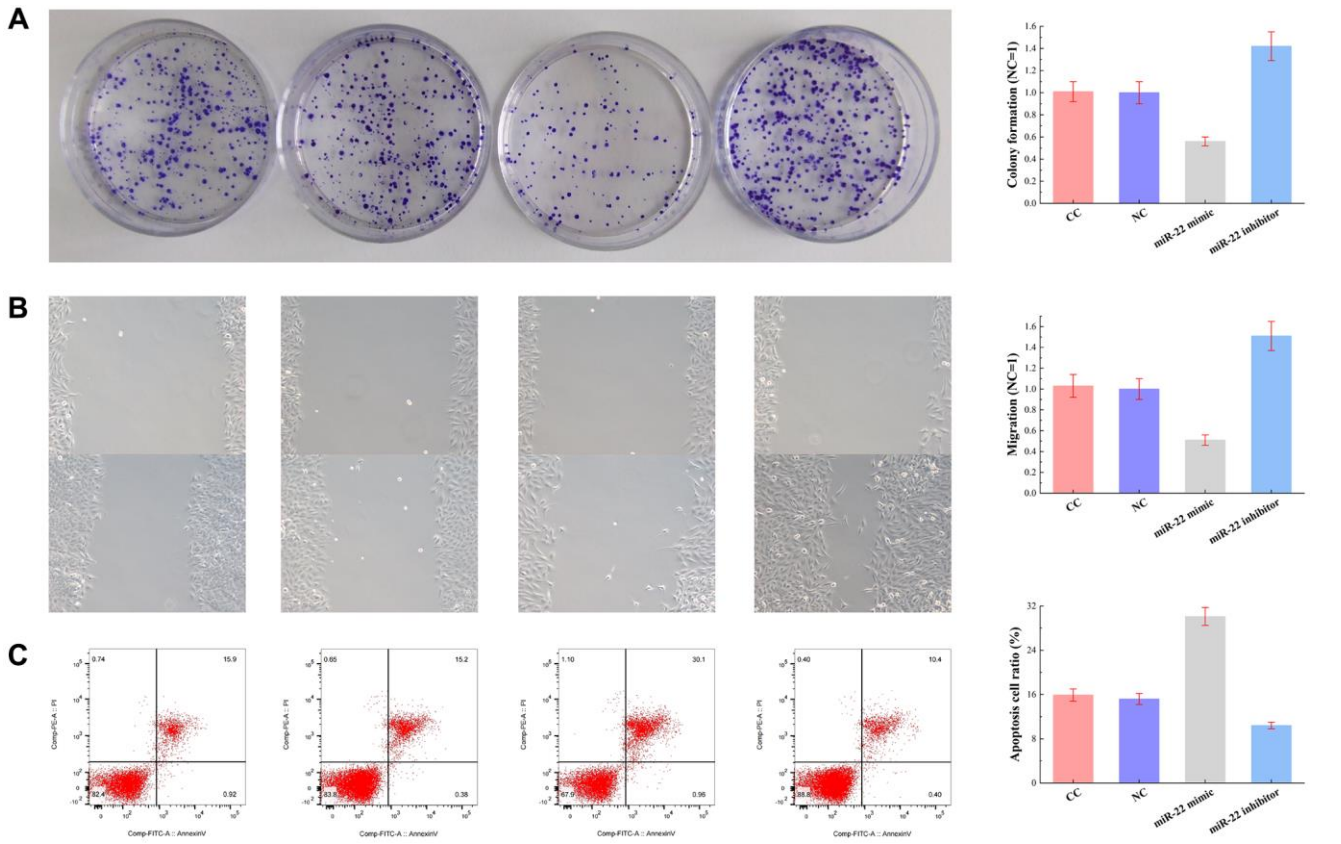

Figure 2. The impact of miR-22 overexpression and suppression of miR-22 expression on Hela cell growth and apoptosis. (**A**) Cell cloning. (**B**) Cell scratch. (**C**) Flow cytometry.

according to the analysis of the dual luciferase reporter genes. In cells co-transfected with TGFBR1-mut and miR-22 mimic, the level of Luciferase activity was not noticeably different. The results demonstrated that miR-22 might target TGFBR1 in particular. This proved that miR-22 specifically targeted TGFBR1 to regulate the TGF-β/Smad3 pathway's expression.

Overexpression of TGFBR1 reversed the effect of overexpression of miR-22 on Hela cells

The effects of overexpression of TGFBR1 to over-expression of miR-22 on the proliferation and apoptosis of Hela cells were verified by cell cloning, cell scratch, and flow cytometry, as shown in Figure 5.

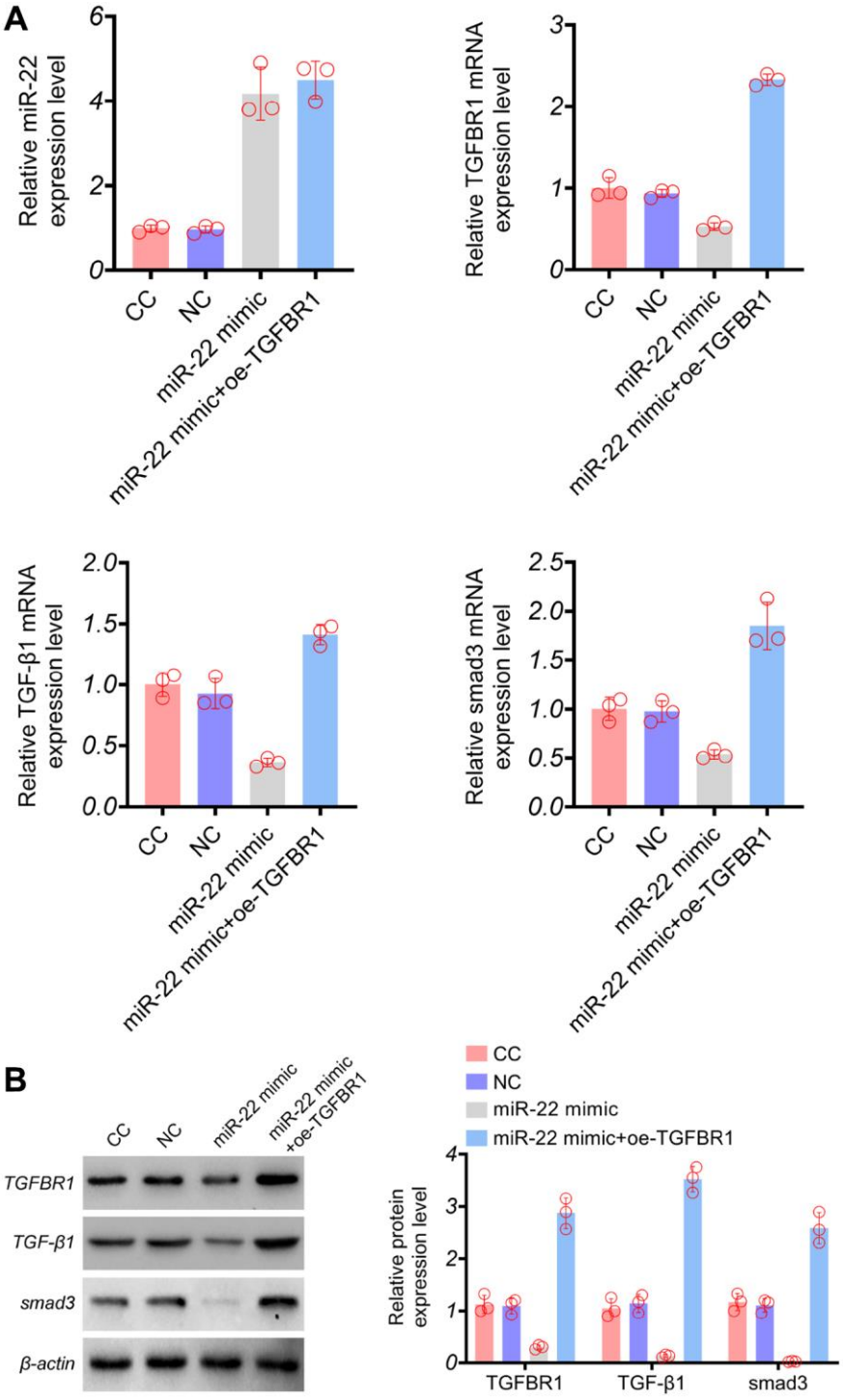

Figure 3. The effects of miR-22 overexpression and suppression of miR-22 expression on TGFBR1, TGF-β1, and Smad3 RNA and protein expression. (**A**) RT-PCR. (**B**) Western blot.

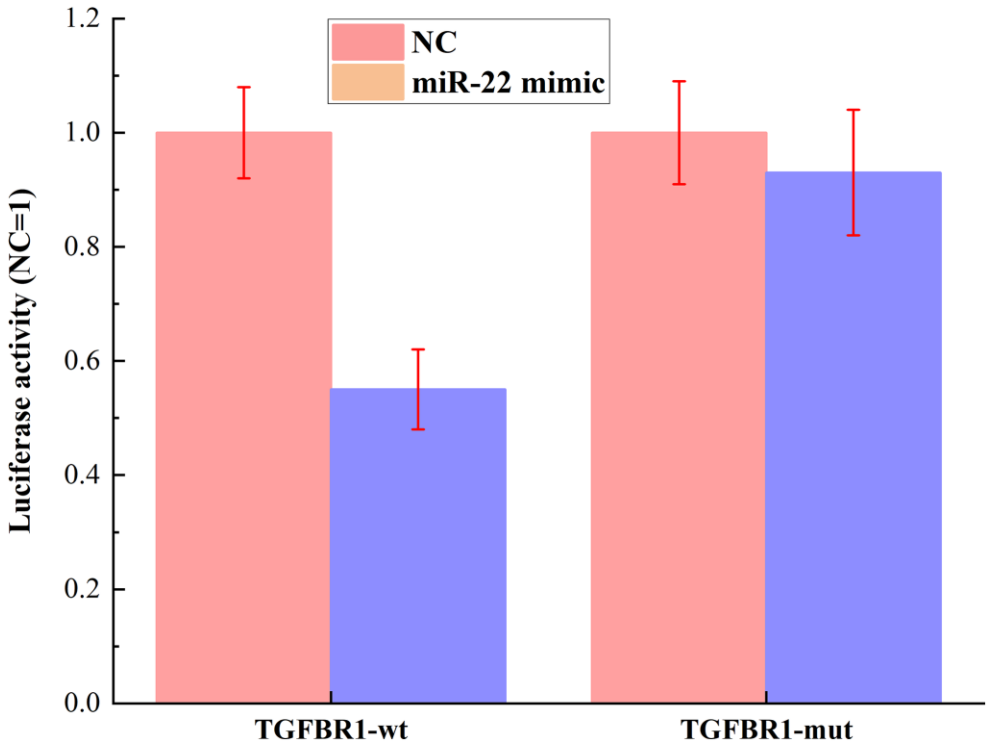

Figure 4. The identification of miR-22 binding to TGFBR1 using dual luciferase reporters.

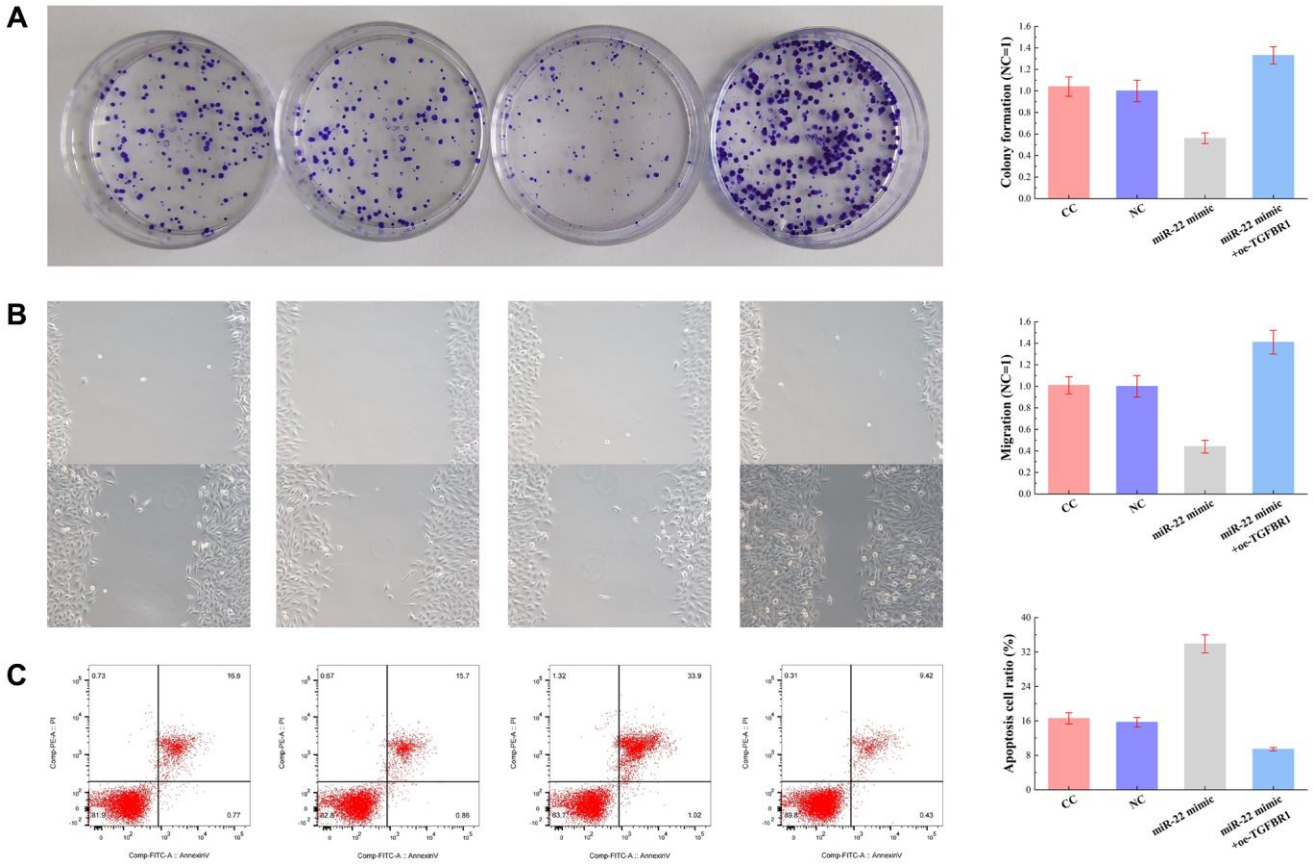

Figure 5. The effects of overexpression of TGFBR1 to overexpression of miR-22 on the proliferation and apoptosis of Hela cells. (**A**) Cell cloning. (**B**) Cell scratch. (**C**) Flow cytometry.

Based on the results of this experiment and similar experiments mentioned earlier, it can be concluded that overexpression of miR-22 significantly inhibited the proliferation and migration of Hela cells, while promoting their apoptosis. Moreover, overexpression of TGFBR1 could reverse the effects of miR-22 overexpression on Hela cells. This fully demonstrated that miR-22 affected the proliferation and apoptosis of Hela cells by targeting TGFBR1.

Overexpression of TGFBR1 reversed the inhibition of TGF-β/Smad3 pathway by overexpression of miR-22

The effects of overexpression of TGFBR1 to overexpression of miR-22 on the RNA and protein expression of TGFBR1, TGF-β1, and Smad3 are shown in Figure 6. Based on the results of this experiment and similar experiments mentioned earlier, it can be

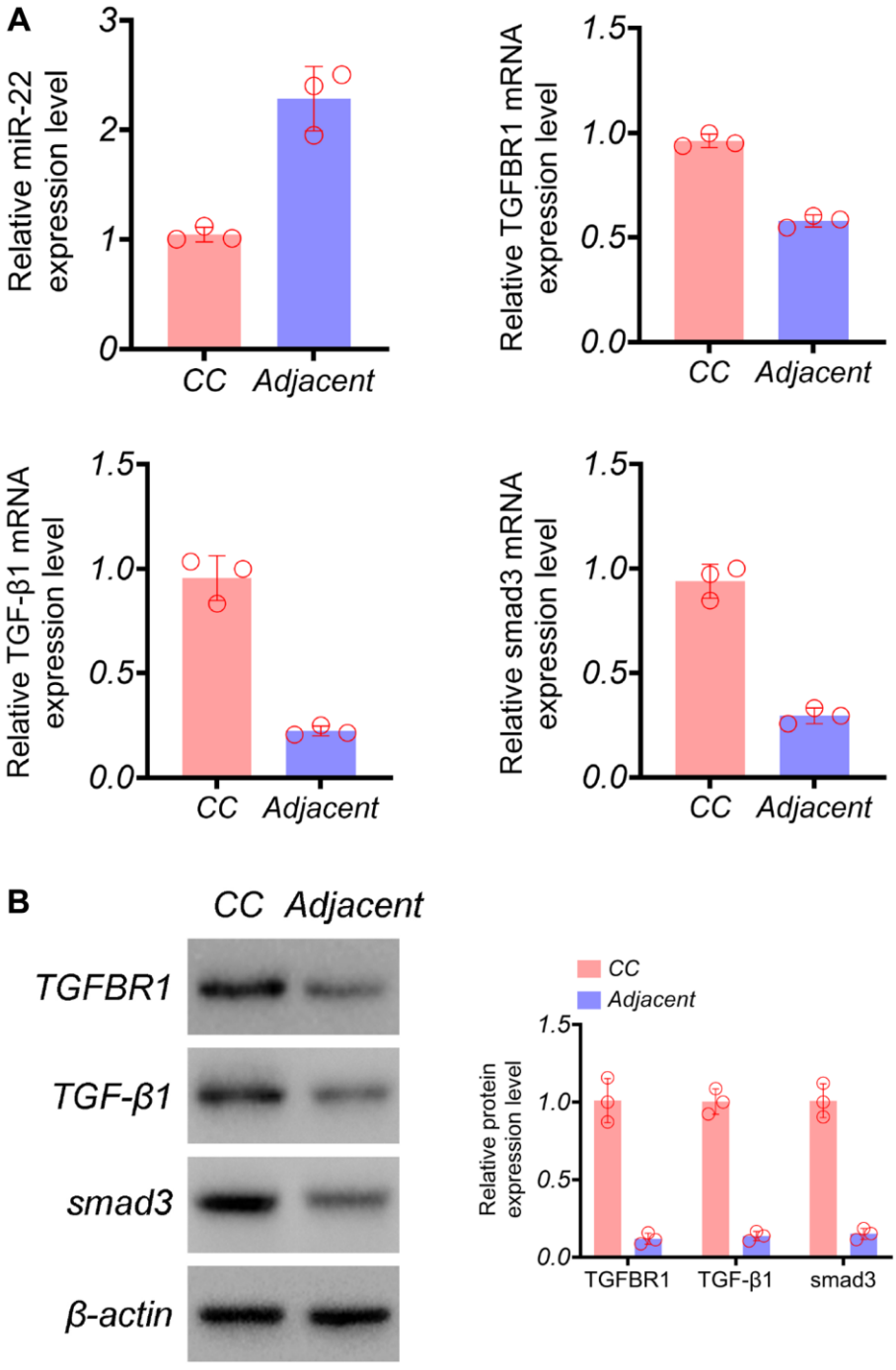

Figure 6. The effects of overexpression of TGFBR1 to overexpression of miR-22 on the RNA and protein expression of TGFBR1, TGF-β1, and Smad3. (**A**) RT-PCR. (**B**) Western blot.

concluded that overexpression of miR-22 could significantly inhibit the RNA and protein expression of TGFBR1, TGF-β1, and Smad3. Furthermore, overexpression of TGFBR1 could reverse the effects of miR-22 overexpression on the expression of TGFBR1, TGF-β1, and Smad3. This once again proved that miR-22 regulated the expression of TGF-β/Smad3 pathway by targeting TGFBR1.

CONCLUSION

The purpose of this work was to show that miR-22 decreased the production of TGF-β/Smad3 pathway by targeting TGFBR1, consequently decreasing the proliferation and increasing apoptosis of cervical cancer cells. This offered fresh treatment options for cervical cancer. More thorough research is still required, nevertheless, to strengthen our findings and better comprehend the processes behind the interaction between miR-22 and the TGF-β/Smad3 pathway as well as the actual use of miR-22 therapy for cervical cancer.

AUTHOR CONTRIBUTIONS

Yuhua Wang conceived and designed experiments; Yuhua Wang performed experiments and data analysis; Mei Wang, Wenfeng Ye, Pingping Tao, Yunzhu Zhang, Ai Zhang, Lingling Feng provided technical support, data collection and analysis; and Yuhua Wang wrote the manuscript. All authors provided final approval for submitted and published version.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest related to this study.

ETHICAL STATEMENT AND CONSENT

The research protocol has been reviewed and approved by the Ethical Committee and Institutional Review Board of The Shanghai Pudong New Area People's Hospital (Approval number: 2023-LW-08). Written consent was obtained from all patients provided tissues.

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