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Research Article
Prediction Value and Mechanism of miR-587 for Cervical Cancer in HPV Infected Population

Aijun Ti, Ruizhi Chen and Mingli Chen

Department of Gynecology, Laizhou People's Hospital, Laizhou, Shandong 261400, China

Abstract

Background and Objective: Cervical cancer is one of the most prevalent gynaecological malignancies, poses a potential menace to patients. To determine the connection between miR-587 and cervical cancer, aiming at providing new ideas for future clinical treatment of cervical cancer. **Materials and Methods:** The study included 41 cervical cancer patients and 49 HPV infected patients from June, 2019 to October, 2021. The miR-587 levels were measured by qRT-PCR. Then CC cells were transfected with miR-587-mimics, miR-587-in inhibition, miR-587-NC, si-BCL2A1 and NC-BCL2A1 to test the alterations in cell multiplication, migration and autophagy protein expression. **Results:** The expression of miR-587 was higher in the Research group than Control group. The miR-587 mimics group has higher cell proliferation, invasion and migration abilities. The si-BCL2A1 group showed the same result. The DLR assay showed that miR-587-mimic inhibited the fluorescence activity of BCL2A1-WT. Moreover, The inhibitory effect of miR-587 on CC cells was completely reversed by Si-BCL2A1. **Conclusion:** The miR-587 is highly expressed in cervical cancer patients, which may be a breakthrough for the diagnosis and treatment of CC in the future.

Key words: Cervical cancer, miR-587, BCL2A1, invasion, migration, DLR assay, HPV

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Corresponding Author: Mingli Chen, Department of Gynecology, Laizhou People's Hospital, Laizhou, Shandong 261400, China

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cervical cancer (CC) is one of the most common gynaecological malignancies with great potential menace. According to the statistics of WTO, the incidence of CC in 2018 was about 13/100,000, with more than 500,000 newly diagnosed CC patients worldwide[1]. And compared with the statistics in 2008, the occurrence of CC shows a steady increase year by year, with an emerging trend of the disease at younger ages (40-50 years old)[2]. The CC is easy to neglect due to the absence of any clinical symptoms in the early stage. However, as the tumour develops and compresses adjacent organs and tissues, it will show obvious symptoms. At this time, the tumour usually has already developed to the middle and late stages, with a greater risk of metastasis[3]. Although the cure rate of early CC is high in clinical practice, the treatment of advanced CC is still not ideal with 5 years mortality exceeding 50%[4]. Therefore, clinically, it is considered that improving the early diagnosis rate of CC is the key to ensuring the life and health of patients and further penetrating its pathogenesis is the basis for finding breakthroughs in CC diagnosis and treatment. Currently, Human Papillomavirus (HPV) infection is believed to be the most critical risk factor for CC and HPV, as a susceptible virus, has an infection rate of 26.8% in women aged 14-59[5]. In China, over 1 million cases of HPV infection are confirmed every year[6], which is also the prime reason for the rising incidence of CC.

In recent years, clinical research on CC has gradually focused on micRNAs-human genetic materials that have been proved to have multiple functions such as regulating development, virus defense, hematopoietic process, organogenesis and cell life cycle[7]. Therefore, micRNAs are of great reference significance for CC, mainly caused by HPV infection. Of them, miR-587 is a recently discovered micRNA family member, which first attracted public attention because it was confirmed to be involved in colorectal carcinogenesis and progression[8]. Subsequently, it was confirmed that it presented obvious abnormal expression in the process of HPV infection[9]. In a recent study, we found that miR-587 was also present when screening micRNAs with abnormal expression in CC[10]. Hence, miR-587 may have important reference significance for CC. Whereas, there is still no reliable research to support our conjecture. Accordingly, in this study, we conducted a preliminary study on the connection between miR-587 and CC, aiming at providing new ideas and references for future clinical treatment of CC and laying a foundation for follow-up research.

MATERIALS AND METHODS

The research project was performed in Laizhou People's Hospital lab, China from June, 2019 to October, 2021.

Samples and croups: A prospective analysis was performed on 41 CC patients (research group, Res group) and 49 HPV infected patients (control group, Con group) treated in our hospital between June, 2019 to October, 2021. All the participants signed the informed consent form by themselves. Res group included: (1) Aged>18, (2) Conforming to the clinical manifestations of CC, with CC diagnosis by pathological biopsy and confirmed persistent infection of high-risk HPV, (3) Complete case data and (4) Pathological stage I-II CC. Patients with multiple tumours and other diseases and Pregnant and lactating patients were also excluded. Con group included patients who were confirmed with persistent infection of high-risk HPV, but no CC.

Cell culture and treatment: Siha and Hela, both human cervical cancer cells supplied by ATCC, were cultivated in DMEM medium +10% fetal bovine serum (FBS) adding 1% penicillin/streptomycin mixture in a 37, 5% CO_2 incubator. When the cells density was near to 70-80%, they were digested with trypsin for 1 min and then cultivated in the medium changed according to the ratio of 1:3 for passage every 2 days. The CC cells were spread on 12-well plates with 1×10^5 cells/well, followed by 48 hrs of transfection with miR-587-mimics, miR-587-in inhibition, miR-587-NC, si-BCL2A, as well as NC-BCL2A1, respectively. The success rate of transfection was verified by qRT-PCR.

qRT-PCR: Invitrogen TRIzol-isolated total RNA was subjected to reverse transcription into cDNA for amplification by PCR under the reaction condition. The expression levels relative to U6 were calculated using the $2^{-\Delta\Delta CT}$ method.

MTT assay: Cells were seeded into 96-well plates with 1×10^5 cells/well and MTT solution was added with 20 µL/well at 0, 24, 48 and 72 hrs, respectively. The supernatant was discarded in 4 hrs and Dimethyl Sulfoxide (DMSO) was added at 150 µL/well. A microplate reader was used to measure the absorbance at a wavelength of 490 nm.

Transwell experiment: The transfected cells were inoculated into the upper chamber of the Transwell chamber and the 10% FBS-containing culture medium was placed into the lower one. After 24 hrs, the transmembrane cells were wiped off with cotton swabs, washed with PBS and stained. Counting the cells under the light microscope.

Wound-healing assay: Cells digested by trypsin were inoculated into 6-well plates at a density of 1×10^6 cells/well and cultured till 90% confluence. About 200 µL pipette tip was used to make scratches from top to bottom, followed by another 24 hrs of cultivation. The moving distance of the leading edge of the cells was measured with an inverted microscope.

Western blotting: The lysed cells were immersed in buffer solution and boiled in boiling water for 10 min. After membrane transfer by electrophoresis, it was blocked with 5% defatted milk, washed and mixed with antibodies (1:1000) for overnight sealing (4). After the addition of the secondary antibody (1:5000), the membrane was processed for development.

Dual-luciferase reporter (DLR) assay: Luciferase reporter vectors BCL2A1-wild type (WT) and BCL2A1-mutant (MUT) were co-transfected with miR-587-mimics into CC cells, respectively and fresh culture medium was replaced 5 hrs later. The fluorescence intensity detected 48 hrs later reflected the binding force of miR-587 to BCL2A1.

Statistical processing: Quantitative materials were recorded by Mean±Standard Deviation (c±s) and categorical materials were described in the form of percentages (n (%)). The statistical analysis was completed by SPSS22.0 software. Independent samples t-test and Chi-square test were used for inter-group comparisons and one-way ANOVA and LSD post hoc test were used for inter-group comparisons. The predictive value was analyzed by ROC curves. The significance level was set at $p<0.05$.

RESULTS

miR-587 expression: The peripheral blood miR-587 was 2.04±0.60 in the Research group, higher than the control group (Fig. 1a). The ROC analysis revealed that when miR-587 was greater than 1.0, the sensitivity and specificity for predicting the occurrence of CC in HPV infected population were 63.41 and 87.76%, respectively (Fig. 1b).

Expression miR-587 in CC cells: By qRT-PCR detection, it is found that the miR-587 expression levels in SiHa and Hela cells were 1.54±0.07 and 1.53±0.13, respectively in the miR-587-mimics group. While the expression of the miR-587-inhibition group in both cells was 0.45±0.04 and 0.37±0.05, which was lower than the expression level of miR-587 in the miR-587-NC group (Fig. 2a), indicating successful transfection.

Subsequently, the MTT experiment showed that the cell proliferation of the miR-587-mimics group in SiHa cells was higher than the other two groups, while the cell proliferation ability in the miR-587-inhibition group was 0.41±0.05, which was lower than the miR-587-NC group (Fig. 2b), while the proliferation ability of miR-587-mimics group in Hela cells was higher than the other two groups (Fig. 2c). The results of the Transwell experiment are shown in Fig. 2d. The number of cell invasions in the miR-587-mimics group was 166.33±11.15, which were higher than the other two groups, while cell invasions number in the miR-587-inhibition group were lower than the miR-587-NC group cell invasion numbers (Fig. 2e). The results of the cell scratch experiment are shown in Fig. 2f. The miR-587-mimics group's cell migration distances were higher than the other two groups, while the

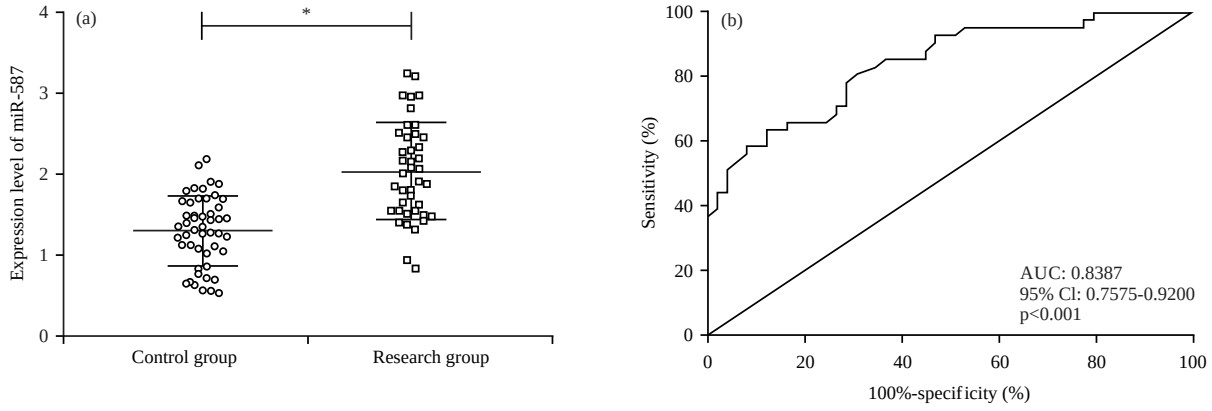

Fig. 1(a-b): Expression level of miR-587, (a) Expression level of miR-587 in the observation group and the control group and (b) ROC curve of miR-587 predicting the occurrence of CC in HPV-infected patients

*p<0.05

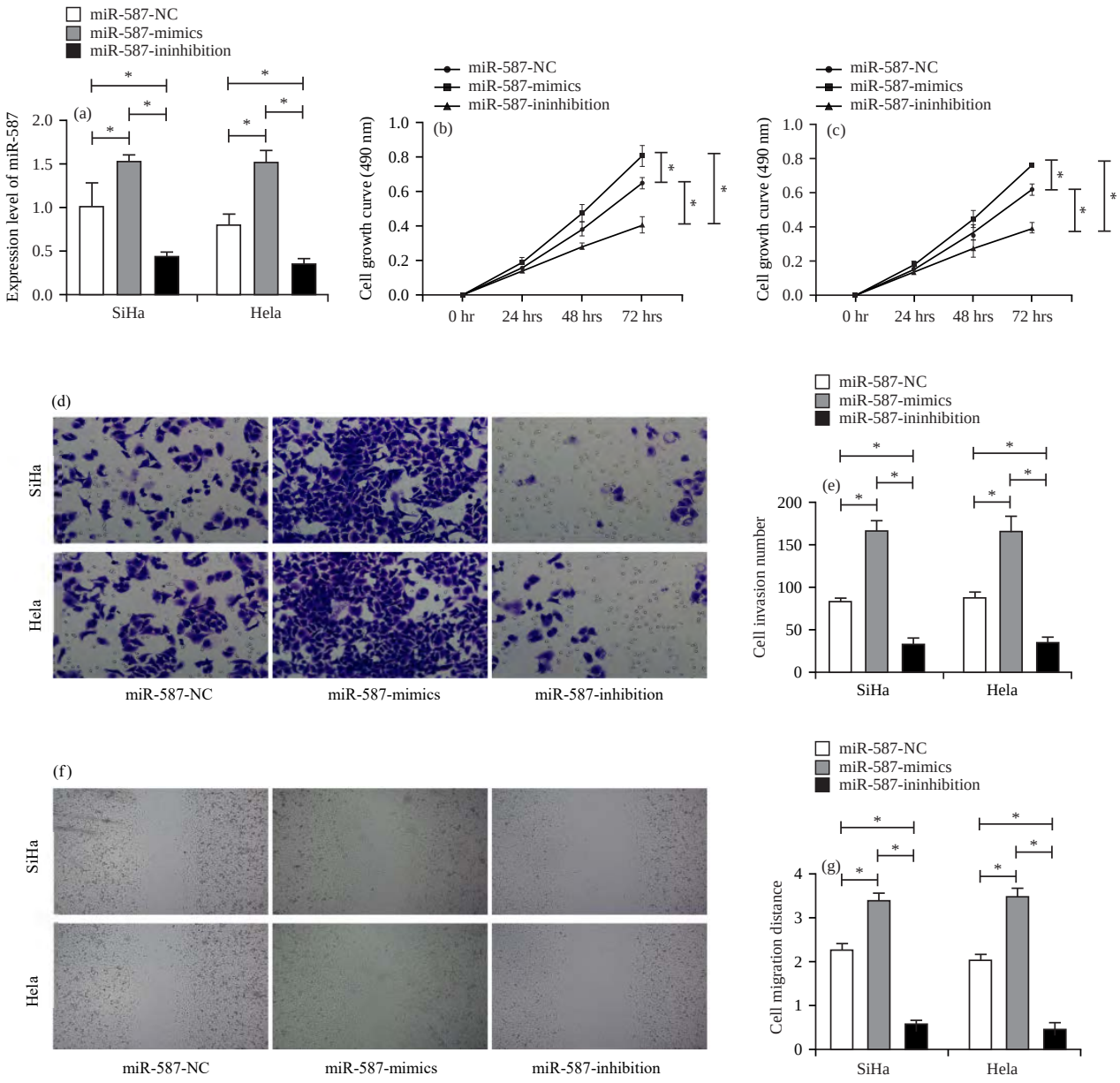

Fig. 2(a-g): Effect of miR-587 on CC cells, (a) qRT-PCR verified the success rate of transfection, (b) SiHa cell growth curve, (c) Hela cell growth curve, (d) Staining of penetrating cells in Transwell experiment, (e) Number of cell invasion, (f) Cell migration in the cell scratch experiment and (g) Cell migration distance

*p<0.05

miR-587-inhibition group were lower than the miR-587-NC group (Fig. 2g). It can be found that increasing the expression of miR-587 in CC cells can promote cell proliferation, invasion and migration while silencing the expression of miR-587 raised the opposite effect.

Affection of BCL2A1 on CC cells: Firstly, the protein expression of BCL2A1 in CC cells was detected by Western blot (Fig. 3a, b). The relative expression of BCL2A1 protein in the si-BCL2A1 group was 0.34±0.07. (Fig. 3c) confirming the successful transfection. Secondly, the proliferation of the si-BCL2A1 group in SiHa cells was 0.81±0.04, which was higher than that in the NC-BCL2A1 group (Fig. 3d), while in Hela cells it was higher than that of the NC-BCL2A1 group (Fig. 3e). The results of the Transwell experiment were shown in Fig. 3f. The cell invasion numbers in the si-BCL2A1 group were higher than

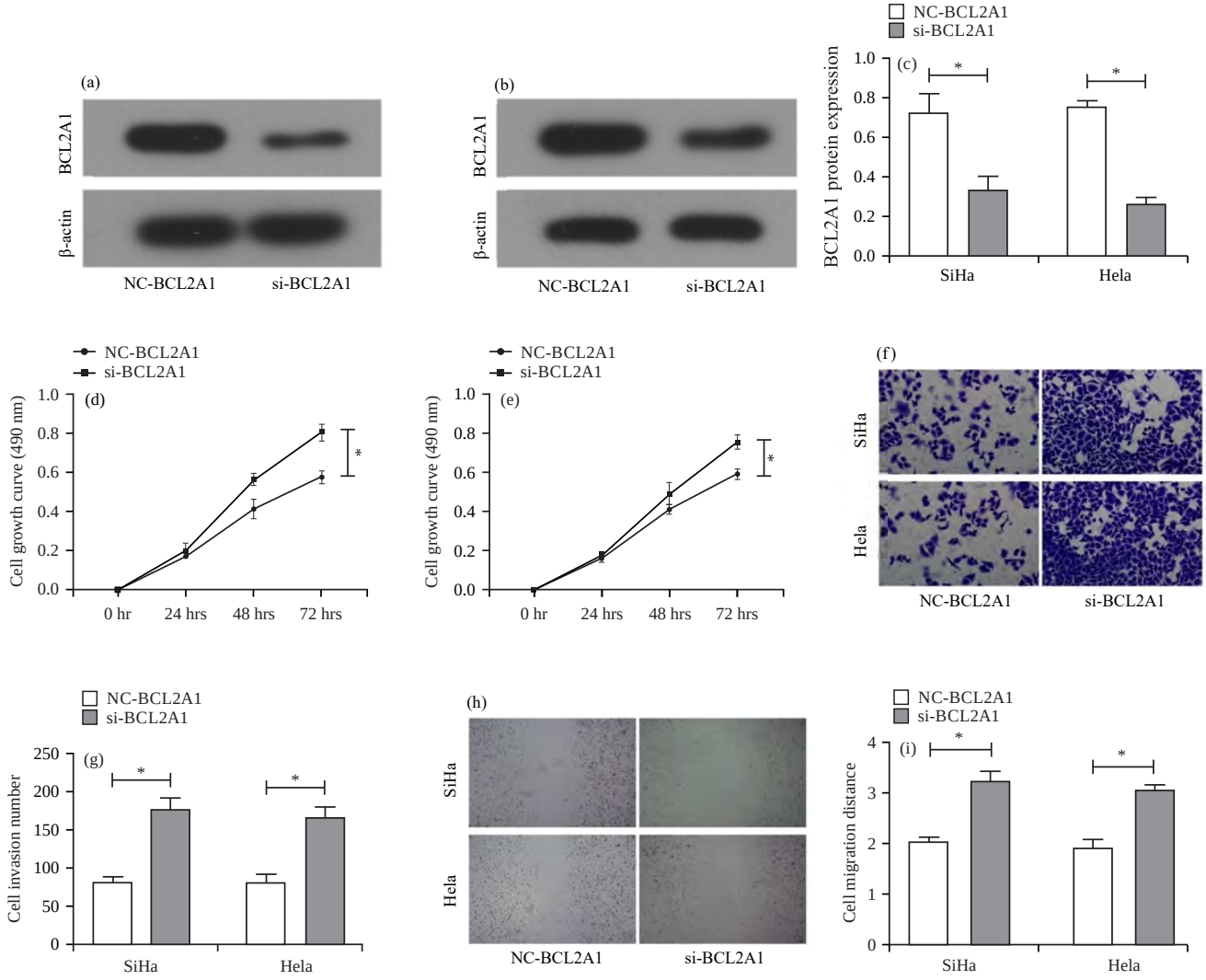

Fig. 3(a-i): Effect of BCL2A1 on CC cells, (a) Western blot map of SiHa cells, (b) Western blot map of Hela cells, (c) Western blot verified the success rate of transfection, (d) Growth curve of SiHa cells, (e) Growth curve of Hela cells, (f) Staining of penetrating cells in Transwell experiment, (g) Cell invasion number, (h) Cell migration in the cell scratch experiment and (i) Cell migration distance, the cell migration ability of si-BCL2A1 group is higher than that of NC-BCL2A1 group
*p<0.05

the NC-BCL2A1 group (Fig. 3g). The results of the cell scratch experiment were shown in Fig. 3h. The migration distances of the cells in the si-BCL2A1 group were higher than the NC-BCL2A1 group (Fig. 3i). It can be found that interfering with the expression of BCL2A1 in CC cells can promote cell proliferation, invasion and migration.

Connection between miR-587 with BCL2A1: In the Target Scan online website, we found that miR-587 and BCL2A1 have complementary sites that can bind (Fig. 4a). Subsequently, we found that the BCL2A1 mRNA level in the miR-587-mimics group was lower than the other two groups, while the BCL2A1 mRNA level in the miR-587-inhibition group was higher than the levels in the miR-587-NC group (Fig. 4b). The dual-luciferase reporter experiment showed that the fluorescence activity of BCL2A1-WT decreased to 0.44±0.03 after miR-587-mimics was transfected, indicating that BCL2A1 was targeted by miR-587 (Fig. 4c).

Rescue assay: In the MTT experiment, the proliferation ability of SiHa cells in the miR-587-inhibition+si-BCL2A1 group was 0.64±0.05, which was no different from the miR-587-NC group 0.67±0.02 and was higher than miR-587-Cell proliferation ability in the inhibition group (Fig. 5a). The cell

proliferation ability of the miR-587-inhibition+si-BCL2A1 group of Hela cells was no different from the NC group and was higher than that of the miR-587-inhibition group (Fig. 5b). The results of the Transwell experiment are shown in Fig. 5c. The cell invasion numbers in the miR-587-inhibition+si-BCL2A1 group were higher than in the NC group (Fig. 5d). The results of the cell scratch experiment are shown in Fig. 5e. The miR-587-inhibition+si-BCL2A1 group cell migration distances were 2.21±0.11 and 2.17±0.20 respectively and the miR-587-NC group cells migrated with no difference in the distance, which were higher than the NC group (Fig. 5f). It can be found that the effect of silencing miR-587 on CC cells can be completely reversed by interfering with the expression of BCL2A1.

(a)

	Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type
Position 142-149 of BCL2A1 3' UTR	5' ...AAUUUUUCUGACUGAUAUGGAAA...	8mer
hsa-miR-587	3' CACUGAGUAGUGGAUACCUUU	
Position 217-224 of BCL2A1 3' UTR	5' ...UUGACCUUCCAGAGUUAUGGAAA...	8mer
hsa-miR-587	3' CACUGAGUAGUGGAUACCUUU	

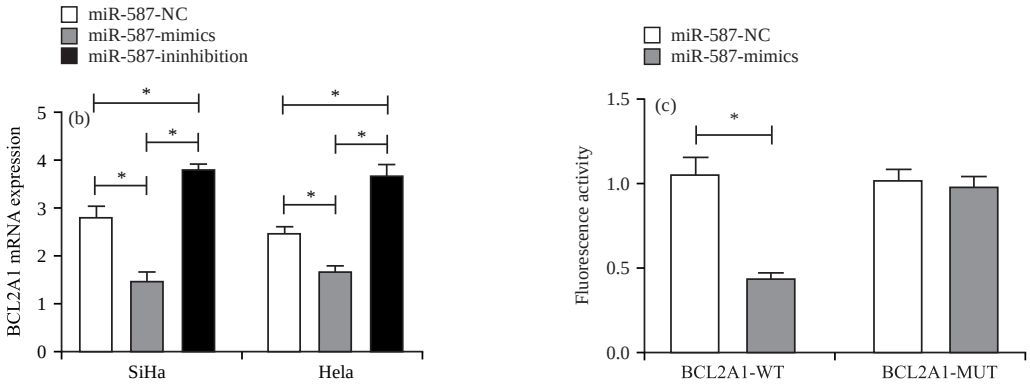

Fig. 4(a-c): Relationship between miR-587 and BCL2A1, (a) Online target gene prediction website analyzes the binding complementary sites of miR-587 and BCL2A1, (b) Effect of miR-587 on the expression level of BCL2A1 in CC cells and (c) Fluorescence activity of dual luciferin reporter enzyme experiment

*p<0.05

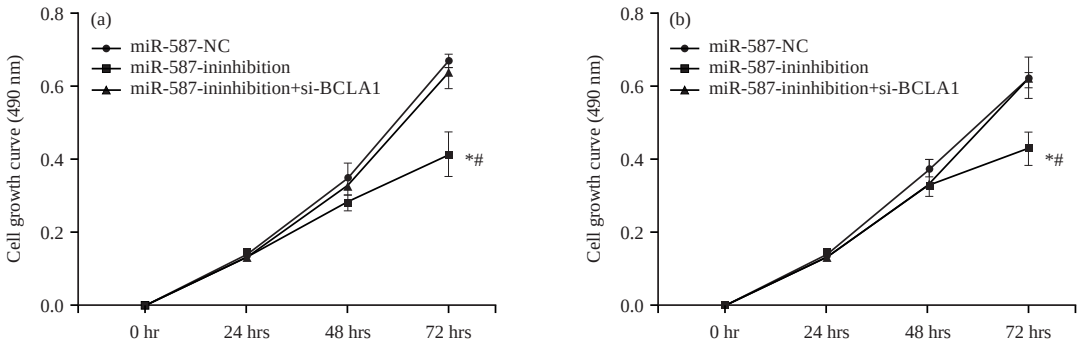

Fig. 5(a-f): Continue

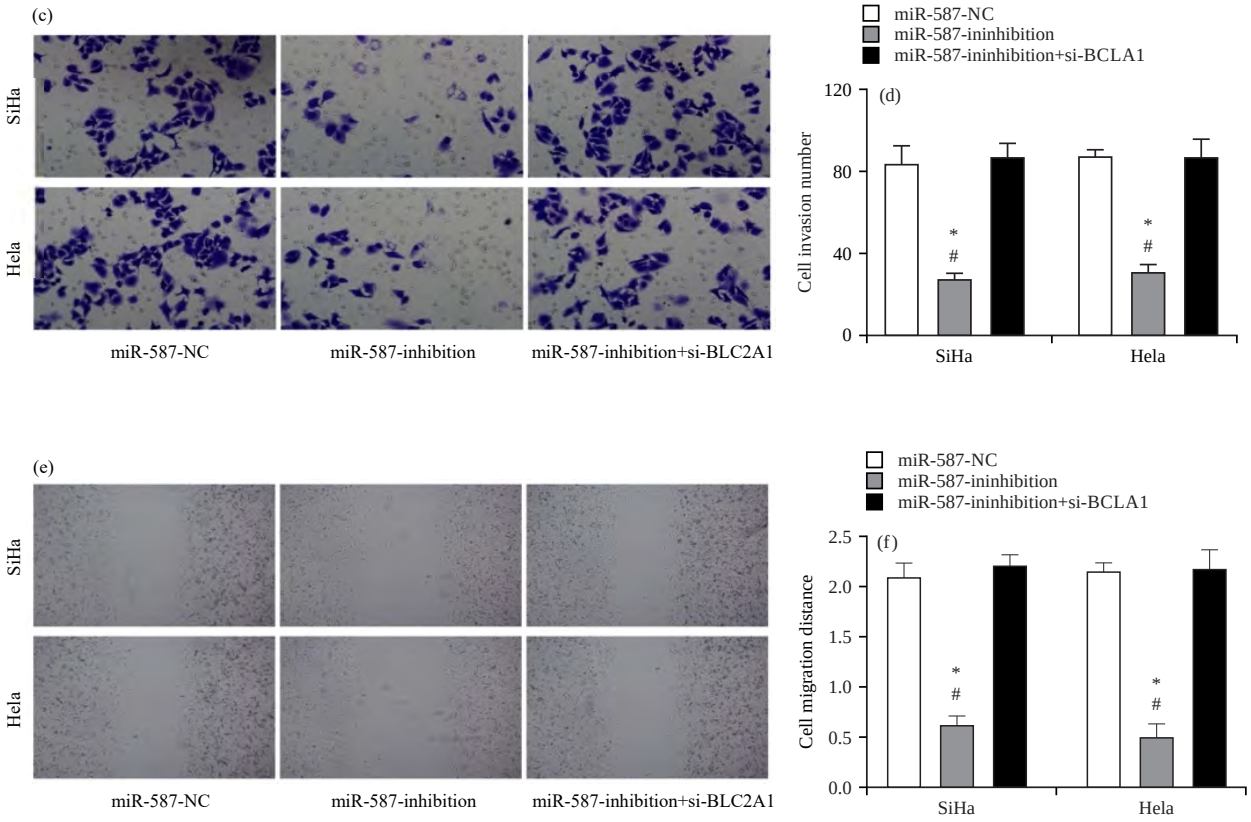

Fig. 5(a-f): Salvage experiments verify the regulatory relationship between miR-587 and BCL2A1, (a) Growth curve of SiHa cells, (b) Growth curve of Hela cells, (c) Staining of transmembrane cells in the Transwell experiment, (d) Cell invasion number, (e) Cell migration in the cell scratch experiment and (f) Cell migration distance

*$p<0.05$ and #$p<0.05$

DISCUSSION

In this study, we discussed the clinical implications and mechanism of miR-587 in CC, which has important reference significance for clinical practice. Firstly, we detected miR-587 expression in CC. The results revealed higher levels in CC patients than in HPV-infected patients, indicating that miR-587 was abnormally highly expressed in CC, which agreed with the previous studies and could support our experimental results[11,12]. The connection between miR-587 and HPV infection has been well demonstrated. As HPV is one of the most critical predisposing factors of CC, there must be some potential connection between miR-587 and CC. However, not all HPV-infected people will develop CC. Therefore, exploring the molecular changes in the process of HPV infection to CC may be a breakthrough in finding a new diagnosis and treatment scheme for CC. In addition, through ROC analysis, we confirmed that miR-587 has an excellent prediction effect for the occurrence of CC in HPV infected population, which further confirmed the significance of miR-587 in clinical detection in the future. Compared with the current diagnosis schemes (tumour markers, imaging techniques, etc.) of CC with multiple limitations, the use of miR-587 not only greatly increases the objectivity of CC diagnosis, but also enhanced the diagnostic specificity. Moreover, the method of using blood samples as detection objects allows for a wide range of clinical screening, which can greatly improve the possibility of early diagnosis of CC, thus ensuring the life safety and outcomes of patients.

Subsequently, to further understand the mechanism of miR-587 in CC, we conducted *in vitro* experiments to confirm its impact on CC cells. The experimental results showed that in CC cells transfected with miR-587 mimic sequence, the multiplication, invasion and migration were enhanced, while in those transfected with miR-587 inhibitor sequence, the opposite results were obtained. This indicates that miR-587, which is up-regulated in CC, plays a role in the oncogenic gene, which is consistent with the results of previous studies[13].

Whereas, further exploration of the downstream signal transduction pathway of miR-587 is warranted to understand its mechanism in CC. It is shown that BCL2A1 had a potential connection with miR-587[14] and in CC, BCL2A1 has also been proved to have important clinical implications[15]. Moreover, BCL2A1 was found when Bao screened the downstream genes of miR-587[16].

Then, through the online target gene prediction website, we confirmed the existence of binding complementary loci between miR-587 and BCL2A1. Therefore, we preliminarily speculated that the impact of miR-587 on CC may be related to BCL2A1. For confirmation, we also tested the influence of BCL2A1 on CC cells and the results identified enhanced CC cell multiplication, invasion and migration after inhibiting BCL2A1, which indicated that down-regulated BCL2A1 could also promote the pathological development of CC. In CC cells transfected with different sequences of miR-587, BCL2A1 was found to be reduced in the miR-587-mimics group while rose in the miR-587-in inhibition group, indicating that miR-587 can inhibit BCL2A1, which can also be confirmed by the results of the above experiments. Furthermore, we found that the fluorescence activity of BCL2A1-WT was inhibited after miR-587-mimics transfection through the DLR assay, suggesting the presence of a targeted regulation relationship between them. Thus, it is clear that miR-587 promotes the multiplication, invasion and migration of CC through targeted inhibition of BCL2A1.

Finally, in the rescue assay, we found that the impact of miR-587 inhibition on CC cells was completely reversed by the inhibition of BCL2A1, which verified the targeting relationship between them, indicating that miR-587 participated in the occurrence and development of CC through targeting BCL2A1.

CONCLUSION

The miR-587 highly expressed in CC has a good predictive value for the occurrence of CC in HPV-infected populations; Moreover, it can accelerate CC cell multiplication, invasion and migration via targeted inhibition of BCL2A1, which may be a breakthrough in CC diagnosis and treatment in the future.

SIGNIFICANCE STATEMENT

This study discovers the miR-587 expressed in cervical cancer patients which will provide new ideas in the field of cervical cancer treatment. This study will help the researcher to uncover the critical areas of the development of gene treatment or prevent disease that many researchers were not able to explore. Thus new theory on miRNA with cervical cancer treatment may be arrived at.

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