

ORIGINAL ARTICLE

MIR31HG regulates the proliferation, migration and invasion of breast cancer by regulating the expression of POLDIP2

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Summary

Purpose: This study aimed to explore the role of MIR31HG and its molecular mechanism in breast cancer (BC).

Methods: The levels of MIR31HG in BC tissues and cell lines were detected. The correlations of MIR31HG expression level with clinical characteristics and prognosis of patients were analyzed. Then we detected the effects of MIR31HG on the proliferative, migrative and invasive abilities of BC cells. Subsequently, we detected the level of the predicted target POLDIP2 in BC. Furthermore, the effect of POLDIP2 on the malignant phenotype of MIR31HG-mediated BC was evaluated through recovery experiments.

Results: MIR31HG was aberrantly up-regulated in BC tissues and cell lines, and its level was in correlation with the patient's tumor diameter, tumor node metastasis (TNM) stage, lymph node metastasis as well as the overall survival rate. Besides, MIR31HG knockdown was able to inhibit the

proliferative ability, migration and invasion of BC cells. Besides, POLDIP2 was aberrantly up-regulated in BC tissues, and its expression level was in positive correlation with the level of MIR31HG. Besides, POLDIP2 overexpression could partially inhibit the proliferative, migrative and invasive abilities of BC cells. Long non-coding (lnc)RNA MIR31HG was aberrantly up-regulated in BC and its expression was associated with poor prognosis of BC patients. Additionally, the levels of MIR31HG and POLDIP2 were positively correlated.

Conclusions: The low expression of MIR31HG or POLDIP2 can inhibit the proliferative, migrative and invasive abilities of BC cells, which provides a new target for the diagnosis along with the treatment of BC.

Key words: breast cancer, MIR31HG, POLDIP2, cell proliferation, cell invasion

Introduction

Breast cancer (BC) is one of the most common malignant tumors worldwide. It is the fifth leading cause of death in females, and its incidence rate is increasing each year. According to statistics, there are about 1.67 million new cases of BC worldwide each year, and the number of deaths is about 520,000, accounting for 25.1% of new global cancer cases and 14.7% of female cancer deaths [1]. Current research shows that the occurrence of BC is related to genetic factors, immune disorders, hormonal disorders, smoking and other factors. Its

occurrence and development are closely related to the imbalance of the proportion of multiple tumor suppressor genes and cancer promoting factors, but its main pathogenesis is not yet clear [2]. Therefore, it is of great importance to explore the pathogenesis of BC and its related molecular mechanisms and find new therapeutic strategies.

Long-chain non-coding RNAs (lncRNAs) and miRNAs are genomic transcripts [3]. Although lncRNAs do not encode proteins, they play a variety of biological regulatory functions including epigenet-

ic regulation through their complex structure and huge variety [4], and participate in epigenetic regulation, cell differentiation and apoptosis [5]. In recent years, lncRNAs have also been shown to exert biological functions in various malignant tumors. For example, down-regulation of lncRNA MEG3 can promote the proliferation of gastric cancer [6]. Besides, down-regulation of lncRNA TP73-AS1 inhibits the proliferation of esophageal squamous carcinoma cells and induces apoptosis [7]. LncRNA LUCAT1 promotes the occurrence of malignant ovarian cancer by regulating the miR-612/HOXA13 pathway [8]. It has been reported that lncRNA participates in the occurrence, development, and even diagnosis and treatment of BC [9]. However, little has been shown about the function and molecular mechanism of lncRNA MIR31HG in BC.

POLDIP2 is a cofactor polymerase interacting proteins present on NADPH oxidase 4 (NOX4), which can interact with human DNA polymerase small subunit P50 and proliferating cell nuclear antigen (PCNA). Previous studies have shown that POLDIP2 interacts with DNA replication and repair, mitochondrial function and extension, and are related to downstream signals of cell adhesion receptors [10]. Previous studies have found that POLDIP2 plays a specific biological function in non-small cell lung cancer [11], but research of POLDIP2 in human malignant tumors, especially BC, is very limited [12]. Therefore, exploring the role of POLDIP2 in BC is very important.

It has been previously indicated that MIR31HG was aberrantly up-regulated in BC, but its biological function and molecular mechanism in BC have not been fully studied [13]. Therefore, we explored the regulatory effect of MIR31HG on BC through a series of *in vitro* experiments, hoping to provide new ideas and targets for the diagnosis as well as the treatment of BC.

Methods

Sample collection

In this study, 50 pairs of BC tissues and normal control ones were collected. All patients in this study had not received radiotherapy or chemotherapy before surgical treatment. All tissues were stored at -80°C. Tumor pathological classification and staging standards were implemented in accordance with the staging standards of the Union for International Cancer Control (UICC). All patients signed a written informed consent form, and this study was approved by the Ethics Committee of Yantai Yuhuangding Hospital.

Cell culture

Human BC cell lines (T47D, BT-474, SUM149-Luc, BT549) and a normal human breast cell line (MCF-10A)

were obtained from the American Center for Cellular Resources (Manassas, VA, USA). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) and 1% penicillin/streptomycin in a humidified incubator at 37°C and 5% CO₂.

Transfection

MIR31HG-specific small interfering RNA (siRNA) and its negative control (si-NC) were obtained from GeneChem (Shanghai, China). When the cell culture adhered to more than 60%, transfection was performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from tissues or cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and reversely transcribed into complementary DNA (cDNA) using PrimeScript RT reagent kit (TaKaRa, Tokyo, Japan). Real-time PCR analysis was performed using SYBR Green (TaKaRa, Tokyo, Japan). The primer sequences are listed below: (5'-3'): MIR31HG: F: TCC-CAGTTTCAGACCACC, R: CCAGGCTATGTCTTCTCTAT; POLDIP2: F: CAAAACAGAATGGAAAATATGAGACCGG, R: TGATTGATGCTCGTGACTGCCCA; U6: F: ATGGGGAAGGTGAAGGTCG, R: GGGGTCATTGATGGCAACAATA; glyceraldehyde 3-phosphate dehydrogenase (GAPDH): F: CTCGCTTCGGCAGCACA, R: ACGCTTCACGAATTTGCGT.

Cell counting kit-8 (CCK-8) experiment

The CCK-8 experiment was performed using the CCK8 kit (CCK-8; Sigma-Aldrich, St. Louis, MO, USA). Ten μ L of CCK8 reagent were added into cells at 0, 24, 48, 72 and 96 h, and then the absorbance of the cells at 450 nm was measured during these periods.

5-Ethynyl-2'-deoxyuridine (EdU) experiment

The cell proliferation ability was measured with EdU kit (RiboBio, Guangzhou, China). Cells were seeded into 96-well plates (3000 cells/well), and then 50 mM EdU solution was added to the medium. After 24 h, the cells were fixed with 4% formaldehyde and permeated with Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA). Afterwards, cells were incubated with EdU reaction mixture and counterstained with 4',6-diamidino-2-phenylindole (DAPI). Five random fields were selected and photographed under fluorescence microscope and the number of EdU positive cells was calculated.

Transwell experiment

The serum-free medium with diluted cells (4×10^5) were inoculated into the upper layer of the matrix gel-containing chamber, and 500 μ L of 10% FBS was placed as a chemical attractant in the lower compartment of the chamber. The transwell was incubated for indicated time, then the lower layer penetrating cells were collected, placed in methanol for 10 min for cell fixation and stained with crystal violet for 30 min. After washed with phosphate buffered saline (PBS), the penetrating cells were counted in 5 random fields (magnification: $\times 200$).

Statistics

SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) and GraphPad software (Version X; La Jolla, CA, USA) were used for statistical analyses of the data. Measurement data were expressed as mean \pm standard deviation, and t-test was used for comparison between groups. The comparison between groups was performed by χ^2 test. $P < 0.05$ showed statistically significant difference.

Results

Abnormally high expression of MIR31HG in BC

In this study, we first detected the level of MIR31HG in 50 pairs of BC tissues as well as normal control tissues by qRT-PCR and found that, compared with normal control tissues, MIR31HG expression was abnormally increased in BC (Figure 1A). Then we detected the relationship between

the level of MIR31HG and the tumor diameter of BC patients. As shown in Figure 1B, we found that the level of MIR31HG was higher in BC patients with tumor diameter ≥ 2 cm. Besides, the level of MIR31HG in high stage (III+IV) was markedly higher than that in low stage tumor (I+II) (Figure 1C). In addition, the results revealed that patients with a higher MIR31HG expression level were more likely to have lymph node metastasis (Figure 1D). Survival analysis suggested that high expression of MIR31HG may result in lower overall survival rate of BC patients. qRT-PCR analysis indicated that MIR31HG level was higher in BC cell lines (T47D, BT-474, SUM149-Luc, BT549) than in the normal breast cell line (MCF-10A) (Figure 1E). The above results suggested that MIR31HG was abnormally up-regulated in BC and was correlated with the prognosis of BC patients.

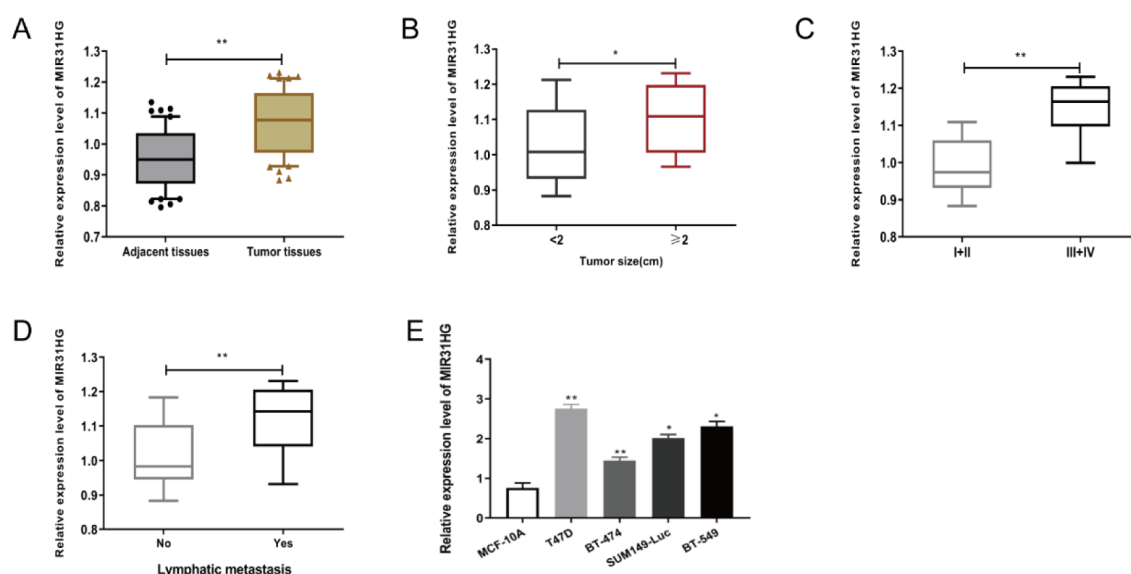


Figure 1. Abnormally high expression of MIR31HG in BC. **A:** qRT-PCR analysis of the level of MIR31HG in breast cancer tissues. **B:** The relationship between MIR31HG level and the tumor diameters. **C:** The relationship between of MIR31HG level and tumor stages. **D:** The relationship between MIR31HG level and lymph node metastasis. **E:** qRT-PCR analysis of the level of MIR31HG in breast cancer cell lines. * $p < 0.05$; ** $p < 0.01$.

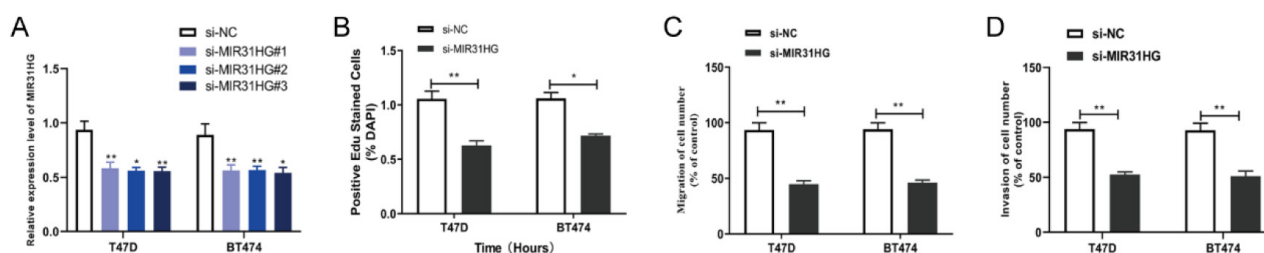


Figure 2. Knockdown of MIR31HG can inhibit the proliferative, migration and invasive ability of BC. **A:** qRT-PCR analysis of the level of MIR31HG after silencing MIR31HG. **B:** EdU experiment was used to detect the proliferation of T47D and BT-474 cells after transfection with si-MIR31HG. **C:** Transwell migration test was used to detect the migration ability of T47D and BT-474 cells after transfection with si-MIR31HG. **D:** Transwell invasion test was used to test the proliferation ability of T47D and BT-474 cells after transfection with si-MIR31HG. * $p < 0.05$; ** $p < 0.01$.

Knockdown of MIR31HG can inhibit the proliferative, migration and invasive ability of BC

To study the biological role of MIR31HG in BC, we knocked down MIR31HG by small interfering RNA (si-MIR31HG) and detected the transfection efficiency by qRT-PCR (Figure 2A). Then we detected the cell proliferation ability through the CCK8 experiment, which showed that the absorbance of BC cells transfected with si-MIR31HG at 450 nm wavelength was lower than that of si-NC. Subsequent EdU experiments revealed that the rate of EdU-positive BC cells transfected with si-MIR31HG was markedly lower than that of si-NC (Figure 2B). At the same time, we performed the Transwell experiment and found that the migration ability as well as invasion of T47D and BT-474 cells transfected with si-MIR31HG was markedly lower than that of si-NC (Figure 2C, 2D). These results indicated that knockdown of MIR31HG can inhibit the proliferative, migrative and invasive abilities of BC.

The expression levels of MIR31HG and POLDIP2 were negatively correlated

In order to study the underlying mechanism of MIR31HG in BC, we predicted the possible target of MIR31HG through bioinformatics website and found that POLDIP2 may be its downstream target gene. To verify this, we first detected the level of POLDIP2 in BC by qRT-PCR, and found that POLDIP2 level was abnormally increased in BC tissues (Figure 3A). Then we analyzed the correlation between the expression levels of MIR31HG and POLDIP2 by Pearson's method, and found that the levels of the two were in positive correlation. Subsequently, we transfected small interfering RNA (si-POLDIP2) against POLDIP2 in BC cells (Figure 3B). Later, the level of POLDIP2 in BC cells after silencing MIR31HG was detected, and it was discovered that the level of POLDIP2 was markedly inhibited (Figure 3C), whereas the level of MIR31HG was markedly reduced after POLDIP2 knockdown (Figure 3D). In addition, MIR31HG

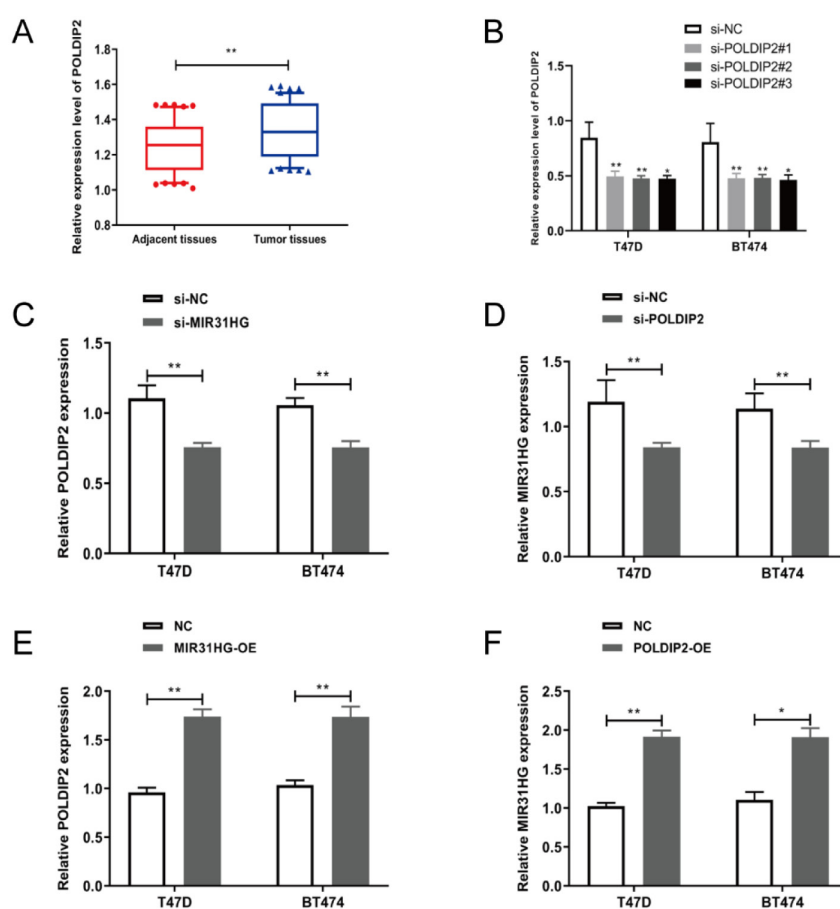


Figure 3. The expression levels of MIR31HG and POLDIP2 were negatively correlated. **A:** qRT-PCR analysis of the level of POLDIP2 in breast cancer tissues. **B:** qRT-PCR analysis was performed to verify the transfection efficiency of si-POLDIP2#1,#2,#3 in BC cells (T47D and BT-474). **C:** qRT-PCR analysis of the level of POLDIP2 expression level in breast cancer cells transfected with si-MIR31HG. **D:** qRT-PCR analysis of the level of MIR31HG in breast cancer cells transfected with si-POLDIP2. **E:** qRT-PCR analysis of the level of POLDIP2 in breast cancer cells transfected with MIR31HG-OE. **F:** qRT-PCR analysis of the level of MIR31HG in breast cancer cells transfected with POLDIP2-OE. *p<0.05; **p<0.01.

overexpression markedly increased the level of POLDIP2 (Figure 3E), while POLDIP2 overexpression in BC cells markedly reduced the expression of MIR31HG (Figure 3F). Therefore, the expression levels of MIR31HG and POLDIP2 were negatively correlated.

Knocking down POLDIP2 can inhibit the proliferative, migration and invasive ability of BC

We then conducted a series of functional tests to explore the biological role of POLDIP2 in BC. First, we tested the proliferative ability of BC cells through CCK8 and EDU experiments, which indicated that the proliferative ability of BC cells was markedly inhibited after knocking down POLDIP2 (Figure 4A). Subsequently, we conducted Transwell

experiments and found that POLDIP2 knockdown could markedly inhibit the migration ability as well as invasion of BC cells (Figure 4B, 4C). The above results demonstrated that POLDIP2 may regulate the proliferative, migrative and invasive abilities of BC.

Knockdown of MIR31HG can partially reverse the promotion effect of POLDIP2 overexpression on the proliferative, migration and invasive ability of BC

In order to further explore the relationship between MIR31HG and POLDIP2, we silenced MIR31HG in POLDIP2-overexpressed BC cells, and found that MIR31HG knockdown could decrease the level of POLDIP2 (Figure 5A). CCK8 and EdU experiments revealed that MIR31HG knockdown in

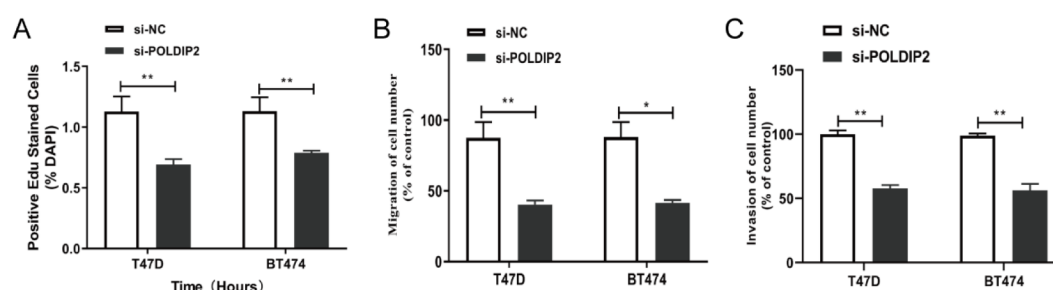


Figure 4. Knocking down POLDIP2 can inhibit the proliferative, migrative and invasive abilities of BC cells. **A:** EdU experiment was used to detect the proliferation of T47D and BT-474 cells after transfection with si-POLDIP2. **B:** Transwell migration test was used to detect the migration ability of T47D and BT-474 cells after transfection with si-POLDIP2. **C:** Transwell invasion test was used to detect the proliferation ability of T47D and BT-474 cells after transfection with si-POLDIP2. * $p < 0.05$; ** $p < 0.01$.

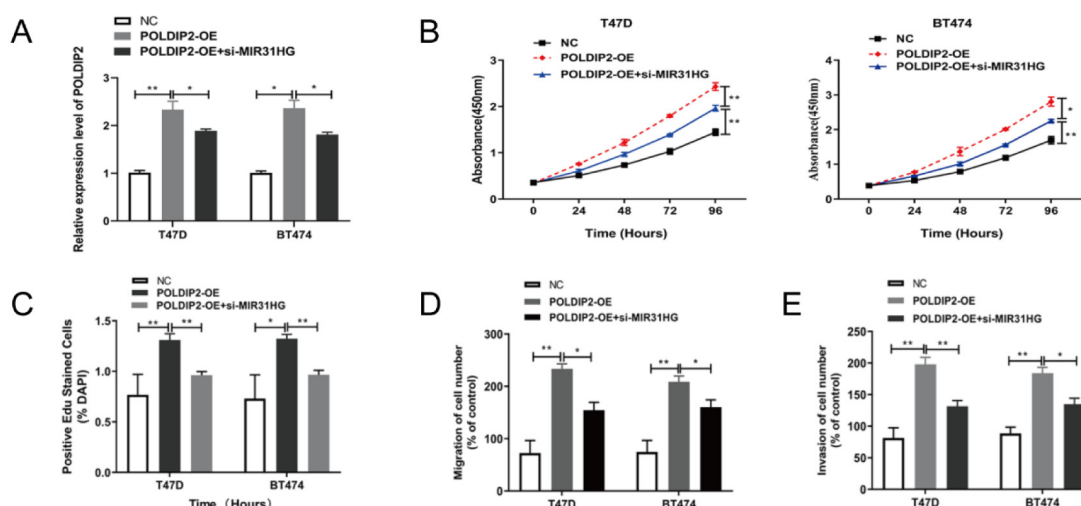


Figure 5. Knockdown of MIR31HG can partially reverse the promotion effect of POLDIP2 overexpression on the proliferative, migration and invasive ability of BC. **A:** QRT-PCR was used to detect the level of POLDIP2 in T47D and BT-474 cells after co-transfection of POLDIP2-OE and si-MIR31HG. **B:** The proliferative ability of T47D and BT-474 cells after co-transfection of POLDIP2-OE and si-MIR31HG was detected by CCK8 experiment. **C:** The proliferative ability of T47D and BT-474 cells after co-transfection of POLDIP2-OE and si-MIR31HG was detected by EdU experiment. **D:** Transwell migration test was used to detect the migration ability of T47D and BT-474 cells after co-transfection of POLDIP2-OE and si-MIR31HG. **E:** Transwell invasion analysis was used to detect the invasion ability of T47D and BT-474 cells after co-transfection of POLDIP2-OE and si-MIR31HG. * $p < 0.05$; ** $p < 0.01$.

BC cells partially inhibited the proliferative ability of cells caused by POLDIP2 overexpression (Figure 5B, 5C). In addition, Transwell experiment also indicated that transfection of si-MIR31HG in BC cells partially inhibited the migrative and invasive abilities of cells caused by POLDIP2 overexpression (Figure 5D, 5E).

Discussion

Breast cancer is one of the most common malignant tumors in women, and its incidence has increased in recent years [14]. Although clinical researchers and basic scientists have conducted extensive research on BC, techniques such as surgical resection, radioisotope therapy, chemical drug therapy, autoimmune cell therapy, and targeted therapy have also been successfully applied to the treatment of BC patients [15]. However, the prognosis of patients with BC is still poor due to metastasis to lung, liver, lymph nodes and other tissues [16]. Therefore, it is quite urgent to explore the underlying mechanism of BC and find new therapeutic targets.

MIR31HG is a type of lncRNA with a length of 2166 bp at 9p21.3. Studies have reported that MIR31HG exerts an important biological function in various human malignant tumors. For instance, MIR31HG regulates ST7L expression through sponging microRNA-575 to inhibit the proliferation and metastasis of liver cancer [17]; in lung adenocarcinoma, the aberrantly up-regulated MIR31HG can be used as a biomarker for poor prognosis and promoting cell proliferation [18,19]. Besides, the level of MIR31HG is related to the aggressive clinical pathological characteristics and poor prognosis of esophageal squamous cell carcinoma. It can be seen that MIR31HG plays an important biological function in a variety of human malignant tumors. To this end, we explored the biological function of MIR31HG in BC through a series of *in vitro* experiments and we found that aberrantly up-regulated MIR31HG was related to poor prognosis of BC patients. At the same time,

through CC8, EdU and Transwell experiments, we found that down-regulation of MIR31HG could inhibit the proliferative, migrative and invasive abilities of BC cells. Therefore, we speculated that MIR31HG may act as an oncogene in BC.

Previously, many lncRNAs have played an important biological function in BC. For example, LINC02273 promotes BC metastasis by increasing the expression of AGR220 epigenetically [20], lncRNA ITGB2-as1 promotes the migration ability as well as the invasion of BC cells by upregulating ITGB2 [21], and lncRNA NEAT1 promotes BC cell migration ability and invasion through silencing miR-133b [22]. However, the specific role of lncRNA MIR31HG has not been fully studied in BC yet.

Previous studies have shown that lncRNA and some specific mRNAs can form a regulatory network to regulate the progress of malignant tumors [23]. Here, we found through the bioinformatics website that the level of POLDIP2 was related to MIR31HG, and verified their mutual relationship through qRT-PCR and Pearson's analysis. The results demonstrated that the levels of MIR31HG and POLDIP2 were positively correlated. Besides, knock down of MIR31HG can partially reverse the promotion effect of POLDIP2 expression on the proliferative, migrative and invasive abilities of BC cells. In sum, MIR31HG can regulate the progress of BC by regulating the expression of POLDIP2.

Conclusions

In conclusion, lncRNA MIR31HG is aberrantly up-regulated in BC, and high expression of MIR31HG can lead to poor prognosis of BC patients. Knocking down MIR31HG can inhibit the proliferative, migrative and invasive abilities of BC cells through mediating POLDIP2. This study may provide a new target for the diagnosis along with the treatment of BC.

Conflict of interests

The authors declare no conflict of interests.

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