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Regular paper

LncRNA MIR31HG promotes cell proliferation and invasive properties of the MCF-7 cell line by regulation of receptor-interacting serine-threonine kinase 4

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LncRNA MIR31HG is involved in many types of cancers, while its roles in breast cancer are still unknown. The current study aimed to explore the function of lncRNA MIR31HG in breast cancer and the underlying mechanisms. Stable expression cell lines were constructed by using lentivirus particles. MTT assay was used to determine cell viability. Wound healing and Transwell assay were used to determine cell migration and invasion, respectively. The changes in biomarkers were determined by using qPR-PCT and Western blotting, respectively. BALB/c nude mice were used to generate a xenograft mouse model. MIR31HG regulated cell proliferation, migration and invasion in MCF7 cells. Besides, MIR31HG regulated N-Cadherin, Vimentin, and E-Cadherin. MIR31HG positively regulated receptor-interacting serine-threonine kinase 4 (RIPK4), as supported by the fact that knockdown of MIR31HG suppressed RIPK4, and the knockdown of RIPK4 did not affect MIR31HG. Additionally, we found that RIPK4 regulated cell proliferation, migration and invasion in MCF7 cells. The changes in RIPK4 regulated N-Cadherin, Vimentin, and E-Cadherin. Consistently, *in vivo* studies showed that the knockdown of MIR31HG or RIPK4 reduced tumor size in xenograft animal models. The roles of lncRNA MIR31HG in breast cancer were associated with its regulatory effects against RIPK4.

Keywords: Breast cancer, LncRNA, cell proliferation, MIR31HG, RIPK4

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Abbreviations: FBS, fetal bovine serum; lncRNAs, long non-coding RNAs; MIR31HG, microRNA-31 host gene; NF-κB, nuclear factor kappa B; RIPK4, receptor-interacting serine-threonine kinase 4; shNC, shRNAs; STAT3, signal transducer and activator of the transcription 3

INTRODUCTION

Breast cancer is known as the most common cancer in women, which accounts for about 12.5% of all cancer cases in the world (Ferlay *et al.*, 2021; Lei *et al.*, 2021). Approximately 2.3 million newly diagnosed breast cancer cases were reported with around 685,000 breast cancer-related deaths in 2020 (Ferlay *et al.*, 2021; Lei *et al.*, 2021; Lima *et al.*, 2021). Breast cancer can be divided into non-invasive or pre-invasive (also termed intraductal carcinoma) and invasive types, where the invasive type accounts for 70~80% of all cases (Matsuno *et al.*, 2007; Sharma *et al.*, 2010). In patients with early stage breast cancer, a 99% of 5-year survival rate can be achieved in patients with localized non-metastasis breast cancer with proper treatment (Page, 2003; Sharma *et al.*, 2010). However, breast cancer can spread to other tissues including liver, lung, bone, and brain tissues. When tumor metastasis occurs, the 5-year survival rate is only 29% (Matsuno *et al.*, 2007; Page, 2003). Therefore, diagnosis of breast cancer in the early stage is crucial for the treatment of breast cancer and the improvement of the survival rate.

The roles of long non-coding RNAs (lncRNAs) have been implicated in many types of cancers such as lung cancer, hepatoma, colorectal cancer, breast cancer, etc. (Fang & Fullwood, 2016; Hauptman & Glavač, 2013). LncRNAs are involved in a series of cellular events such as cell proliferation, migration and invasion, and genetic stability, in part, by the regulation of various genes (Fang & Fullwood, 2016; Spizzo *et al.*, 2012). Moreover, lncRNAs are also known as functional transcripts, which are associated with tumor progression and metastasis (Spizzo *et al.*, 2012; Zhang *et al.*, 2013). Targeting lncRNAs becomes a promising strategy for cancer therapy (Zhang *et al.*, 2013). LncRNA microRNA-31 host gene (MIR31HG) was identified as an oncogene in twelve different types of cancers including bladder cancer, head and neck cancer, osteosarcoma, cervical cancer, gastric cancer, etc. (Wei *et al.*, 2022). Besides, the changes of MIR31HG can also be used as a prognosis biomarker for patients with digestive system cancer (Zhou *et al.*, 2020). However, it is still unclear whether MIR31HG is involved in breast cancer. Therefore, this study aims to determine the role of MIR31HG in breast cancer and its molecular mechanisms.

A receptor-interacting serine-threonine kinase (RIPK) 4 is known to be associated with epidermal homeostasis and development by the regulation of cell-cell adhesion (Oberbeck *et al.*, 2019; Xu *et al.*, 2020). It also acts as a transcriptional target of TP63 and plays important roles in transcriptional factor- nuclear factor kappa B (NF-κB) activation (Liu *et al.*, 2018; Xu *et al.*, 2020). Recently, the roles of RIPK4 in cancer have drawn much attention from scientists (Li *et al.*, 2021; Liu *et al.*, 2015; Liu *et al.*, 2021). In 2015, Liu and colleagues have reported that the RIPK4 can be used as a prognostic and diagnostic biomarker for patients with cervical cancer (Liu *et al.*, 2015). In 2021, Li and colleagues have found that RIPK4 regulates cell migration and metastasis in hepatoma by the regulation of signal transducer and activator of the transcription 3 (STAT3) signaling pathway (Li *et al.*, 2021). However, the roles of RIPK4 are still unknown. Herein,

the roles of MIR31HG in breast cancer *in vitro* and *in vivo* were clarified in our study.

MATERIALS AND METHODS

Antibody and reagents

Antibodies against Vimentin, E-Cadherin, N-Cadherin, and GADPH were obtained from Cell Signaling Technology (Danvers, MA). Antibodies against RIPK4 and Ki67 were obtained from Thermo Fisher Scientific (Waltham, MA). Predesigned shRNAs targeting MIR31HG and RIPK4, control shRNAs (shNC), and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Cell culture and cell viability

Breast cancer cell line MCF7 was purchased from the ATCC (Rockville, MD). The cells were cultured in recommended medium with 10% fetal bovine serum (FBS) and penicillin/streptomycin (100 U/ml) under 37°C with 95% humidity and 5% CO_2. MTT assay was used to determine cell viability. In brief, MTT solution (5 mg/mL, 10 μL) was added and incubated for another 2 h. Next, DMSO (100 μL) was used to dissolve the purple formazan crystal followed by shaking for 30 sec. Next, the plate was read under a wavelength of 570nm with a reference wavelength of 630 nm.

Construction of stable cell lines

To construct stable MIR31HG and RIPK4 knockdown cell lines, MCF7 cells were transfected with MIR31HG shRNA (Targeting sequence: GCTGCTGATGACGTAAAGT), RIPK4 shRNA (Targeting sequence: CGTTCGTTTCTCGTTGCCTAA) or empty vector (shNC) lentivirus as previously described. After 72 h, a medium containing puromycin (2 μg/mL) was added and incubated for another 72 h for the selection of the cells. To construct MIR31HG and RIPK4 overexpressing cell lines, pcDNA MIR31HG, pcDNA RIPK4, or empty plasmid containing shNC sequence was used to construct pLKO.1 plasmid followed by the generation of lentivirus. Puromycin (2 μg/mL) was used for the selection of stable overexpressing cell lines.

Cell invasion and wound healing assays

Cell invasion assay was determined by using Transwell chamber. In the upper chamber, the cells were seeded and cultured with a serum-free medium. In the lower chamber, a medium containing 10% FBS was added. After 72 h, cells that were in the lower chamber were stained. Five random fields were selected, and the number of cells were counted. In the wound healing assay, cells were seeded into a 6-well plate. When 80–90% confluence was observed, a sterile tip (1 mm width, 200 μL) was used to scratch the cell monolayer. After 72 h, cells were observed by using a microscope, and wound healing rate was calculated. The Transwell chamber was purchased from BD (Franklin Lakes, NJ, USA).

Western blotting assay

Protein samples were extracted from the cells by using cold RIPA buffer (1% phenylmethanesulfonyl fluoride) and qualified by using BCA assay. Protein samples were loaded on a 10% SDS page followed by transferring onto the PVDF membrane. After that, the membrane was blotted with 5% non-fat milk for 1 h at room temperature. The primary antibodies were added and incubated overnight at 4°C. After that, the secondary antibodies conjugated with HRP were added and blotted for another 2 h at room temperature. A chemiluminescence substrate was added, and the protein intensity was compared to the internal control GADPH.

qRT-PCR

Total RNAs were extracted by using the phenol-chloroform method. In this study, the sequences of primers were described below.

TAP-1 forward primer: 5'-GCCCACTACCACGTCAAGAT-3', and reverse primer: 5'-TTCACCATG ATGTGCAGGAT-3'; MIR31HG forward primer: 5'-GTTTCTGGTCCTCATACCGTGTGGTT-3', and reverse primer: 5'-CTTGGAATGAATCCTCTGTCCTCC-3'; GADPH forward primer: 5'-TCAACGACCACTTTGTCAAGCTCA-3', and reverse primer: 5'-GCTGGTGGTCCAGGGGTCTTACT-3'. A reverse transcription reaction was then performed by using real-time PCR kits (Takara Bio Inc, San Jose, CA).

MCF7 Xenograft animal model

BALB/c nude mice (age 4~6 weeks) were purchased from GemPharmatech (Nanjing, China) and kept in specific pathogen-free facilities. In this study, animal procedures were approved by the First Affiliated Hospital of Bengbu Medical College. Mice were subcutaneously injected with MCF7 cells (MIR31HG-KD, MIR31HG-OE, or MCF7) at a density of 5×10^6 cells, and tumor size was measured once a week in accordance with a formula: tumor volume=length×width^2/2.

Data analysis

The results were represented as means ± standard deviation (S.D.). A significant difference was obtained by using GraphPad prism 7 based on either one-way or two-way ANOVA test followed with a post hoc test. When a p-value was smaller than 0.05, the statistical significance was decided.

RESULTS

MIR31HG regulated cell proliferation in MCF7 cells

Our results showed an elevation of MIR31HG in MIR31HG-OE cells and a reduction of MIR31HG in the MIR31HG-KD cells, indicating the successful construction of MIR31HG stable cell lines (Fig. 1A). MTT assay showed that cell proliferation was significantly increased in the MIR31HG-KD cells, whereas cell proliferation was diminished in the MIR31HG-OE cells (Fig. 1B). Consistently, we found that Ki67 decreased in the MIR31HG-KD cells. Interestingly, the protein expressions of Ki67 were enhanced in the MIR31HG-OE cells (Fig. 1C).

MIR31HG regulated wound healing and cell invasion

The effects of MIR31HG on cell migration and invasion were then determined. We observed a decrease in the closure rate and a reduction in the invaded cell numbers in the MIR31HG-KD cells, whereas an elevation in closure rate and invaded cell numbers was observed in the MIR31HG-OE cells (Fig. 2A and B). Additionally, overexpression of MIR31HG enhanced the protein ex-

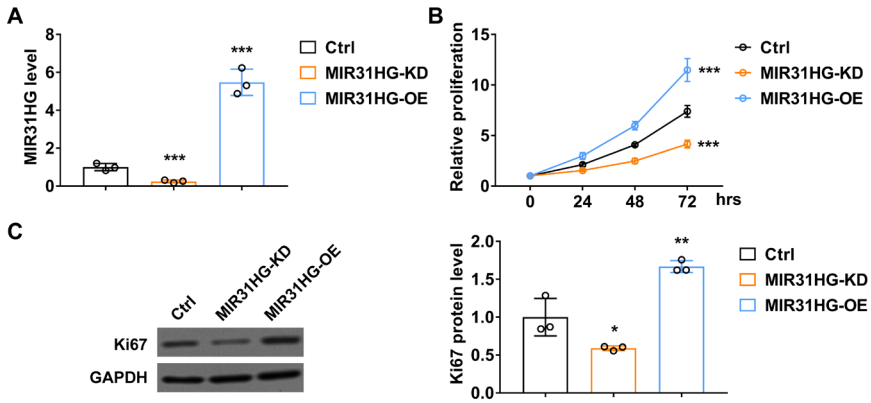

Figure 1. MIR31HG regulated the proliferation of MCF7 cells.
(**A**) qRT-PCR was used to determine the mRNA levels of MIR31HG in control (Ctrl), MIR31HG knockdown (KD), and MIR31HG overexpressing (OE) MCF7 cells. (**B**) MTT assay was used to determine the cell proliferation in control, MIR31HG-KD, and MIR31HG-OE MCF7 cells. (**C**) Western blotting was used to determine the protein levels of Ki67 in control, MIR31HG-KD, and MIR31HG-OE MCF7 cells (n=3).

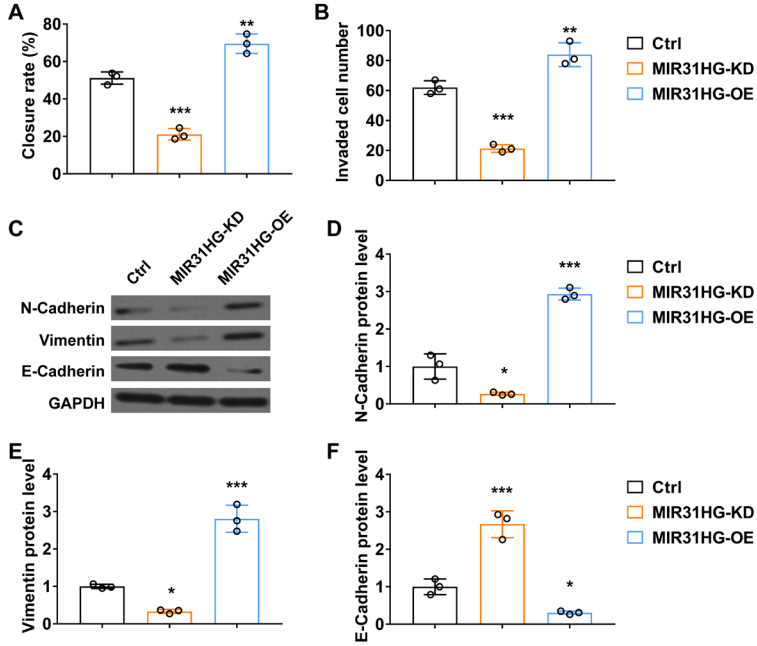

Figure 2. MIR31HG regulated the migration and invasion of MCF7 cells.
(**A**) A wound healing assay was used to determine the closure rate of control, MIR31HG-KD, and MIR31HG-OE MCF7 cells. (**B**) The invasion assay was used to determine the numbers of invaded cells in control, MIR31HG-KD, and MIR31HG-OE MCF7 cells. (**C–F**) Western blotting was used to determine the protein levels of EMT-related markers including N-Cadherin (**C and D**), Vimentin (**C and E**), and E-Cadherin (**C and F**) in control, MIR31HG-KD, and MIR31HG-OE MCF7 cells (n=3).

pressions of N-Cadherin and Vimentin, whereas knockdown of MIR31HG suppressed the protein expressions of N-Cadherin and Vimentin in MCF7 cells (Fig. 2C–E). Moreover, overexpression of MIR31HG suppressed the protein expressions of E-Cadherin, whereas knockdown of MIR31HG enhanced the protein expressions of E-Cadherin (Fig. 2C and F).

MIR31HG regulated the levels of RIPK4

We further determined the impact of MIR31HG on RIPK4 in MCF7 cells. An elevation of RIPK4 in the MIR31HG-KD cells, and a reduction of RIPK4 were observed in the MIR31HG-OE cells (Fig. 3A and B). Next, we successfully constructed RIPK-KO and RIPK-OE cells (Fig. 3D). Interestingly, no significant change of MIR31HG was found among RIPK-KO, RIPK-OE, and control MCF7 cells (Fig. 3C). We also found that a reduction in RIPK4 in the RIPK-KO cells, whereas an elevation of RIPK4 was observed in the MIR31HG-OE cells both at the mRNA and protein levels (Fig. 3D and E). These results indicate that MIR31HG regulated the levels of RIPK4 in MCF7 cells.

RIPK4 mediated cell proliferation in breast cancer cells

We determined the effects of RIPK4 on cell proliferation. Our results showed that cell proliferation was enhanced in the MIR31HG-KD cells as compared to the MIR31HG-OE cells (Fig. 4A). Consistently, we found that Ki67 decreased in the MIR31HG-KD cells. Interestingly, Ki67 increased in the MIR31HG-OE cells (Fig. 4B).

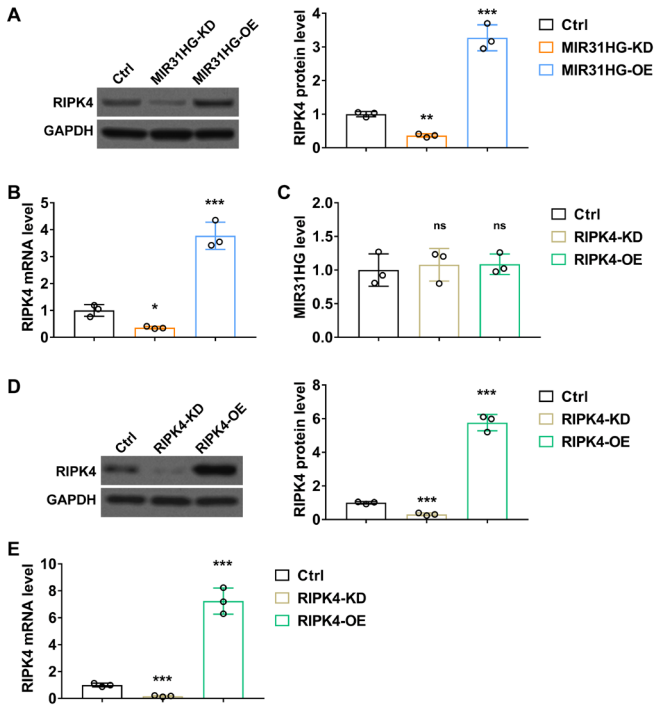

Figure 3. MIR31HG regulated the levels of RIPK4 in MCF7 cells.
(**A–B**) Western blotting and qRT-PCR were used to determine the protein (**A**) and mRNA (**B**) levels of RIPK4 in control, MIR31HG-KD, and MIR31HG-OE MCF7 cells. Besides, (**C**) the mRNA levels of MIR31HG were determined in control, RIPK4 knockdown (KD), and RIPK4 overexpressing (OE) MCF7 cells. (**D–E**) The protein and mRNA levels of RIPK4 in control, RIPK4-KD, and RIPK4-OE MCF7 cells (n=3).

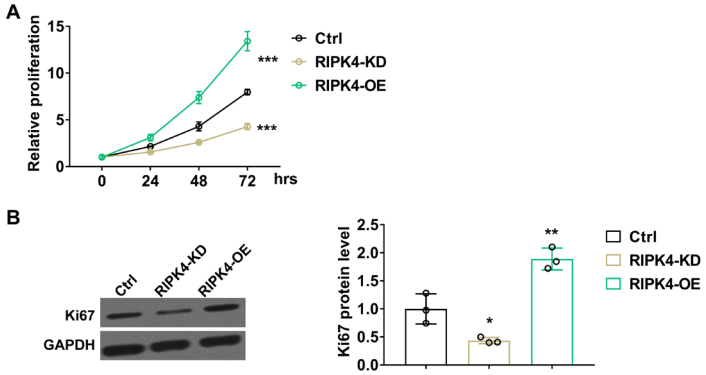

Figure 4. RIPK4 mediated the proliferation of breast cancer cells *in vitro*.
(**A**) MTT assay was used to determine cell viability of control, RIPK4-KD, and RIPK4-OE MCF7 cells (**B**) Western blotting was used to determine the protein levels of Ki67 in control, RIPK4-KD, and RIPK4-OE MCF7 cells (n=3).

RIPK4 regulated cell migration and invasion

Moreover, we investigated the impact of RIPK4 change on cell migration and invasion. A decrease in closure rate as well as a reduction in invaded cell numbers were observed in the RIPK4-KD cells, whereas both closure rate and invaded cell numbers were increased in RIPK4-OE cells (Fig. 5A and B). Additionally, overexpression of RIPK4 increased N-Cadherin and Vimentin, whereas knockdown of RIPK4 suppressed N-Cadherin and Vimentin (Fig. 5C-E). Moreover, overexpression of RIPK4 suppressed E-Cadherin, whereas knockdown of RIPK4 increased E-Cadherin (Fig. 5C and F).

Knockdown of MIR31HG or RIPK4 repressed MCF7 tumor growth in mice

To confirm the observations *in vitro*, we observed the effects of MIR31HG or RIPK4 knockdown on tumor growth in the animal cancer xenograft model. Interestingly, we found that tumor size was significantly reduced in the MIR31HG-KD and RIPK4-KD groups (Fig. 6A). The protein expressions of Ki67 were also significantly decreased in the MIR31HG-KD and RIPK4-KD groups (Fig. 6B). These results indicate that the knockdown MIR31HG or RIPK4 suppressed tumor growth *in vivo*. Next, we investigated the levels of Epithelial-to-mesenchymal transition (EMT)-related biomarkers. A reduction of N-Cadherin and Vimentin was observed in the MIR31HG-KD and RIPK4-KD group as compared to the control group (Fig. 6C–E). Besides, an elevation of E-Cadherin was observed in the MIR31HG-KD and RIPK4-KD group (Fig. 6F), suggesting the regulatory effects of MIR31HG and RIPK4 on EMT.

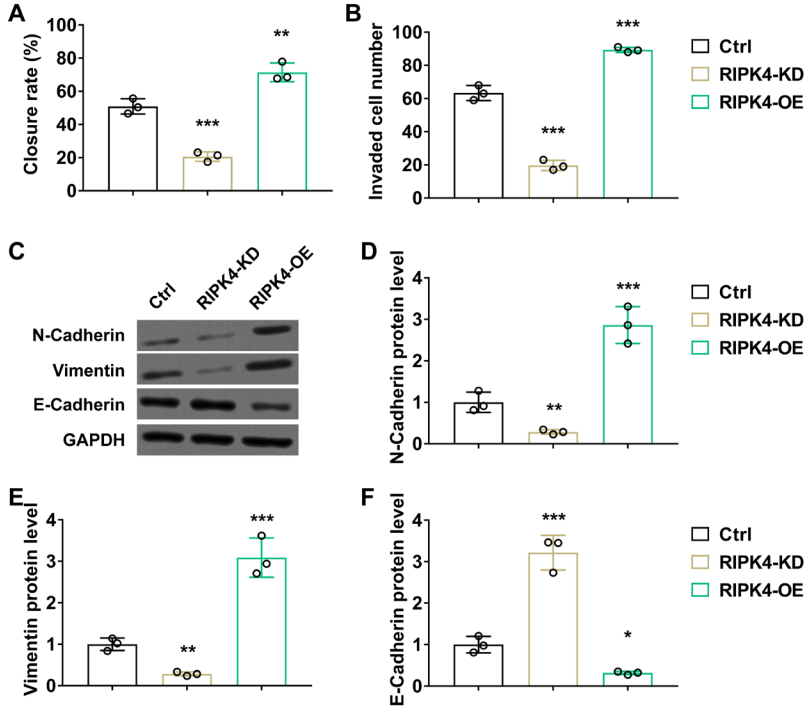

Figure 5. RIPK4 regulated the migration and invasion of MCF7 cells.
(**A**) A wound healing assay was used to determine the closure rate of control, RIPK4-KD, and RIPK4-OE MCF7 cells. (**B**) Invasion assay was used to count the numbers of the invaded cells of control, RIPK4-KD, and RIPK4-OE MCF7 cells. (**C–F**) Western blotting was used to determine the protein levels of EMT-related markers including N-Cadherin (**C and D**), Vimentin (**C and E**), and E-Cadherin (**C and F**) in control, RIPK4-KD and RIPK4-OE MCF7 cells (n=3).

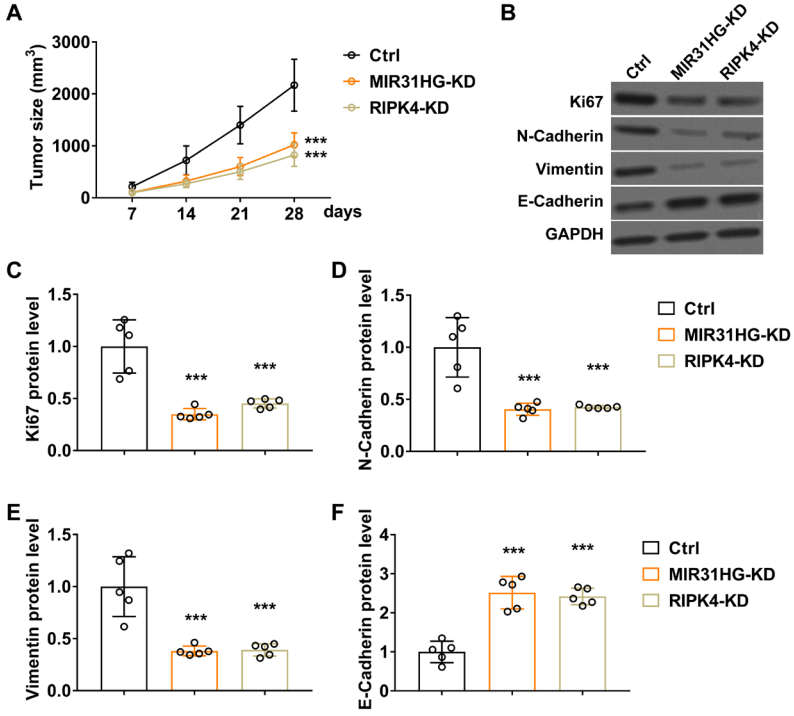

Figure 6. Knockdown of MIR31HG or RIPK4 repressed MCF7 tumor growth in mice.
(**A**) Tumor size was measured in the xenograft mice of the control, MIR31HG-KD, and RIPK4-KD MCF7 group. (**B–**F) Western blotting was used to determine the protein levels of Ki67 (**C**), N-Cadherin (**D**), Vimentin (**E**), and E-Cadherin (**F**; n=5 mice).

DISCUSSION

This study revealed the roles of MIR31HG in the MCF7 breast cancer cell lines and breast cancer xenograft mouse model. MIR31HG promoted cancer growth by regulating a series of cellular events including cell proliferation, migration and invasion. Additionally, the roles of MIR31HG in breast cancer were associated

with its regulatory effects against EMT, as suggested by the changes in N-Cadherin, Vimentin, and E-Cadherin that were observed in these MIR31HG overexpressing or knockdown cell lines. Underlying mechanism studies identified RIPK4 as a downstream target of MIR31HG, as supported by MIR31HG positively regulating the levels of RIPK4, whereas knockdown of RIPK4 did not significantly affect MIR31HG. These results suggested that MIR31HG might serve as an oncogenic gene in breast cancer by its regulatory effects against RIPK4.

MIR31HG is involved in cancer occurrence and progression (Wei *et al*., 2022). Moreover, the alteration of MIR31HG is observed in twenty types of cancers (Wei *et al*., 2022). For instance, Zheng and colleagues reported that MIR31HG promotes cell proliferation and invasion in lung cancer (Zheng *et al*., 2019). The downregulation of MIR31HG is associated with the alternation of EMT in non-small cell lung cancer. In another study, the overexpression of MIR31HG is associated with a low overall survival rates and advanced tumor, node, and metastasis stage in patients with bladder cancer (Wu *et al*., 2019). More recently, Ko and colleagues demonstrated that the overexpression of MIR31HG is associated with poor disease-free survival rate in patients with pancreatic ductal adenocarcinoma (Ko *et al*., 2022). These results suggested the oncogenic roles of MIR31HG in cancer. However, it is still unclear whether MIR31HG promoted tumor growth in breast cancer. Herein, our study reported the roles of MIR31HG in breast cancer. Consistently, our results revealed that the overexpression of MIR31HG promoted cancer cell growth and invasion in MCF7 cells as well as tumor growth in the cancer xenograft mouse model.

EMT is frequently observed in breast tumor invasion and metastasis (Ye *et al*., 2017). Moreover, the activation of EMT is linked with drug resistance in breast cancer (Kotiyal & Bhattacharya, 2014). Previous studies indicated the regulatory effects of MIR31HG on EMT in many types of cancers (Zheng *et al*., 2019). For instance, MIR31HG is known to promote EMT in pancreatic cancer cells induced by the transforming growth factor β and to induce EMT phenotype in non-small lung cancer cells (Ko *et al*., 2022). Therefore, we explored whether the roles of MIR31HG in breast cancer were associated with EMT. Interestingly, our data suggested that overexpression of MIR31HG increased the protein levels of N-Cadherin and Vimentin, whereas changes in MIR31HG regulated these EMT-related biomarkers in MCF7 cells and breast tumor tissues.

The roles of MIR31HG in cancers are associated with its regulatory effects on various biological processes, signaling pathways, and proteins (Ko *et al*., 2022; Wei *et al*., 2022; Zheng *et al*., 2019). For instance, MIR31HG promotes non-small cell lung cancer growth, in part, by regulating Wnt/β-catenin signaling pathways (Zheng *et al*., 2019). Another study revealed that MIR31HG is upregulated in colorectal tumor tissues and associated with proteins relevant to glycolysis (ex. hexokinase 2 and glucose transporter 1) and angiogenesis (ex. vascular endothelial growth factor A and tissue inhibitor matrix metalloproteinase 1) (Wang *et al*., 2022). Wang and colleagues reported that MIR31HG promotes tumor growth in head and neck cancer via its regulation of hypoxia-inducible factor A and cyclin-dependent kinase inhibitor p21 (Wang *et al*., 2018). Our study found the role of MIR31HG in breast cancer, at least partly, by regulating RIPK4. Interestingly, we found RIPK4 served as a downstream target of MIR31HG, since changes in MIR31HG regulated the levels of RIPK4, whereas the knockdown of RIPK4 did not significantly affect the levels of MIR31HG.

RIPK4 is involved in many types of cancers by affecting a series of biological functions and signaling transduction proteins (Li *et al*., 2021; Qi *et al*., 2018). For instance, Li and colleagues reported that RIPK4 inhibits hepatoma cancer cell invasion and metastasis by the regulation of STAT3 signaling pathways (Li *et al*., 2021). As an immune regulatory biomarker, the abnormal expressions of RIPK4 are observed in many types of cancers including gynecologic cancer, melanoma, cervical squamous cell carcinoma, etc. (Liu *et al*., 2015; Liu *et al*., 2021; Madej *et al*., 2021). Liu and colleagues observed that an elevation of RIPK4 in cervical squamous cell carcinoma is correlated to poor overall survival rate, and knockdown of RIPK4 suppresses cell migration and invasion (Liu *et al*., 2015). In this study, we identified RIPK4 as a downstream target of MIR31HG in breast cancer. RIPK4 regulated cell proliferation, invasion, and migration as well as EMT-related biomarkers. Consistently, *in vivo* studies showed that the knockdown of RIPK4 reduced tumor size in cancer xenograft animal models. These results suggested that targeting RIPK4 might be a good strategy for breast cancer therapy.

CONCLUSION

MIR31HG positively regulates cell proliferation, invasion and migration, and EMT. In addition, MIR31HG positively regulates RIPK4, as supported by the fact that changes in MIR31HG affect RIPK4, whereas RIPK4 knockdown does not affect MIR31HG. Consistently, *in vivo* studies showed that the knockdown of MIR31HG or RIPK4 reduces tumor size in tumor xenograft animal models. These data suggest that the roles of lncRNA MIR31HG in breast cancer are associated with its regulatory effects against RIPK4.

Declarations

Disclosure of potential conflicts of interest. The authors declare that they have no competing interests.

Ethical Approval. The animal procedures were approved by the First Affiliated Hospital of Bengbu Medical College. This study was performed in strict accordance with the NIH guidelines for the care and use of laboratory animals (8th edition, NIH).

Data availability statement. The raw data supporting the conclusions of this article will be made available on request to the corresponding author by email, as requested by our department.

Consent for publication. Current study is available from the corresponding author on reasonable request.

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