Original Article

In vitro study of piwi interaction *RNA-31106* promoting breast carcinogenesis by regulating *METTL3*-mediated *m6A* RNA methylation

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Background: Breast cancer is the most common gynecological malignancy and the leading cause of cancer-related deaths in women. P-element induced wimpy testis (PIWI)-interacting RNAs (piRNAs) are novel non-coding RNAs whose abnormal expressions have been closely associated with multiple cancers. This study explored the roles and possible mechanisms of *piRNA-31106* in breast cancer.

Methods: The expression of *piRNA-31106* in breast cancer tissues and cells was detected by reverse transcription polymerase chain reaction (RT-PCR). The pcDNA vector containing *piRNA-31106* (pcDNA-*piRNA-31106*) and a short hairpin (sh)RNA containing *piRNA-31106* (shRNA-*piRNA-31106*) were used to interfere with *piRNA-31106* expression in breast cancer cells. The effects on cell proliferation, apoptosis/cell cycle, invasion, and metastasis were detected via Cell Counting Kit-8 (CCK-8), flow cytometry, transwell assays, and scratch tests, respectively. The protein expressions of murine double minute 2 (MDM2), cyclin-dependent kinase 4 (CDK4), and cyclinD1 were detected by Western blot analysis. The N6-methyladenosine (*m6A*) RNA methylation level and the binding relationship between *piRNA-31106* and *METTL3* were analyzed. The role of *METTL3* in the regulation of breast cancer by *piRNA-31106* was further analyzed by using small interfering (si)RNA targeting *METTL3*.

Results: *PiRNA-31106* was highly expressed in breast cancer tissues and cell lines MDA-MB-231 and MCF-7. Overexpression of *piRNA-31106* promoted the viability, invasion, and migration of breast cancer, inhibited apoptosis, and promoted the expressions of MDM2, CDK4, and cyclinD1. Inhibition of *piRNA-31106* showed the opposite effect. In addition, *piRNA-31106* promoted the *m6A* methylation levels and facilitated methyltransferase-like 3 (*METTL3*) expression in MDA-MB-231 and MCF-7 cells. RNA immunoprecipitation (RIP) assays confirmed the binding relationship between *piRNA-31106* and *METTL3*. Further experiments demonstrated that *si-METTL3* could inhibit the regulatory effects of *piRNA-31106* on breast cancer.

Conclusions: *PiRNA-31106* was significantly highly expressed in breast cancer and could promote breast cancer progression by regulating *METTL3*-mediated *m6A* RNA methylation.

Keywords: *piRNA-31106*; breast cancer; *METTL3*; methylation

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Introduction

Breast cancer is the most common gynecological cancer in women worldwide (1). Among newly diagnosed cases of cancer worldwide, female breast cancer has the highest incidence, and its mortality rate ranks first among all female cancer-related deaths globally (2). Although early detection and more effective treatment options have greatly reduced the death rate from breast cancer, it still kills more than half a million people worldwide each year (3). Due to the complicated pathological progression of breast cancer, the recurrence rate and distant metastasis rate show an increasing trend (4). Therefore, understanding the molecular regulation and molecular mechanisms involved in the occurrence and development of breast cancer will be useful in the identification of novel molecular biomarkers and the development of effective targeted therapies for breast cancer.

Noncoding RNAs (ncRNAs) regulate cancer-related molecular networks through transcription or post-transcriptional silencing of gene expression and play a unique regulatory role in cancer (5). PIWI-interacting RNAs (piRNAs) are novel endogenous ncRNAs, and the association between piRNA and tumorigenesis has been frequently reported (6-8). Research has found that *piRNA-31106* is highly expressed in breast cancer and is associated with poor prognosis of breast cancer, thus, it may represent an effective biomarker in cancer (8). A recent study using 227 breast cancer tissues for small RNA sequencing screening showed that *piRNA-31106* may be a potential candidate biomarker for breast cancer (9). However, the expression and regulatory mechanism of *piRNA-31106* in breast cancer warrants further exploration.

Epigenetic regulation is thought to play a crucial role in the biological behavior of cancer (10,11). PiRNAs mainly induce transposon silencing and epigenetic regulation (12), and have been shown to be involved in the regulation of epigenetic mechanisms in tumorigenesis (13). N6-methyladenosine (*m6A*) RNA methylation is one of the most prevalent types of internal epigenetic regulation in mammalian transcriptome and is involved in the processes of breast cancer (14,15). Hypermethylation and inactivation of genes can affect multiple cellular processes that may be involved in cancer development (16). Methyltransferase-like 3 (*METTL3*), as the main methyltransferase, is responsible for the formation of *m6A* (17). PiRNAs have been reported to regulate disease progression by controlling *METTL3*-dependent *m6A* methylation (18). In addition, there is increasing evidence in recent years that *METTL3* plays a key role in multiple cancers (19). However, it remains unclear whether *piRNA-31106* is involved in breast cancer processes through *METTL3*-mediated *m6A* methylation.

This study explored the expression of *piRNA-31106* in breast cancer tissues and cells, its regulation of malignant biological behavior in breast cancer, and its role in *METTL3*-mediated *m6A* methylation. The results demonstrated that *piRNA-31106* is involved in the development of breast cancer through *METTL3*-mediated *m6A* RNA methylation, suggesting that *piRNA-31106* may be a potential biomarker of breast cancer and/or a cancer therapeutic target. We present this article in accordance with the MDAR reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-790/rc).

Highlight box

Key findings

- This study explored the roles and possible mechanisms of *piRNA-31106* in breast cancer.

What is known and what is new?

- PIWI-interacting RNAs (piRNAs) are novel non-coding RNAs whose abnormal expressions have been closely associated with multiple cancers. The role and mechanism of *piRNA-31106* in breast cancer are still unclear.
- This article reveals that *piRNA-31106* was significantly highly expressed in breast cancer and could promote breast cancer progression by regulating *METTL3*-mediated *m6A* RNA methylation.

What is the implication, and what should change now?

- *piRNA-31106* may be a potential target for the treatment of breast cancer.

Methods

Tissue sample collection

Breast cancer samples were obtained from patients who underwent surgery at the Affiliated Hospital of Guangdong Medical University. All patients signed an informed consent form, and all patients conformed to surgical conditions and pathological tissue samples were routinely collected. Based on postoperative pathological results, a total of 6 pairs of breast cancer tissues and adjacent non-cancer tissues, as confirmed by 2 or more pathologists in our hospital, were selected for reverse transcription polymerase chain reaction (RT-PCR) analysis. This study was approved by the Ethics

Committee of the Affiliated Hospital of Guangdong Medical University (PJKT2023-064) and was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Cell culture and transfection

Breast cancer cell lines MDA-MB-231 (cat. no. CL-0150B) and MCF-7 (cat. no. CL-0149), and the human normal mammary epithelial cell line MCF 10A (cat. no. CL-0525) were purchased from Procell (Wuhan, China). All cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Procell, China) containing 10% fetal bovine serum (FBS; Vica Cell, New Zealand) in a 5% CO_2 incubator at 37 ℃. The overexpression pcDNA vector containing *piRNA-31106* (pcDNA-*piRNA-31106*), the short hairpin (sh)RNA of *piRNA-31106* (shRNA-*piRNA-31106*), and the small interfering (si)RNA targeting *METTL3* (*si-METTL3*) were designed and synthesized by RiboBio (China) for transfection into MDA-MB-231 and MCF-7 cells. For plasmid transfection, Opti-MEM™ I (Gibco, USA) was used to dilute the plasmid DNA and the Lipofectamine™ 2000 transfection reagent (Gibco, USA), and let stand at room temperature for 5 minutes. The plasmid was mixed with the transfection reagent, left at room temperature for 5 minutes, and then added to the cells for transfection. For siRNA transfection, the siRNA storage solution was diluted with RiboFect™ CP buffer (RiboBio, China) and added to RiboFect™ CP transfection reagent (RiboBio, China) and incubated at room temperature for 15 minutes. The prepared transfection complexes were then added to the cell culture medium for transfection. Fresh medium was replaced 6 hours after transfection, and follow-up experiments were performed 48 hours after transfection.

Western blot analysis

The protein expression of cyclin-dependent kinase 4 (CDK4), cyclinD, murine double minute 2 (MDM2), and *METTL3* were detected by Western blot analysis. Total protein was obtained using RIPA lysis solution (cat. no. G2002-100ML; Servicebio, China). The lysate was collected and centrifuged at 4 ℃ at 12,000 rpm for 10 minutes. After centrifugation, the supernatant was collected and the protein concentration was determined using the BCA protein quantitative kit (cat. no. P0009; Beyotime, China). The quantified protein was denatured with loading buffer using a thermocycler at 95C for 15 minutes. The denatured proteins were separated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) membranes (cat. no. ISEQ00010; Sigma-Aldrich, USA), and sealed with 5% skim milk for 2 hours. The PVDF membranes were incubated with the following primary antibodies at 4 ℃ overnight: CDK4 (1:2,000; cat. no. A21317, abclonal, China), cyclinD (1:2,000; cat. no. A19038, abclonal, China), MDM2 (1:2,000; cat. no. A13327, abclonal, China), *METTL3* (1:2,000; cat. no. A8370, abclonal, China), and β-actin (1:5,000; cat. no. AC026, abclonal, China). Membranes were then incubated with the secondary antibody biotinylated goat anti-rabbit IgG (H + L) (1:5,000; cat. no. AS014, abclonal, China) at room temperature for 2–3 hours. The ECL kit (cat. no. 17046, zen-bio, China) and chemiluminescent gel imager (Tanon, China) were used to visualize the protein bands. β-actin was used as internal reference, and the results were expressed as the relative expression level of the target protein.

RT-PCR

Total RNA was extracted using TRIzol Reagent (Invitrogen, USA). For the detection of mRNA, the Prime Script RT reagent kit (Takara, China) was used for reverse transcription reactions to obtain the cDNA, and RT-PCR was performed in a 20 μL reaction system using the SYBR Green kit (Invitrogen, USA). To quantify *piRNA-31106*, RT-PCR was performed using specific miRNA/piRNA stem-loop RT primers and Bulge-Loop qPCR kit (RiboBio, China) in accordance with the manufacturer's instructions. The primers used in this study were as follows: *METTL3*: F: 5'-CAAGGCTTCAACCAGGGTCT-3', R: 5'-GGTTTCCAAGGGTGATCCAGT-3'; ACTIN: F: 5'-GAAGATCAAGATCATTGCTCC-3', R: 5'-TACTCCTGCTTGCTGATCCA-3'; *piRNA-31106*: F: 5'-AGCCCTGATGATGCCCACTC-3', R: 5'-TGGCAAAGAGCACAATGAAG-3'; and U6: F: 5'-GGAACGATACAGAGAAGATTAGC-3', R: 5'-TGGAACGCTTCACGAATTTGCG-3'. Actin was the internal control of mRNA, and U6 small nuclear RNA was the internal control of *piRNA-31106*. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Cell proliferation assay

MDA-MB-231 and MCF-7 cells at the logarithmic growth stage were collected, plated into 96-well plates with an

adjusted density of 4×10^3 cells/well, and incubated at 37 ℃ and 5% CO_2. After transfection for 48 hours, diluted Cell Counting Kit-8 (CCK-8) working solution (cat. no. BS350A; Biosharp, China) was added and incubated for 2 hours at 37 ℃ and 5% CO_2. The light absorption values of each well were measured at a wavelength of 450 nm using a microplate reader (SpectraMax Plus 384, Molecular Devices, China).

Cell apoptosis assay

After different treatments, MDA-MB-231 and MCF-7 cells were collected and centrifuged at 250 g for 5 minutes. The supernatant was discarded and washed with phosphate buffered saline (PBS). The cells were the resuspended in 500 µL Binding Buffer, 5 µL Annexin V, and 5 µL propidium iodide (PI; cat. no. KGA1030; KeyGEN BioTECH, China) and incubated at room temperature for 15 minutes in the dark, followed by flow cytometry analyses (CytoFLEX, Beckman, USA).

Invasion and migration analysis

Cell invasion was assessed using transwell assays. After matrigel (CORNING, USA) preparation of the transwell invasion wells, complete medium containing 20% FBS was added into the lower chamber. Transfected MDA-MB-231 and MCF-7 cells, which were starved before the experiment, were seeded into the transwell chamber at a density of 4×10^4 cells/well. After incubation for 24 hours, cells were fixed at room temperature with precooled ethanol for 30 minutes, followed by staining with 0.5% crystal violet dye (cat. no. JC3913; BOMEI, China) at room temperature for 30 minutes. After proper air drying, the number of invasive cells was counted under the microscope (LEICA, Germany).

Cell migration was detected using the scratch assay. First, a marker pen was used to draw horizontal lines evenly behind the 6-well plates. MDA-MB-231 and MCF-7 cells, at logarithmic growth, were inoculated in 6-well plates at a density of 2×10^5 cells/well. When the cells had grown to a single layer, the supernatant was removed and a pipette tip was used to scratch the cell layer in a line perpendicular to the back horizontal line. The scratched cells were gently washed with PBS and re-cultured at 37 ℃ and 5% CO_2 for 24 h. The scratch status of cells in each well was photographed under the microscope (LEICA, Germany) at 0 hour and again at 24 hours.

Cell cycle assay

Cells from different treatment groups were collected, washed with PBS, and centrifuged to collect cell precipitates. A 75% pre-cooled ethanol solution was added to the cell pellet and fixed overnight at 4 ℃. After washing with PBS, the cells were resuspended with 500 µL PI/RNase A staining solution (cat. no. KGA512; KeyGEN BioTECH, China) and incubated at room temperature for 30 minutes away from light. Samples were then analyzed using flow cytometry (CytoFLEX, Beckman, USA).

Quantitative detection of m6A RNA methylation

Total RNA was isolated and purified from breast cancer cell lines, and the global *m6A* RNA methylation levels were measured using the EpiQuik *m6A* RNA methylation quantitative kit (EpigenTek, USA) according to the manufacturer's instructions. Briefly, the isolated RNA was first bound to the reaction wells, and after washing, diluted capture antibody was added and incubated for 1 hour at room temperature. After washing again, the developer solution was added and absorbance was measured at 450 nm using a microplate reader.

RNA immunoprecipitation (RIP) assay

The binding relationship between *piRNA-31106* and *METTL3* was verified using the Magna RIP RNA-binding protein immunoprecipitation kit (Millipore, USA), in accordance with the manufacturer's protocol. The collected cells were lysed with RIP lysis buffer, and 100 µL lysate was incubated with magnetic bead RIP buffer containing *METTL3* antibody (cat. no. ab240595, abcam, USA) or control normal mouse IgG (Millipore, USA). The protease K buffer was then used to digest the protein. Finally, the bound RNA was isolated by Trizol reagent (Invitrogen, USA) and the purified *piRNA-31106* was detected by RT-PCR.

Statistical analysis

All the experiments were replicated biologically six times, and SPSS 22.0 statistical software was used for statistical analyses. Data are expressed as mean ± standard deviation, the means of two groups were tested by Student's *t*-test, and comparison among multiple means was performed by one-way analysis of variance. A P value <0.05 was considered

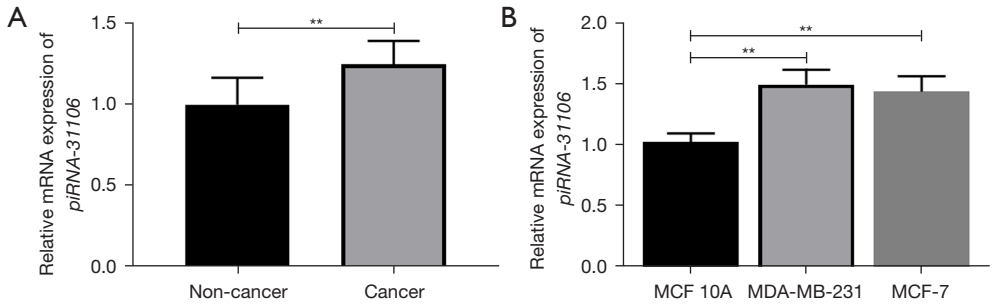

Figure 1 The expression of *piRNA-31106* in breast cancer was analyzed by RT-PCR. (A) The expression of *piRNA-31106* was higher in breast cancer tissues compared to adjacent non-cancer tissues (n=6). (B) The expression of *piRNA-31106* in breast cancer cells MDA-MB-231 and MCF-7 was higher than that in MCF 10A normal breast epithelial cells (n=6). **, P<0.01. MCF, Michigan Cancer Foundation; MDA-MB-231, human breast cancer cells; RT-PCR, reverse transcription polymerase chain reaction.

statistically significant.

Results

piRNA-31106 was upregulated in breast cancer tissues and cell lines

To clarify the role of *piRNA-31106* in breast cancer, the expression of *piRNA-31106* was analyzed. The expression of *piRNA-31106* in breast cancer tissues was significantly higher than that in non-cancer tissues (P<0.01, *Figure 1A*). Similarly, compared with human normal mammary epithelial cells MCF 10A, the expression of *piRNA-31106* was significantly increased in the breast cancer cell lines MDA-MB-231 and MCF-7 (P<0.01, *Figure 1B*).

Overexpression of piRNA-31106 promoted malignant biological behavior of breast cancer cells

The pcDNA vector was used to construct a *piRNA-31106* overexpression plasmid to analyze the effect of upregulation of *piRNA-31106* on the function of breast cancer cells. After transfection of MDA-MB-231 and MCF-7 cells with pcDNA-*piRNA-31106* or its negative control pcDNA-NC, the transfection efficiency was confirmed by RT-PCR. The results showed that the expression of *piRNA-31106* in the pcDNA-*piRNA-31106* group was significantly increased compared with that in the pcDNA-NC group in MDA-MB-231 and MCF-7 cells (P<0.001, *Figure 2A*). Further analysis of cell function showed that pcDNA-*piRNA-31106* could significantly promote cell proliferation (P<0.001, *Figure 2B*), inhibit cell apoptosis (P<0.001, *Figure 2C,2D*), and promote cell invasion and migration (P<0.001, *Figure 2E-2H*). In addition, cell cycle results demonstrated that pcDNA-*piRNA-31106* significantly decreased the percentage of G1 phase cells and promoted cell cycle progression (P<0.001, *Figure 2I,2J*). These results confirmed that overexpression of *piRNA-31106* could promote the proliferation, invasion, and migration of breast cancer cells, and inhibit apoptosis.

Interference with piRNA-31106 inhibited cell proliferation and invasion, and promoted apoptosis

To investigate the effects of *piRNA-31106* interference on proliferation, invasion, apoptosis, and cell cycle of breast cancer cells, MDA-MB-231 and MCF-7 cells were transfected with shRNA-*piRNA-31106*. The results showed that the expression of *piRNA-31106* was significantly decreased in cells transfected with shRNA-*piRNA-31106* (P<0.001, *Figure 3A*), indicating successful intervention. Compared with the sh-NC group, shRNA-*piRNA-31106* inhibited cell proliferation (P<0.001, *Figure 3B*), promoted cell apoptosis (P<0.001, *Figure 3C,3D*), and inhibited invasion (P<0.001, *Figure 3E,3F*) and migration (P<0.001, *Figure 3G,3H*). In addition, after *piRNA-31106* expression was silenced, the proportion of cells in the G1 phase was significantly increased, while the proportions of cells in the S phase and G2 phase were decreased (P<0.001, *Figure 3I,3J*), indicating that the interference of *piRNA-31106* stalled cells in the G1 phase, and cellular DNA synthesis and DNA replication were relatively reduced.

piRNA-31106 promoted the expression of oncogenes

Further analysis of the expression of oncogenes showed that

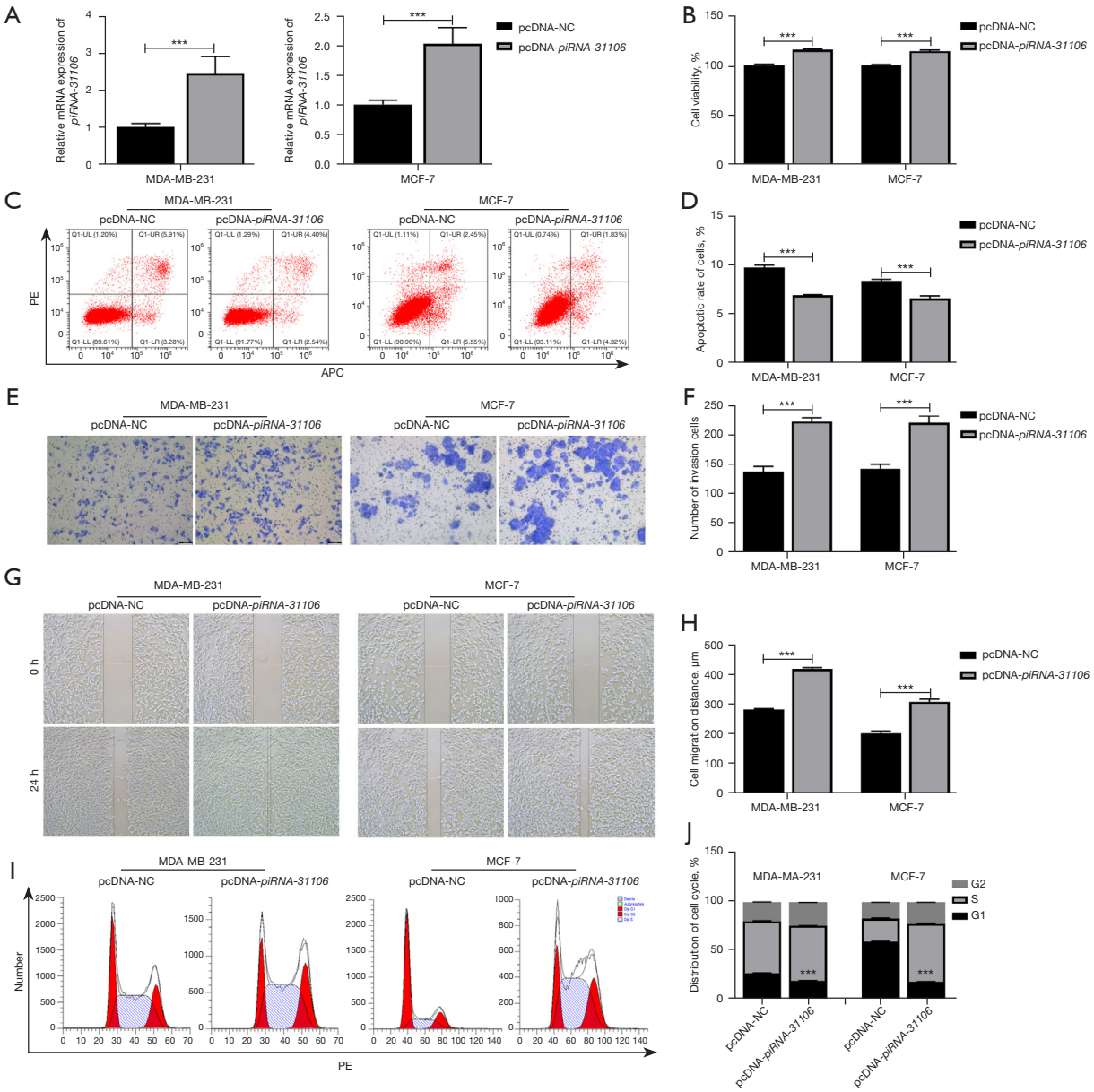

Figure 2 The effects of *piRNA-31106* overexpression on the biological behavior of breast cancer cells. (A) Transfection of pcDNA-*piRNA-31106* into breast cancer cell lines MDA-MB-231 and MCF-7 significantly increased the expression of *piRNA-31106* compared to cells transfected with the pcDNA-NC, as shown by RT-PCR. (B) CCK-8 assay revealed that the cell proliferation activity was increased in cells transfected with pcDNA-*piRNA-31106* compared to cells transfected with pcDNA-NC. (C) Flow cytometry analysis demonstrated decreased cell apoptosis in cells transfected with pcDNA-*piRNA-31106* compared to cells transfected with pcDNA-NC. (D) A histogram showing the percentage of apoptotic cells. (E) Transwell assays demonstrated significant cell invasion ability in cells transfected with pcDNA-*piRNA-31106* compared to cells transfected with pcDNA-NC. Staining with crystal violet. Magnification ×100. (F) Statistical analysis of the number of invasive cells shown in (E). (G) The scratch assay revealed that the cell migration distance was significantly greater in cells transfected with pcDNA-*piRNA-31106* compared to cells transfected with pcDNA-NC. Observation under light microscope. Magnification ×100. (H) A histogram showing the statistical results of cell migration distance. (I) The proportion of cells in each phase of the cell cycle was detected by flow cytometry. (J) Cell cycle distribution statistics. ***, P<0.001; n=6. MDA-MB-231, human breast cancer cells; MCF, Michigan Cancer Foundation; pcDNA, pcDNA vector; NC, negative control; G2, gap2; S, synthesis phase; G1, gap1; PE, phycoerythrin; RT-PCR, reverse transcription polymerase chain reaction; CCK-8, Cell Counting Kit-8.

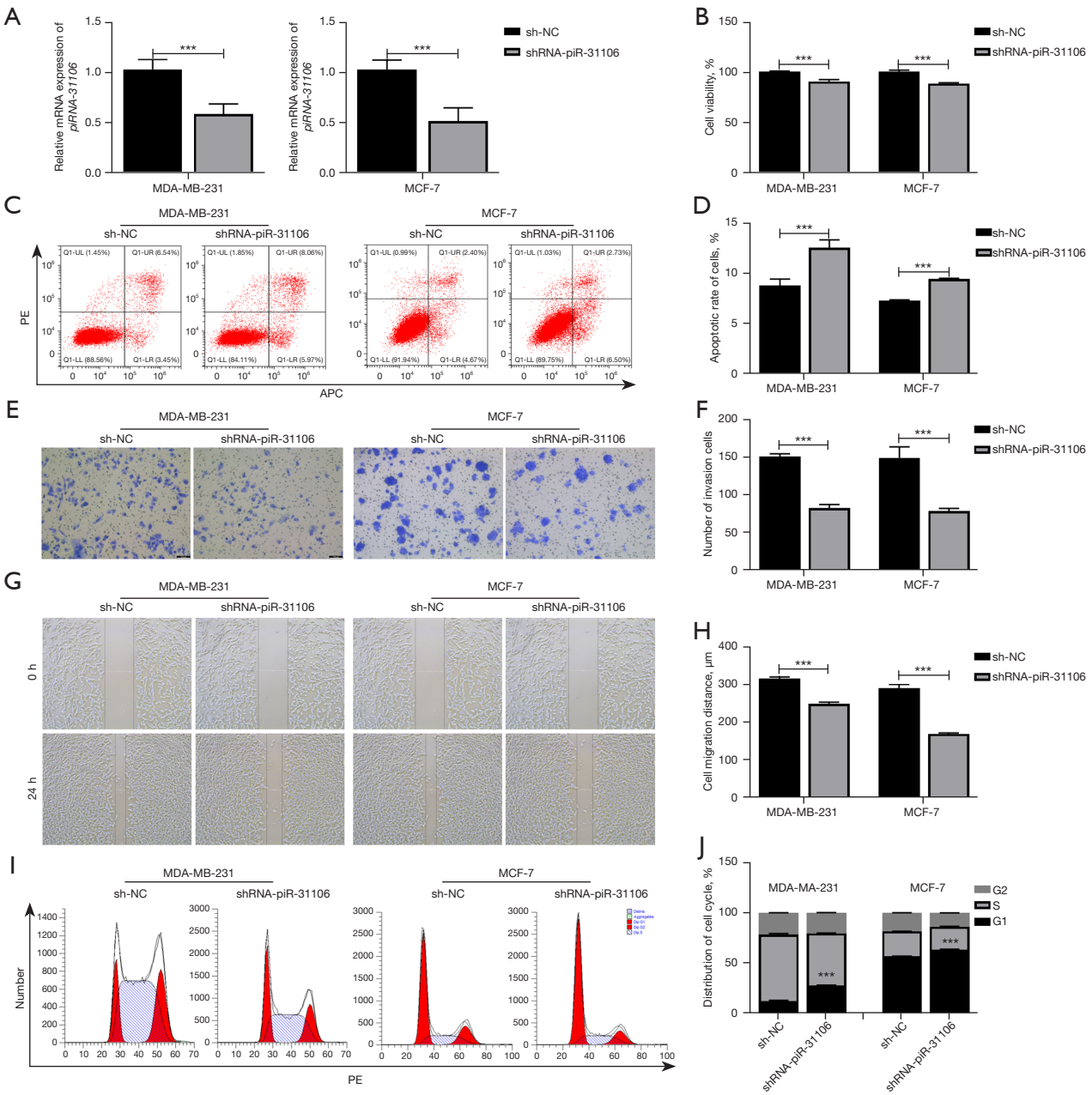

Figure 3 The effects of intervention of *piRNA-31106* on the biological behavior of breast cancer cells. (A) After transfection of short hairpin (sh)RNA-*piRNA-31106* into breast cancer cell lines MDA-MB-231 and MCF-7, the expression of *piRNA-31106* was significantly decreased. (B) Cell proliferation activity as detected by CCK-8 was reduced in cells transfected with shRNA-*piRNA-31106*. (C) The rate of cell apoptosis was increased in cells transfected with shRNA-*piRNA-31106*, as detected by flow cytometry. (D) A histogram showing the percentage of apoptotic cells. (E) Transwell assays revealed that the invasion ability of cells was reduced after transfection with shRNA-*piRNA-31106*. Staining with crystal violet. Magnification ×100. (F) Statistical analysis of the number of invasive cells. (G) The cell migration distance was reduced after transfection with shRNA-*piRNA-31106*, as detected by the scratch assay. Observation under light microscope. Magnification ×100. (H) Statistical analysis of the cell migration distance. (I) The cell cycle was assessed by flow cytometry. (J) Cell cycle distribution statistics. ***, P<0.001; n=6. MDA-MB-231, human breast cancer cells; MCF, Michigan Cancer Foundation; sh-NC, short hairpin (sh)RNA-negative control; APC, allophycocyanin; PE, phycoerythrin; CCK-8, Cell Counting Kit-8.

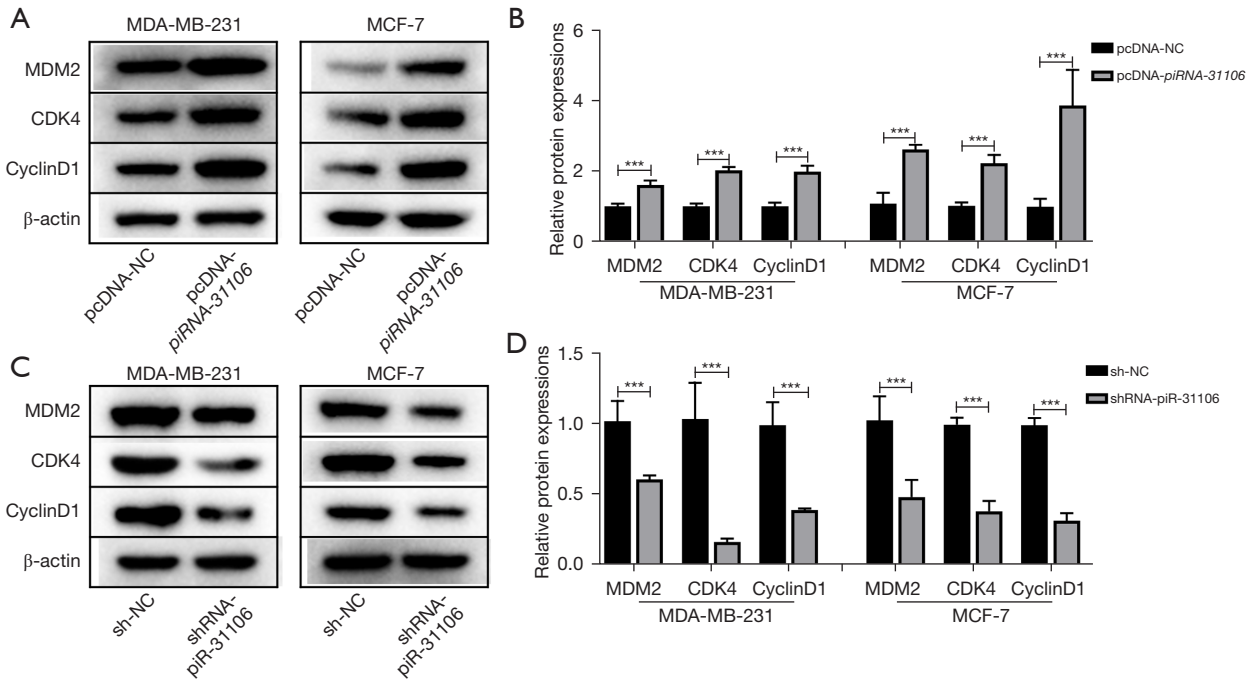

Figure 4 The effects of *piRNA-31106* on oncogene expression in breast cancer cells. (A) Western blot was used to detect the protein expressions of MDM2, CDK4, and cyclinD1 in the breast cancer cell lines MDA-MB-231 and MCF-7 transfected with pcDNA-*piRNA-31106* or pcDNA-NC. (B) Statistical histogram showing the protein expressions. (C) Expression of MDM2, CDK4, and cyclinD1 after transfection with shRNA-*piRNA-31106* or shRNA-NC. (D) Statistical histogram showing the protein expressions. ***, P<0.001; n=6. MDA-MB-231, human breast cancer cells; MCF, Michigan Cancer Foundation; pcDNA-NC, pcDNA-negative control; shRNA-NC, short hairpin (sh)RNA-negative control.

the protein levels of MDM2, CDK4, and cyclinD1 were significantly increased in cells transfected with pcDNA-*piRNA-31106* compared to cells transfected with pcDNA-NC, and significantly lower in cells transfected with shRNA-*piRNA-31106* compared to cells transfected with sh-NC (P<0.001, *Figure 4*). These results suggested that *piRNA-31106* could promote the expression of oncogenes.

piRNA-31106 promoted METTL3-mediated m6A methylation

PiRNAs mainly induce transposon silencing and epigenetic regulation (12). To further investigate the potential mechanism of *piRNA-31106* in regulating the behavior of breast cancer, the global *m6A* levels in the *piRNA-31106* transfected cells were analyzed. The results revealed that compared with the pcDNA-NC/sh-NC groups, levels of *m6A* methylation were upregulated in the pcDNA-*piRNA-31106* group and downregulated in the shRNA-piR-36741 group (P<0.001, *Figure 5A*), suggesting that *piRNA-31106* could affect *m6A* methylation levels in breast cancer. *METTL3*, as the main methyltransferase, is responsible for the formation of *m6A* (17). The results of this study showed that mRNA expression of *METTL3* in the pcDNA-*piRNA-31106* group was significantly increased compared with that in the pcDNA-NC group. Furthermore, compared with the sh-NC group, mRNA expression of *METTL3* was significantly decreased in the shRNA-*piRNA-31106* group (P<0.001, *Figure 5B*). RIP results showed that *METTL3* could combine with *piRNA-31106* to form a complex (*Figure 5C*). These studies suggested that *piRNA-31106* could promote *METTL3*-mediated *m6A* methylation.

Intervention of METTL3 antagonized the malignant-promoting effects of piRNA-31106 on breast cancer cells

To further clarify the role of *piRNA-31106* in regulating *METTL3* in breast cancer, a si-RNA interference sequence targeting *METTL3* was constructed and transfected into

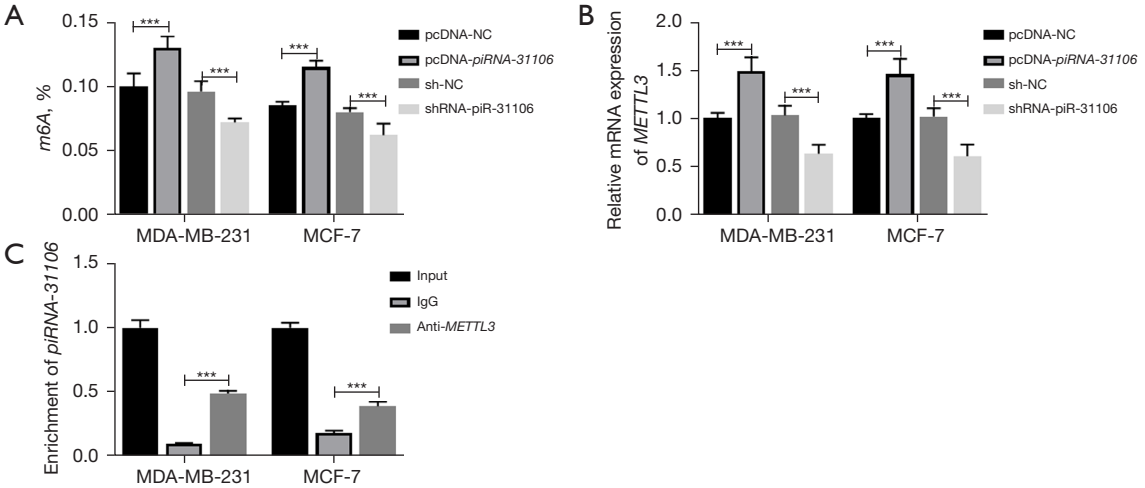

Figure 5 The effects of *piRNA-31106* on *METTL3*-mediated *m6A* methylation in breast cancer cells. (A) The global *m6A* level as detected by *m6A* RNA methylation quantitative detection kit was increased in cells transfected with pcDNA-*piRNA-31106*, and was reduced in cells transfected with shRNA-*piRNA-31106*. (B) The gene expression of *METTL3* as detected by RT-PCR was significantly increased in cells transfected with pcDNA-*piRNA-31106*, and was decreased in cells transfected with shRNA-*piRNA-31106*. (C) The combination of *METTL3* and *piRNA-31106* was verified by RIP experiment. ***, P<0.001; n=6. MDA-MB-231, human breast cancer cells; MCF, Michigan Cancer Foundation; pcDNA-NC, pcDNA-negative control; sh-NC, short hairpin (sh)RNA-negative control; IgG, immunoglobulin G; RT-PCR, reverse transcription polymerase chain reaction; RIP, RNA immunoprecipitation.

breast cancer cells. Western blot analysis and RT-PCR were used to verify the intervention effects (P<0.001, *Figure 6A*,*6B*). Breast cancer cells were then transfected with pcDNA-*piRNA-31106* and *si-METTL3* or their corresponding negative controls. Western blot and RT-PCR analysis showed that pcDNA-*piRNA-31106* could significantly promote the protein and gene expression of *METTL3*, and also significantly reverse the decreased expression of *METTL3* in cells caused by *si-METTL3* (P<0.001, *Figure 6C*,*6D*). However, intervention with *METTL3* had no significant effect on the expression of *piRNA-31106* (*Figure 6E*). In addition, CCK-8 results showed that *si-METTL3* could significantly inhibit cell proliferation and reverse the promotional effect of pcDNA-*piRNA-31106* on cell viability (P<0.001, *Figure 7A*). Flow cytometry showed that *si-METTL3* could significantly promote cell apoptosis and reverse the inhibitory effect of *piRNA-31106* overexpression on apoptosis (P<0.001, *Figure 7B*,*7C*). Similarly, *si-METTL3* inhibited cell invasion and migration, and reversed the increased cell invasion and migration caused by pcDNA-*piRNA-31106* (P<0.001, *Figure 7D-7G*). Furthermore, it reversed the pcDNA-*piRNA-31106* regulation of the cell cycle (P<0.001, *Figure 7H*,*7I*). Western blot results showed that *si-METTL3* significantly inhibited the protein expression of MDM2, CDK4, and cyclinD1, and also reversed the promotional effect of pcDNA-*piRNA-31106* on these proteins (P<0.001, *Figure 8*).

Discussion

Breast cancer is one of the most common gynecological tumors in the clinic, with high morbidity and mortality, which seriously threatens human life and health (20). PiRNAs are small non-coding RNAs expressed in a tissue-specific manner in a variety of human tissues that regulate key signaling pathways at the transcriptional or post-transcriptional level (21). Increasingly, studies have detected abnormal expression of piRNAs in many cancers, including breast cancer, and its expression is closely related to the maintenance of genomic stability and epigenetic regulation (22). It has been reported that *piRNA-31106* is dysregulated in breast cancer (9). This study confirmed that *piRNA-31106* is significantly highly expressed in breast cancer. Overexpression of *piRNA-31106* significantly promoted the proliferation, invasion, and migration of MDA-MB-231 and MCF-7 cells, and inhibited apoptosis, while intervention of *piRNA-31106* had the opposite effect.

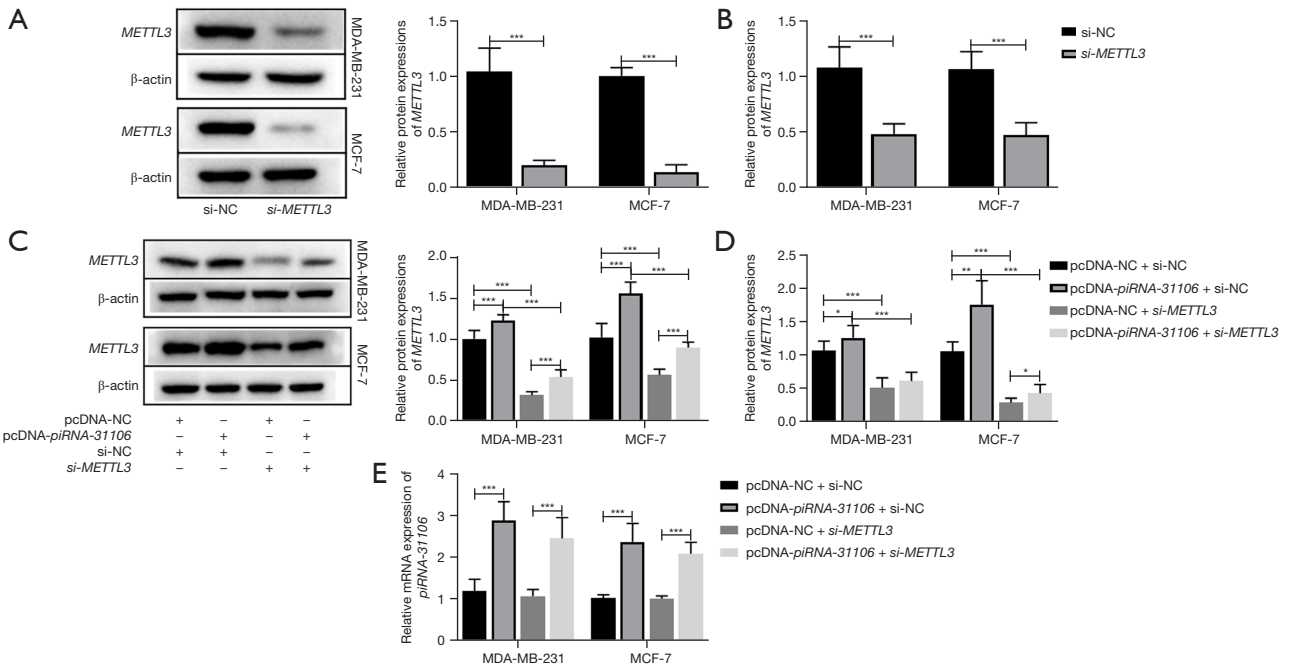

Figure 6 Protein and gene expression of *METTL3*. The breast cancer cell lines MDA-MB-231 and MCF-7 were transfected with pcDNA-*piRNA-31106* and/or *si-METTL3*. (A,B) Western blot and RT-PCR were used to detect the protein and gene expressions of *METTL3* in *si-METTL3* transfected cells to verify the transfection efficiency. (C) The protein expression of *METTL3* was detected by Western blot. (D) The gene expression level of *METTL3* was detected by RT-PCR. (E) The gene expression level of *piRNA-31106* was detected by RT-PCR. *, P<0.05; **, P<0.01; ***, P<0.001; n=6. si-NC, small interfering RNA-negative control; MDA-MB-231, human breast cancer cells; MCF, Michigan Cancer Foundation; pcDNA-NC, pcDNA-negative control; RT-PCR, reverse transcription polymerase chain reaction.

In addition, *piRNA-31106* significantly promoted the *m6A* level and *METTL3* gene expression in breast cancer, and there was an interaction between *piRNA-31106* and *METTL3*. Further study (23) confirmed that intervention of *METTL3* expression could significantly inhibit the progression of breast cancer cells, and inhibit the promotional effect of *piRNA-31106* on breast cancer.

PiRNAs were initially isolated from testes, and the length of the mature piRNA is slightly longer than that of microRNAs (miRNAs), ranging from 26 to 31 nucleotides (23,24). As a new class of small non-coding RNAs, piRNA has been reported to have important biological functions in regulating the proliferation, cycle operation, apoptosis, migration and invasion of tumor cells, and has a carcinogenic or cancer-suppressing regulatory effect (25). Normally, the piRNA/PIWI protein is maintained at a stable level through a physiological balance between synthesis and degradation in germ cells and somatic cells. However, when the expression of piRNA or PIWI proteins is disturbed, they lose their normal function and may lead to the development of cancer (26). Differential expression of piRNA is considered to be a biomarker and a new therapeutic target for early diagnosis and prognosis of malignant tumors (27). Study has shown that *piR-5937* and *piR-28876* are upregulated in colon cancer and are promising diagnostic biomarkers for colon cancer (28). The up-regulated expression of piR-1245 in gastric cancer tissues is correlated with the stage of tumor lymph node metastasis and is a potential prognostic marker for gastric cancer (29). This study found that *piRNA-31106* was significantly highly expressed in breast cancer and may be one of the key targets in breast cancer. Overexpression of *piRNA-31106* promoted cancer cell growth, while inhibition of *piRNA-31106* inhibited the progression of breast cancer and the protein expression of MDM2, CKD4, and cyclinD. MDM2 is a newly discovered proto-oncogene, a member of the apoptosis suppressor gene family, which has the strongest inhibitor effect on apoptosis and is expressed in most human tumor tissues (30). CyclinD and CDK4 are important positive regulators of the cell

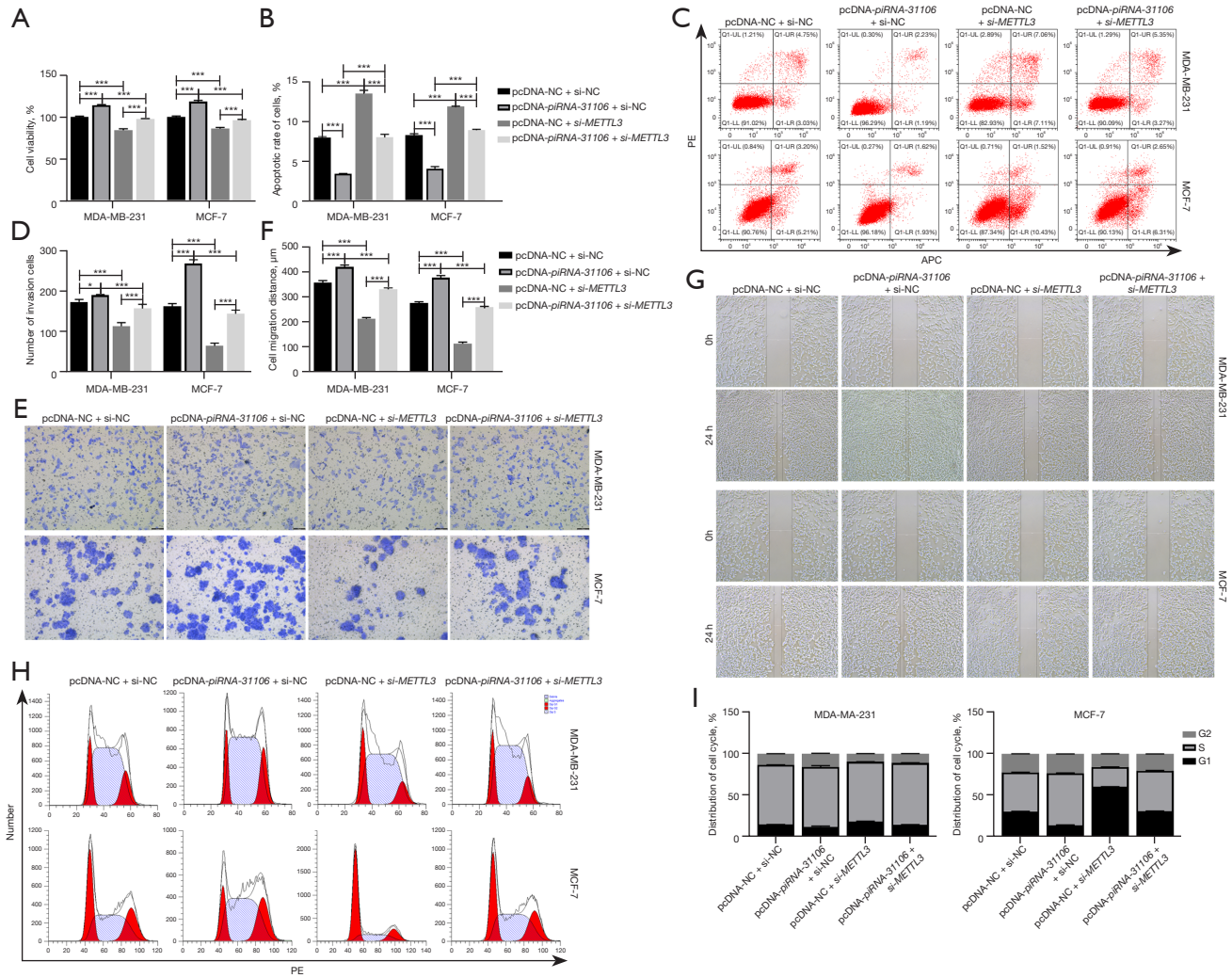

Figure 7 The effects of *METTL3* intervention on the regulation of the biological behavior of breast cancer cells by *piRNA-31106*. (A) Cell proliferation activity was detected by CCK-8. (B,C) Flow cytometry was used to detect cell apoptosis. (D,E) Transwell was used to detect the invasion ability of cells. Staining with crystal violet. Magnification ×100. (F,G) Cell migration distance was detected by scratch assay. Observation under light microscope. Magnification ×100. (H,I) Flow cytometry was used to detect cell cycle. *, P<0.05; ***, P<0.001; n=6. MDA-MB-231, human breast cancer cells; MCF, Michigan Cancer Foundation; pcDNA-NC, pcDNA-negative control; si-NC, small interfering RNA-negative control; PE, phycoerythrin; APC, allophycocyanin; G2, gap2; S, synthesis phase; G1, gap1; CCK-8, Cell Counting Kit-8.

cycle, and participate in the occurrence and development of cancers (31). In tumor tissues, increased amplification or expression of these genes can promote tumor cell proliferation, DNA damage repair, invasion and metastasis, and is an important cause of cancer (32,33). The results of this study suggest that *piRNA-31106* can promote the expression of oncogenes by up-regulating the expression of MDM2, CKD4 and cyclinD and lead to the occurrence of breast cancer.

The classical function of piRNA is mainly epigenetic methylation regulation. *m6A* methylation is the most common post-transcriptional modification in eukaryotic mRNA, which regulates the expression of proto-oncogenes and tumor suppressor genes at the epigenetic level, thus affecting the occurrence and development of tumors (34). *m6A* methylation has been found to be closely related to the development of breast cancer (35). The key proteins targeting *m6A* and *m6A* modification are expected to

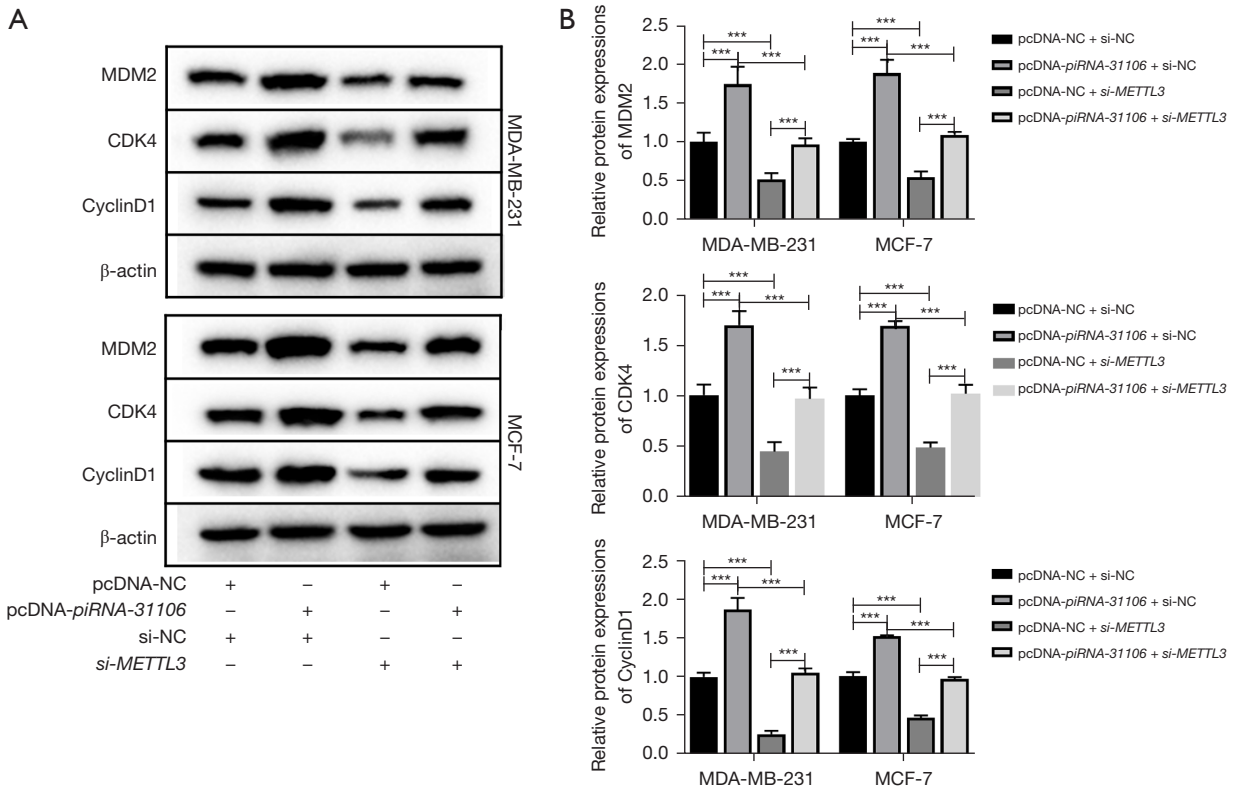

Figure 8 The effects of *METTL3* intervention on *piRNA-31106* regulation of oncogene expression. (A) The protein expressions of MDM2, CDK4, and cyclinD1 in MDA-MB-231 and MCF-7 cells transfected with different plasmids or siRNA were detected by western blot. (B) Statistical histogram of protein expressions. ***, P<0.001; n=6. pcDNA-NC, pcDNA-negative control; si-NC, small interfering RNA-negative control; MDA-MB-231, human breast cancer cells; MCF, Michigan Cancer Foundation.

become potential molecular targets for cancer diagnosis and treatment (36). Similarly, piRNA may be involved in the occurrence of tumors through the *m6A* methylation mechanism (37). This study found that *piRNA-31106* could significantly promote the *m6A* levels of breast cancer cells. The realization of *m6A* RNA methylation function first requires *m6A* modification of adenine on RNA under the action of *m6A* methyltransferase (38). *METTL3* is one of the major methyltransferases involved in the biological processes of RNA splicing, transport, translation, and degradation that is regulated by *m6A*, and is also believed to be closely related to cancers (39). Study has shown that *METTL3* is dysregulated and carcinogenic in a variety of malignant tumors (40). Knockdown of *METTL3* can effectively inhibit the proliferation, migration, and invasion ability of gastric cancer cells, and overexpression can increase its carcinogenic function (41). *METTL3* has also been shown to play an important role in breast cancer, where its expression is upregulated and *METTL3* knockout could reduce methylation levels, reduce cancer cell proliferation, accelerate apoptosis of necrotic cells, and inhibit tumor growth (42,43). This study found that *piRNA-31106* could significantly promote the gene expression of *METTL3*. In addition, there was a binding relationship between *piRNA-31106* and *METTL3*. Inhibition of *METTL3* significantly inhibited the proliferation, invasion, and metastasis of breast cancer cells, decreased the expression of MDM2, CKD4, and cyclinD, and reversed the promotional effect of *piRNA-31106* on the malignant biological behavior of breast cancer.

Conclusions

In conclusion, this study demonstrated that *piRNA-31106* was upregulated in breast cancer tissues and cell lines. Overexpression of *piRNA-31106* promoted the proliferation, invasion, and oncogene expression of breast cancer cells, and inhibited apoptosis, while interference

with *piRNA-31106* played a completely opposite role. Further studies confirmed that *piRNA-31106* promoted *METTL3* expression and *m6A* methylation in breast cancer cells. SiRNA intervention with *METTL3* antagonized the promotional effects of *piRNA-31106* on the malignant function of breast cancer cells. This study confirmed that *piRNA-31106* plays an important role in breast cancer progression by regulating *METTL3*-mediated *m6A* RNA methylation. *piRNA-31106* may be a potential target for the treatment of breast cancer.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-790/rc

Data Sharing Statement: Available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-790/dss

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-790/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the Ethics Committee of the Affiliated Hospital of Guangdong Medical University (PJKT2023-064). All patients signed an informed consent form.

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