

# Hsa-miR-221-3p promotes proliferation and migration in HER2-positive breast cancer cells by targeting LASS2 and MBD2

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**Summary.** Background. Human epidermal growth factor receptor (HER2)-positive breast cancers account for nearly 20% of all breast cancer cases and microRNAs (miRNAs) play crucial roles in disease progression. The study was aimed to explore the role of miR-221-3p in HER2-positive breast cancer.

**Methods.** Differentially expressed miRNAs were identified by high-throughput sequencing. Quantitative real-time PCR was used to evaluate mRNA levels of corresponding genes. CKK8 and transwell assays were performed to evaluate cell viability and migration. The translation binding was assessed by luciferase assay.

**Results.** Hsa-miR-221-3p was highly upregulated in HER2-positive breast cancer samples, particularly in patients with advanced or metastatic disease, as compared to healthy controls. miR-221-3p upregulation using mimics promoted cell proliferation and migration in HER2-positive cell lines, whereas miR-221-3p suppression had the opposite effect. Additionally, miR-221-3p mimics reduced the expression levels of LASS2 and MBD2 in HER2-positive breast cancer cells; conversely, miR-221-3p inhibition upregulated LASS2 and MBD2. miR-221-3p inhibited the translation of LASS2 and MBD2 by directly binding to their 3'-untranslated regions. Forced expression of LASS2 and MBD2 significantly attenuated the ability of miR-221-3p mimics to enhance cell growth and migration in HER2-positive but not in HER2-negative breast cancer cells. In HER-2-positive breast cancer patients, the levels of miR-221-3p were negatively correlated with the mRNA levels of *LASS2* and *MBD2*.

**Conclusions.** Upregulation of hsa-miR-221-3 in

HER2-positive breast cancer contributes to cancer cell proliferation and migration by directly targeting the tumor suppressors LASS2 and MBD2. Therefore, the hsa-miR-221-3 may serve as a promising and actionable therapeutic target in HER2-positive breast cancer.

**Key words:** HER2-positive breast cancer, Hsa-miR-221, LASS2, MBD2, Proliferation and migration

## Introduction

Breast cancer is the second leading cause of cancer deaths in women. Between 2012 and 2016, the incidence rate of breast cancer increased by 0.3% per year (Siegel et al., 2018; DeSantis et al., 2019). Breast cancer is a heterogeneous disease encompassing four major molecular subtypes: luminal A, luminal B, HER2-positive, and basal-like (Deng et al., 2017). Among the four subtypes, HER2-positive breast cancer is the most invasive and has the highest rate of recurrence (Witton et al., 2003; Ross et al., 2009). Although the advent of trastuzumab has notably improved the postoperative recurrence rate, the development of drug resistance remains a major clinical challenge in patients with HER-2-positive breast tumors (Wong et al., 2011; Zhang et al., 2015). Therefore, the identification of robust biomarkers and the development of effective targeted therapies for HER-2 positive breast tumors are urgent clinical needs.

Accumulating evidence suggests that the expression of certain microRNAs (miRNAs) is dysregulated in HER2-positive breast cancer; these miRNAs have a potential value as diagnostic or prognostic biomarkers (Jia et al., 2019). miR-221 plays a critical role in

**Abbreviations.** HER2, Human epidermal growth factor receptor; ER, estrogen receptor; LASS2, longevity assurance homolog 2; MBD2, Methyl-CpG-binding domain 2 protein.

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tumorigenesis, and miR-221-3p has been proposed as a potential prognostic biomarker in basal-like breast cancer (Shah and Calin, 2011; Deng et al., 2017). miR-221 is expressed at significantly higher levels in patients with basal-like breast cancer than in patients with luminal breast cancers (Stinson et al., 2011). In luminal-type breast cancer, miR-221 has been shown to regulate cell proliferation and invasion (Dentelli et al., 2014). High miR-221 expression levels have been linked to tamoxifen resistance in estrogen receptor (ER)-positive breast cancer (Yoshimoto et al., 2011; Hanna et al., 2012; Wei et al., 2014). Hence, miR-221 is considered a potential biomarker that predicts survival in patients with breast cancer and may serve as a novel therapeutic target (Eissa et al., 2015). However, the role of miR-221 in the progression of HER2-positive breast tumors remains unclear.

The expression of *Homo sapiens* longevity assurance homolog 2 (LASS2) in breast cancer is highly heterogeneous. High LASS2 expression levels are associated with a lower risk of lymph node metastasis in breast cancer patients (Wang et al., 2013). Furthermore, LASS2 is expressed at higher levels in non-invasive breast cancer cells than in highly metastatic cells (Zhang et al., 2019). LASS2 overexpression suppresses cell proliferation and invasion but enhances apoptosis in triple-negative breast cancer (Su et al., 2007). Methyl-CpG-binding domain 2 protein (MBD2) also regulates breast cancer cell growth and invasion (Cheishvili et al., 2014). MBD2 is highly expressed in triple-negative breast cancer cells (Bao et al., 2017), enhancing tumor malignancy (Teslow et al.). Interestingly, no MBD2 expression differences between ER-positive and ER-negative breast cancers have been reported (Muller et al., 2003), suggesting that MBD2 translation is not affected by ER status. Intriguingly, both LASS2 and MBD2 are potential binding targets of miR-221. miR-221 inhibition has been shown to upregulate MBD2 expression and suppress colorectal cancer cell growth and metastasis in mice (Yuan et al., 2013). Conversely, miR-221 overexpression promotes cell migration and invasion by selectively targeting MBD2 in human oral squamous cell carcinoma cells (Robert et al., 2015). Forced expression of miR-221/222 enhances cell viability in cervical cancer cells by repressing MBD2 (Pan et al., 2019). LASS2 has also been identified as a direct target of miR-221/222. miR-221/222 upregulation promotes Schwann cell proliferation and migration by directly binding to the 3'-untranslated region of LASS2, causing reduced LASS2 mRNA and protein levels (Yu et al., 2012). Nevertheless, there exist no reports on the role of MBD2 or LASS2 in the ability of miR-221-3p to regulate the progression of HER2-positive breast cancer.

In this study, we investigated the differentially expressed miRNAs in HER2-positive breast cancer cells and identified hsa-miR-221-3p as a highly expressed miRNA in this cell type. Importantly, hsa-miR-221-3p enhanced the proliferation and migration of HER2-positive breast cancer cells by directly targeting LASS2

and MBD2. These findings suggest that miR-221-3p is involved in the pathologic progression of HER2-positive breast cancer.

## Materials and methods

### Subjects and experimental design

In this study, we enrolled 50 patients with HER2-positive breast cancer and 50 healthy volunteers. Serum samples from five cancer patients and five healthy volunteers were subjected to high-throughput sequencing. Differentially expressed miRNAs were validated by quantitative real-time PCR (qRT-PCR). Thirty patients (aged between 45 and 58) with HER2-positive breast cancer were treated with Herceptin for three or six consecutive months. The levels of miR-221-3p and CA153 were determined by qRT-PCR and ELISA, respectively. Informed consent was obtained from all patients, and the study was approved by the local ethics committee of the Chinese Academy of Sciences.

### Cell culture and transfection

AU565, SKBR3, and MCF-7 cells were purchased from Shanghai Tongpai and were cultured in RPMI complete medium containing 10% fetal bovine serum (Gibco, 10099-141) at 37°C in a 5% CO<sub>2</sub> incubator. For cell transfection, cells were seeded in six-well plates at a density of 5×10<sup>5</sup> cells per well. After incubation overnight, 5 µL of lipofectamine 2000 (Invitrogen, 11668-019) were mixed in 250 µL of Opti-MEM (Gibco, 31985) at room temperature for 5 min. Subsequently, 2 µL miR-221-3p mimics (50 nM; forward: 5'-AGCUACAUGUCUGCUGGUGUUUC-3', reverse: 5'-AACCCAGCAGACAAUGUAGCUUU-3') or miR-221-3p inhibitor (100 nM; 5'-GAAACCCAGCAGACAAUGUAGCU-3') were diluted in 250 µL Opti-MEM and transfected into AU565, SKBR3, and MCF-7 cells. The sequences of the negative controls for miR-221-3p mimics and inhibitor were 5'-UUCUCCGAACGUGUCACGUTT-3' and 5'-ACGUGACACGUUCGGAGAATT-3'. For plasmid construction, LASS2 and MBD2 cDNA fragments were inserted into a pEGFP-N1 plasmid (Emamian et al., 2021). The CDS fragments were translationally fused to GFP. Empty pEGFP-N1 served as a negative control. Recombinant plasmids and miR-221-3p mimics were co-transfected into AU565 and MCF-7 cells. Tumor cell growth and invasion were assessed by CCK8 and transwell assays.

### High-throughput sequencing

High-throughput sequencing was performed in accordance with instructions from Illumina. Serum from patients and healthy volunteers and the TruSeq Small RNA Sample Prep Kit (Illumina, San Diego, USA) were used to generate small RNA sequencing libraries. Subsequently, PCR products (140-160 bp) were isolated

by 6% polyacrylamide-borate-EDTA gel electrophoresis. An Illumina HiSeq 2000/2500 was used to sequence the constructed library (reading length of 50 bp). ACGT101-miR (LC Sciences, Houston, Texas, USA) was used for miRNA data analysis.

#### qRT-PCR

TRIzol reagent (Aidlab, 252250AX) and an RNeasy Mini Kit (Qiagen) were used to extract total RNA. mRNA was reverse-transcribed using random primers and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). PCR amplification was performed using the All-in-One miRNA qRT-PCR Detection Kit (GeneCopoeia) or AceQSYBR qPCR Master Mix (Vazyme, q111-02). Reactions were analyzed on a CFX96 instrument (Bio-Rad). The sequences of the primers used are provided in Table 1. Expression data were normalized to the levels of the *U6* or *GAPDH* reference genes.

#### CCK8 assay

AU565 and MCF-7 cells transfected with negative controls, miR-221-3p mimics, miR-221-3p inhibitor alone, or miR-221-3p mimics combined with LASS2 and MBD2-overexpressing plasmids were seeded into 96-well plates. After incubation for 48 h, 10  $\mu$ L of CCK-8 solution (Biosharp, BS350B) was added to each well, and the cells were cultured at 37°C for an additional 4 h. The optical absorbance at 450 nm was then determined using a microplate reader (Thermo, Multiskan MK3).

#### Transwell assay

The migration abilities of AU565 and MCF-7 cells were assessed using transwell chambers (BD Biosciences, 353097). In brief, the bottom surface of the upper chamber was coated with 1 mg/mL Matrigel (BD, 354230). Then, 800  $\mu$ L of complete medium was added into the lower chambers. Cells (200  $\mu$ L;  $2 \times 10^5$  cells/mL) resuspended in DMEM were added to the top chamber and were allowed to migrate (37°C, 5% CO<sub>2</sub>, 24 h). After that, the upper surface of each membrane was cleaned with a cotton swab. Cells attached to the bottom surface of each membrane were stained with 0.5% crystal violet dye (Sigma) and counted using a DMR inverted microscope (Olympus, IX51). Assays were performed three times using triplicate wells.

#### Western blot analysis

Total protein was extracted using RIPA buffer. Protein concentrations were measured and adjusted to ensure equal loading onto polyacrylamide gels. Equal amounts of protein were subjected to 10% polyacrylamide gel electrophoresis and then transferred onto PVDF membranes. The membranes were incubated overnight with primary antibodies against LASS2 (Abcam, Ab176709; 1:2,000) or MBD2 (Abcam, Ab38646; 1:500). Anti-GADPH antibody (Hangzhou Xianzi Biological co., LTD., AB-P-R 001, 1:1,000) served as an internal control. The bands were visualized using an ECL luminescent reagent (Millipore, Billerica, USA) after incubation with secondary antibody for 1 h. The membranes were visualized using the ChemiDoc™ MP Imaging System (BIO-RAD).

#### ELISA

The levels of CA153 in the sera of patients with HER2-positive breast cancer after Herceptin treatment were determined by ELISA. Briefly, peripheral blood was collected in a coagulant tube and stored at 4°C overnight. Serum was obtained by centrifugation at 9,000 rpm for 3 min at 4°C. Serum was diluted in ELISA diluent, and the levels of CA153 were determined according to the ELISA kit instructions.

#### Luciferase assay

The wild-type and mutant 3'-UTR sequences of LASS2 (WT: caAGCUUGGCAUCAUGAUGUAGCa; MUT: caAGCUUGGCAUCAUGUUCUUGGa) and MBD2 (WT: gacuguuucuaaaGUGUGUAGCa or gcAAUCUACUGGA-AAUGUAGCa; MUT: gacuguuucuaaaGUGAGAACCca and gcAAUCUACUGGA-UAAGAACCca) were amplified from genomic DNA and inserted directly downstream of a stop codon in the luciferase gene of a luciferase reporter vector (pGL4-Basic system). Lipofectamine 2000 (Invitrogen) was used to transfect AU565 cells with the reporter plasmids and Renilla control plasmid. 24 h after the transfection of reporter plasmids, miRNA mimics and antagonists were transfected into cells. After 48 h of incubation, firefly and Renilla luciferase activities in the cell lysates were measured using the dual-luciferase reporter assay system (Promega, Madison, WI). The luciferase activity was measured in a Victor 3 V multilabel plate reader.

**Table 1.** Primers sequences in qPCR analysis.

| Primer name      | Forward (5'-3')         | Reverse (5'-3')        |
|------------------|-------------------------|------------------------|
| hsa-miR-221-3p-F | GCGCAGCTACATTGTCTGCTG   | GTGCAGGGTCCGAGGT       |
| U6               | CTCGCTTCGGCAGCACATATACT | ACGCTTCACGAATTTGCGTGTG |
| GAPDH-F          | GGTCTCCTCTGACTTCAACA    | GAGGTCTCTCTCTTCTCT     |
| LASS2-F          | TCTCCTGGTTTGCCAATTACG   | CCGGGCAGGGACCCTCATCA   |
| MBD2-F           | ACGAATGAATGAACAGCCACG   | TGCTACCTGGACCAACTCCT   |

(Perkin Elmer).

### Statistical analysis

The correlation between miR-221-3p and LASS2 levels in HER2-positive breast cancer was evaluated using SPSS (Version 25.0); T correlation coefficient  $r$  was used to evaluate the strength of the correlation. The data are expressed as means  $\pm$  standard deviation. Student's  $t$ -test or one-way analysis of variance (ANOVA) were used to analyze the differences between groups.  $P$ -values lower than 0.05 were considered statistically significant.

## Results

### Relationship between miRNAs and treatment outcomes in patients with HER2-positive breast cancer

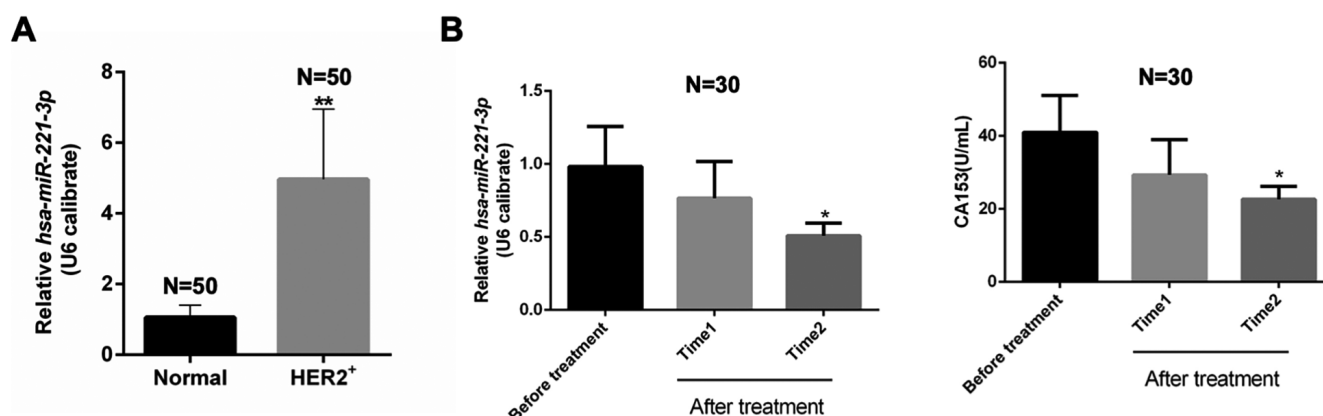
High-throughput sequencing was employed to identify differentially expressed miRNAs in the serum of healthy volunteers and of patients with HER2-positive breast cancer. We identified ten miRNAs expressed at

significantly different levels between breast cancer patients and healthy volunteers: has-miR-103a-3p\_R-2, hsa-miR-107\_R-2, hsa-let-7d-5p\_R-1, hsa-let-7a-5p\_R-1, hsa-miR-22-3p, hsa-miR-221-3p\_R-1, hsa-let-7f-5p\_R-1, hsa-miR-18a-5p\_R-3, hsa-miR-142-5p, and hsa-miR-144-3p (Table 2). The expression levels of these miRNAs were significantly associated with the proliferation and invasion of tumor cells. Among the differentially expressed miRNAs, hsa-miR-221-3p exhibited the most drastic differences between the two groups; hence, hsa-miR-221-3p was selected for subsequent validation in a larger number of clinical samples. The serum levels of hsa-miR-221-3p in patients with HER2-positive breast cancer were four times higher than those in healthy volunteers (Fig. 1A). To further assess the prognostic value of hsa-miR-221-3p in patients with HER2-positive breast cancer, we enrolled 30 patients treated with Herceptin for HER2-positive breast cancer. The serum levels of hsa-miR-221-3p and a known prognostic factor, CA153, were significantly decreased by Herceptin treatment in a time-dependent manner (Fig. 1B). Patients with advanced disease (grades III and IV) had higher miR-221 levels than

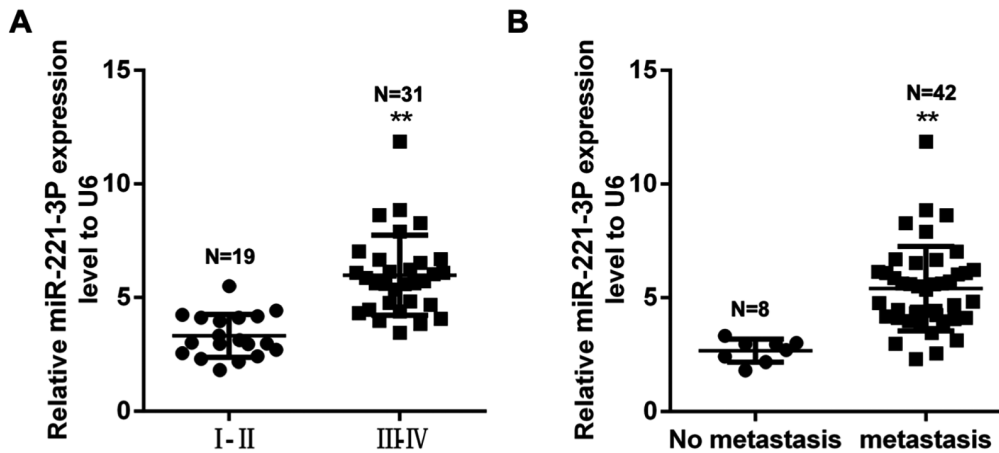
**Table 2.** Differentially expressed miRNAs.

| miR_name            | miR_seq                | up/down | fold_change | pvalue(T_test) | Con(mean) | Test(mean) |
|---------------------|------------------------|---------|-------------|----------------|-----------|------------|
| hsa-miR-103a-3p_R-2 | AGCAGCATTGTACAGGGCTAT  | down    | 0.56        | 2.58E-03       | 3,692     | 2,064      |
| hsa-miR-107_R-2     | AGCAGCATTGTACAGGGCTAT  | down    | 0.56        | 2.58E-03       | 3,692     | 2,064      |
| hsa-let-7d-5p_R-1   | AGAGGTAGTAGTTGCATAGT   | down    | 0.45        | 1.19E-02       | 1,890     | 855        |
| hsa-let-7a-5p_R-1   | TGAGGTAGTAGTTGTATAGT   | down    | 0.51        | 1.25E-02       | 3,305     | 1,689      |
| hsa-miR-221-3p_R-1  | AGCTACATTGTCTGCTGGGTTT | up      | 1.68        | 1.26E-02       | 969       | 1,624      |
| hsa-miR-22-3p       | AAGCTGCCAGTTGAAGAACTGT | up      | 1.32        | 2.58E-02       | 5,541     | 7,337      |
| hsa-let-7f-5p_R-1   | TGAGGTAGTAGATTGTATAGT  | down    | 0.56        | 3.45E-02       | 2,806     | 1,569      |
| hsa-miR-18a-5p_R-3  | TAAGGTGCATCTAGTGCAGA   | down    | 0.55        | 3.58E-02       | 1,189     | 652        |
| hsa-miR-142-5p      | CATAAAGTAGAAAGCACTACT  | down    | 0.59        | 3.96E-02       | 4,230     | 2,491      |
| hsa-miR-144-3p      | TACAGTATAGATGATGTA     | down    | 0.55        | 4.87E-02       | 53,527    | 29,527     |

Note: con(mean) and test(mean) represented for the number of reads in control group and HER2-positive samples.



**Fig. 1.** Association of serum hsa-miR-221-3p with HER2-positive breast cancer patients. **A.** Serum hsa-miR-221-3p in HER-2-positive breast cancer patients and control samples were analyzed by qRT-PCR. N=50. **B.** The expression levels of hsa-miR-221-3p and CA153 in serum of HER-2-positive breast cancer patients after treatment with Herceptin (N=30). One-way ANOVA and t-test were used for data statistics, and the error line represented standard deviation (SD). \* means  $p < 0.05$  when comparing before treatment group vs after treatment group. \*\* means  $p < 0.01$  when comparing HER-2 positive vs normal group. Normal means healthy, and HER-2+ means HER-2 positive breast cancer patients.



**Fig. 2.** Associations between miR-221 and tumor stage and metastasis of HER2-positive breast cancer patients. **A.** HER2-positive breast cancer patients with grade I/II (N=19) and grade III/IV (N=31) were enrolled and divided into two groups according to tumor grade, and expression analysis of miR-221 as conducted by qRT-PCR. **B.** Metastatic HER2-positive breast cancer patients (N=42) and non-metastatic patients (N=8) were enrolled and divided into two groups according to metastasis status, and expression analysis of miR-221 as conducted by qRT-PCR. \*\* represents  $p < 0.01$ .

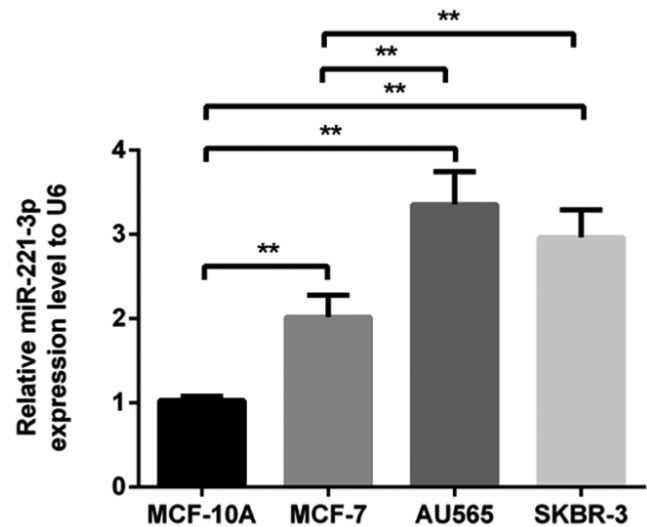
patients with early-stage tumors (grades I and II). Additionally, miR-221 levels were significantly higher in patients with metastatic disease than in patients with no metastases (Fig. 2A-2B). These data suggest that hsa-miR-221-3p may be a useful diagnostic and prognostic factor in patients with HER2-positive breast cancer.

#### *Hsa-miR-221-3p enhances the proliferation and migration of HER2-positive breast cancer cells*

miR-221 was highly expressed in breast cancer cells, particularly in the HER2-positive breast cancer cell lines AU565 and SKBR3 (Fig. 3A). To elucidate the role of hsa-miR-221-3p in HER2-positive breast cancer cells, we used hsa-miR-221-3p mimics and inhibitors to modulate hsa-miR-221-3p expression levels in AU565 cells (Fig. 4A). Increased levels of hsa-miR-221-3p significantly enhanced cell viability in HER2-positive cells (Fig. 4B). Conversely, hsa-miR-221-3p inhibition profoundly inhibited cell growth in AU565 and SKBR3 cells (Fig. 4B). Similarly, hsa-miR-221-3p mimics significantly enhanced cell migration, whereas hsa-miR-221-3p inhibition had the opposite effect (Fig. 4C and Fig. 3A-B). These findings suggest that hsa-miR-221-3p increases the malignancy of HER2-positive cells.

#### *Hsa-miR-221-3p inhibits the expression of LASS2 and MBD2 in HER2-positive breast cancer cells*

MBD2 and LASS2, commonly regulated by miRNAs, are involved in the proliferation and migration of breast cancer cells (Mian et al., 2011; Fan et al., 2015). LASS2 mRNA levels were decreased by hsa-miR-221-3p mimics and increased by the hsa-miR-221-3p inhibitor (Fig. 5A). Similar trends were observed for MBD2 mRNA levels (Fig. 5B). Consistently, hsa-miR-221-3p mimics significantly reduced the levels of LASS2 and MBD2 proteins; conversely, ablation of hsa-miR-221-3p markedly increased the levels of LASS2 and MBD2 proteins in AU565 and SKBR3 cells (Fig. 5C-D). Given that LASS2 and MBD2 regulate cell

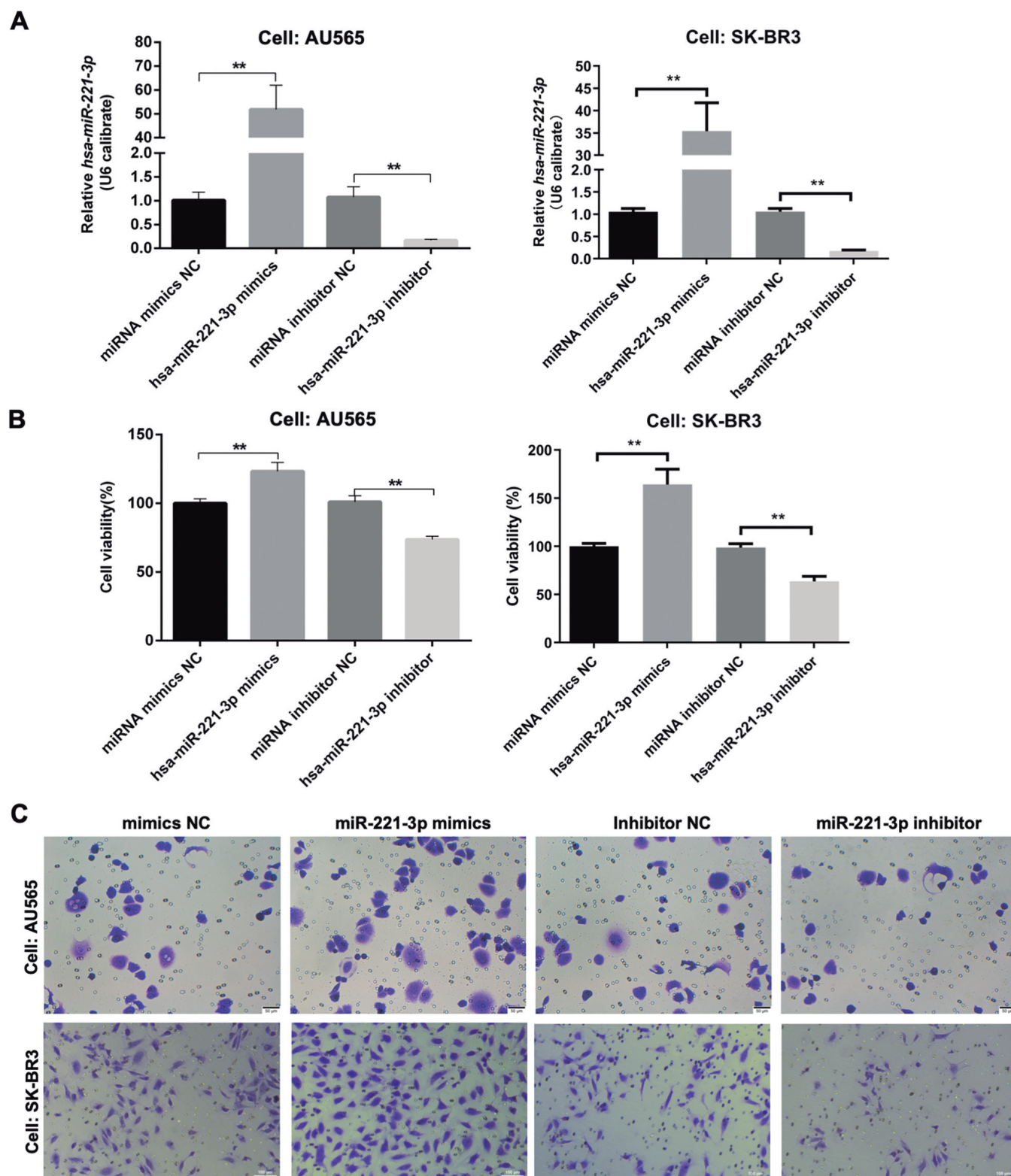


**Fig. 3.** Regulatory role of hsa-miR-221-3p on the expression of LASS2 and MBD2 in SKBR3 cells. miR-221 expression in normal mammary epithelial cells MCF-10A, MCF-7, AU565 and SKBR3 cells as determined by qRT-PCR. \*\* represents  $p < 0.01$ .

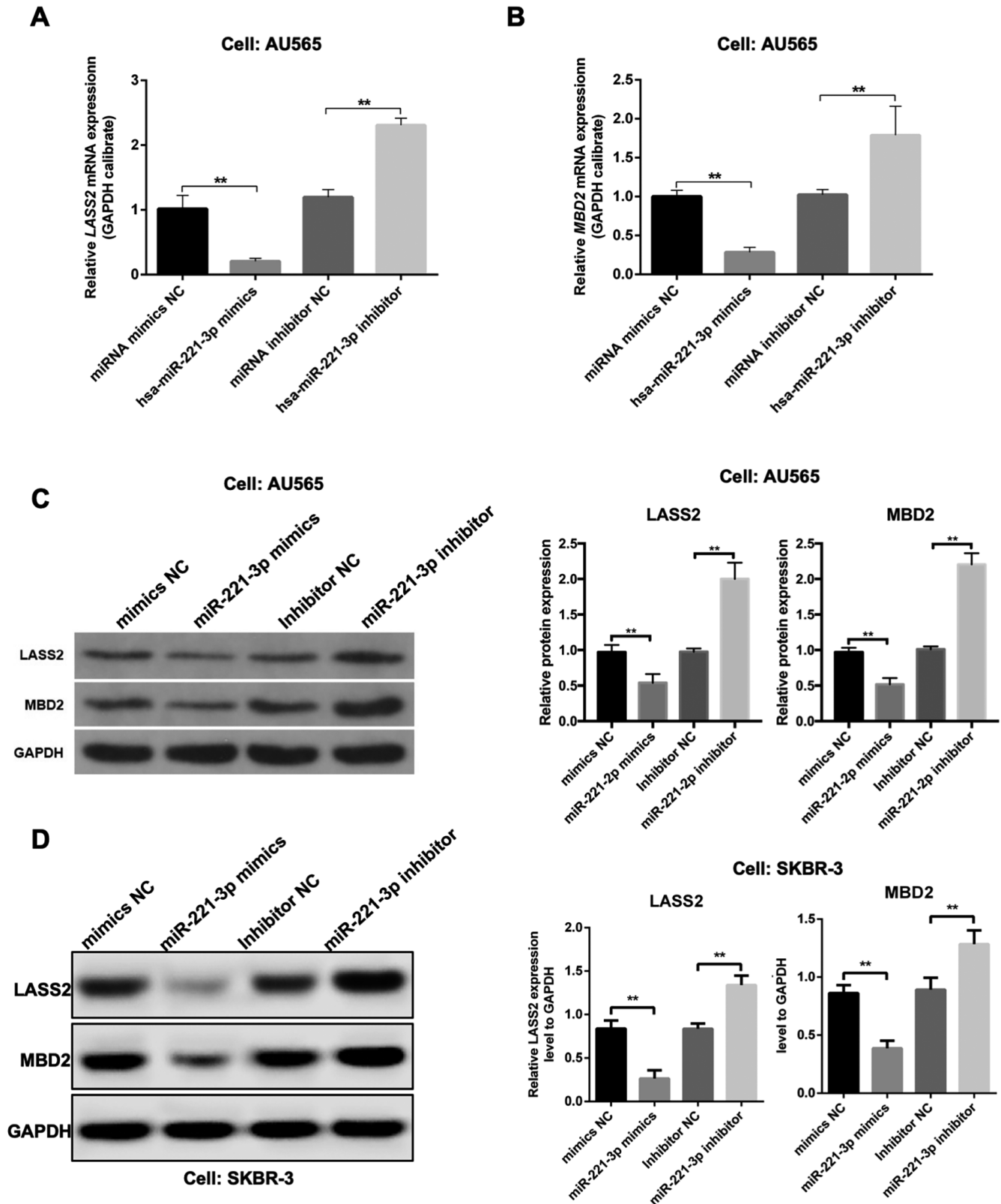
viability and migration, the effects of hsa-miR-221-3p on the malignancy of HER2-positive breast cancer cells might be mediated by LASS2 and MBD2.

#### *Hsa-miR-221-3p directly targets the 3'-untranslated regions of LASS2 and MBD2*

Hsa-miR-221-3p regulates cell proliferation and migration by targeting MBD2 and LASS2 in various types of cells. Since hsa-miR-221-3p affected the expression levels of MBD2 and LASS2 in HER2-positive breast cancer cells, we next explored the relationship between hsa-miR-221-3p, MBD2, and LASS2 using plasmids expressing the wild-type or mutant 3'-UTRs of MBD2 or LASS2. Luciferase assay revealed no differences in luciferase activity when cells



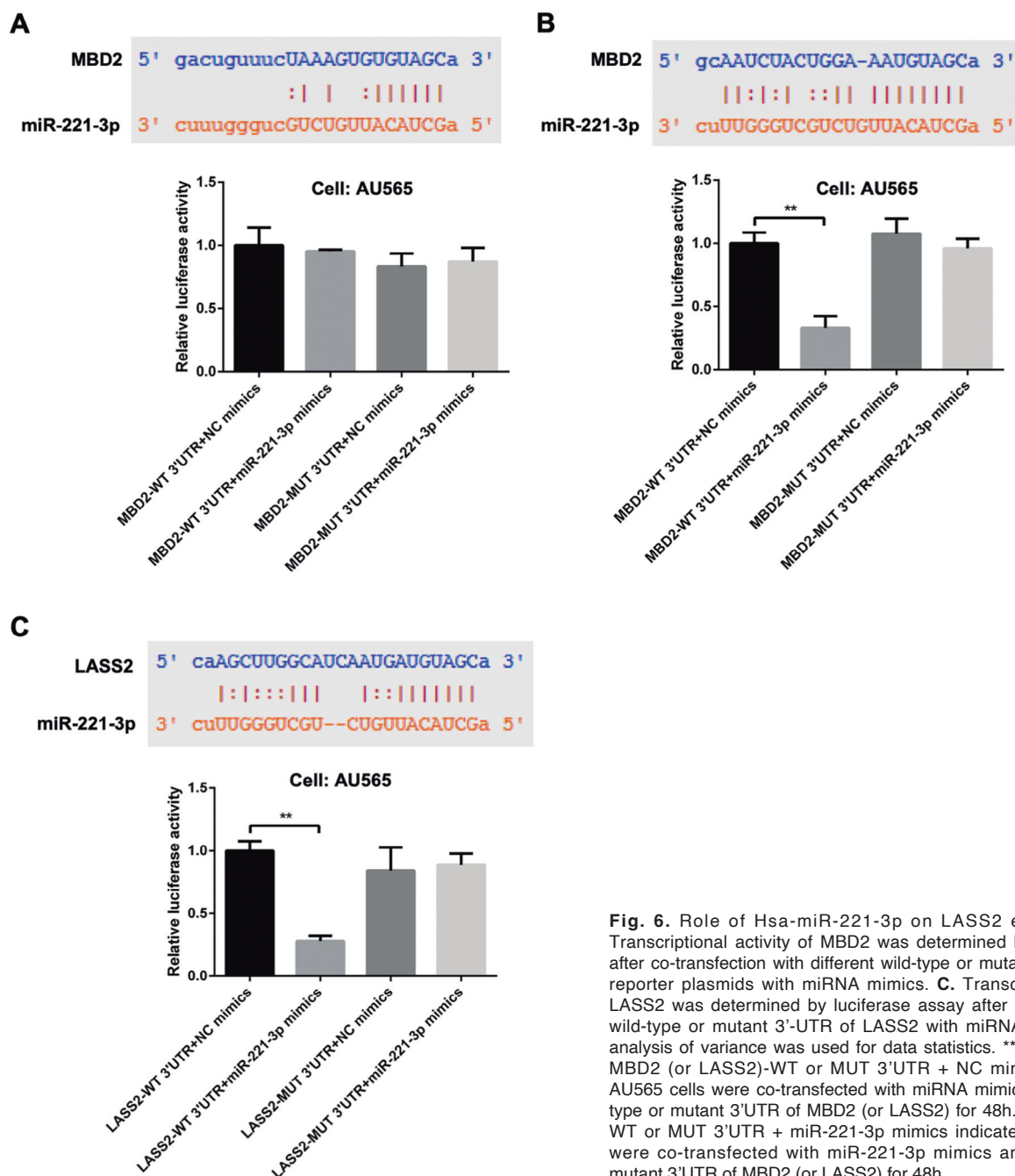
**Fig. 4.** Effects of hsa-miR-221-3p on proliferation and invasion of HER-2-positive breast cancer cells. **A.** Validation of hsa-miR-221-3p expression as measured by qRT-PCR in cells transfected with hsa-miR-221-3p mimics and inhibitor. **B.** Cell viability as detected by CCK8 assay in AU565 and SKBR3 cells. **C.** Detection of cell invasion capacity in AU565 and SKBR3 cells after hsa-miR-221-3p mimics and inhibitor of transfection. One-way analysis of variance was used for data statistics. \*\* represents  $p < 0.01$ . miRNA mimics NC, hsa-miR-221-3p mimics, inhibitor NC and hsa-miR-221-3p inhibitor indicated that AU565 cells were transfected with corresponding small RNAs for 48h. Scale bars: 50  $\mu$ m.



**Fig. 5.** Regulatory role of hsa-miR-221-3p on the expression of LASS2 and MBD2. Detection of LASS2 mRNA (**A**) and MBD2 mRNA (**B**) in AU565 cells transfected with hsa-miR-221-3p mimics, inhibitor and their negative control miRNA. **C.** AU565 cells were transfected with negative control mimics, miR-221-3p mimics, negative inhibitor and miR-221-3p inhibitor. Subsequently, LASS2 and MBD2 protein as measured by western blotting. Relative quantitative analysis of protein expression of LASS2 and MBD2 in panel C as presented on the right. **D.** SKBR3 cells were transfected with negative control mimics, miR-221-3p mimics, negative inhibitor and miR-221-3p inhibitor. Subsequently, LASS2 and MBD2 protein levels as measured by western blotting in the above group cells. Relative quantitative analysis of protein expression of LASS2 and MBD2 in panel D as presented on the right. One-way analysis of variance was used for data statistics. \*\* represents  $p < 0.01$ .

were co-transfected with miR-221-3p mimics and the wild-type or mutant 3'-UTR of MBD2 (gacuguuucuaaa GUGUGUAGCa) (Fig. 6A). However, different results were obtained when another MBD2 reporter plasmid (gcAAUCUACUGGA-AAUGUAGCa) was used. The relative luciferase activity was lower in cells transfected with the wild-type 3'-UTR of MBD2; this trend was not observed in cells transfected with the mutant 3'-UTR of

MBD2 (Fig. 6B). Co-transfection of cells with the miR-221-3p precursor and luciferase constructs containing the wild-type 3'-UTR of LASS2 resulted in a lower luciferase activity than that in control cells; miR-221-3p mimics had no effect on the transcriptional activity of mutant LASS2 3'-UTR (Fig. 6C). These results indicate that miR-221-3p negatively regulates LASS2 and MBD2 expression by binding to their 3'-UTRs.

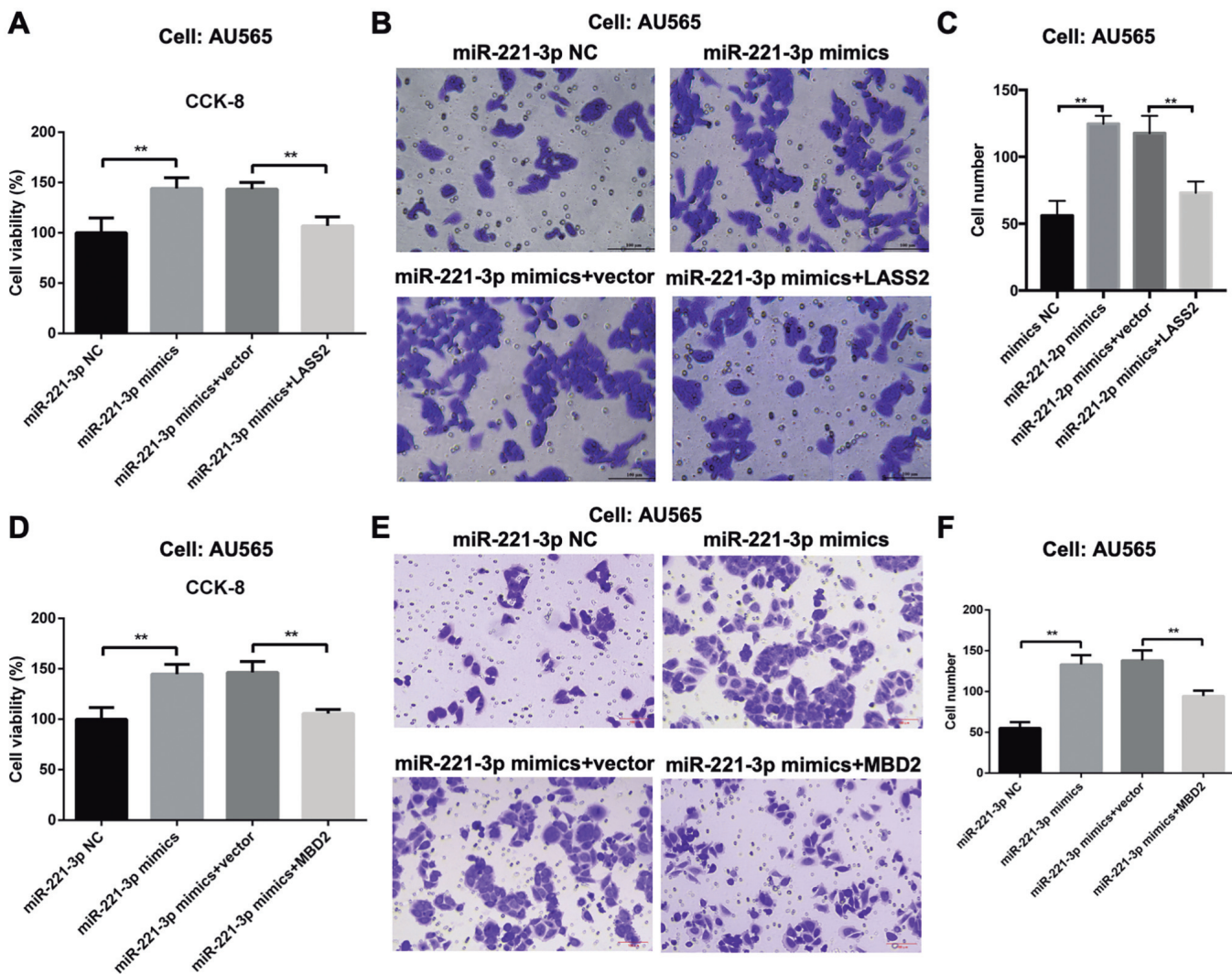


**Fig. 6.** Role of Hsa-miR-221-3p on LASS2 expression. **A, B.** Transcriptional activity of MBD2 was determined by luciferase assay after co-transfection with different wild-type or mutant 3'-UTR of MBD2 reporter plasmids with miRNA mimics. **C.** Transcriptional activity of LASS2 was determined by luciferase assay after co-transfection with wild-type or mutant 3'-UTR of LASS2 with miRNA mimics. One-way analysis of variance was used for data statistics. \*\* represents  $p < 0.01$ . MBD2 (or LASS2)-WT or MUT 3'UTR + NC mimics indicated that AU565 cells were co-transfected with miRNA mimics NC and the wild-type or mutant 3'-UTR of MBD2 (or LASS2) for 48h. MBD2 (or LASS2)-WT or MUT 3'UTR + miR-221-3p mimics indicated that AU565 cells were co-transfected with miR-221-3p mimics and the wild-type or mutant 3'-UTR of MBD2 (or LASS2) for 48h.

### Upregulation of LASS2 and MBD2 reverses hsa-miR-221-3p-induced cell proliferation and migration

Compared with control AU565 cells, cells transfected with miR-221-3p mimics exhibited increased cell viability (Fig. 7A). However, forced expression of LASS2 significantly reversed the ability of miR-221-3p mimics to increase cell viability (Fig. 7A). Forced expression of LASS2 also attenuated the ability of miR-221-3p mimics to increase cell migration (Fig. 7B-C). Similarly, MBD2 overexpression suppressed hsa-miR-

221-3p-induced cell proliferation and migration (Fig. 7D-F). In contrast, compared with control cells, MCF-7 (HER2-negative) cells transfected with miR-221-3p mimics exhibited increased cell viability (Fig. 8A). However, forced expression of LASS2 did not alter cell viability in cells transfected with miR-221-3p mimics (Fig. 8A). miR-221-3p mimics enhanced MCF-7 cell migration, and LASS2 forced expression had no effect on the ability of miR-221-3p to regulate cell migration (Fig. 8B-C). Similarly, MBD2 overexpression failed to attenuate the ability of miR-221-3p mimics to increase



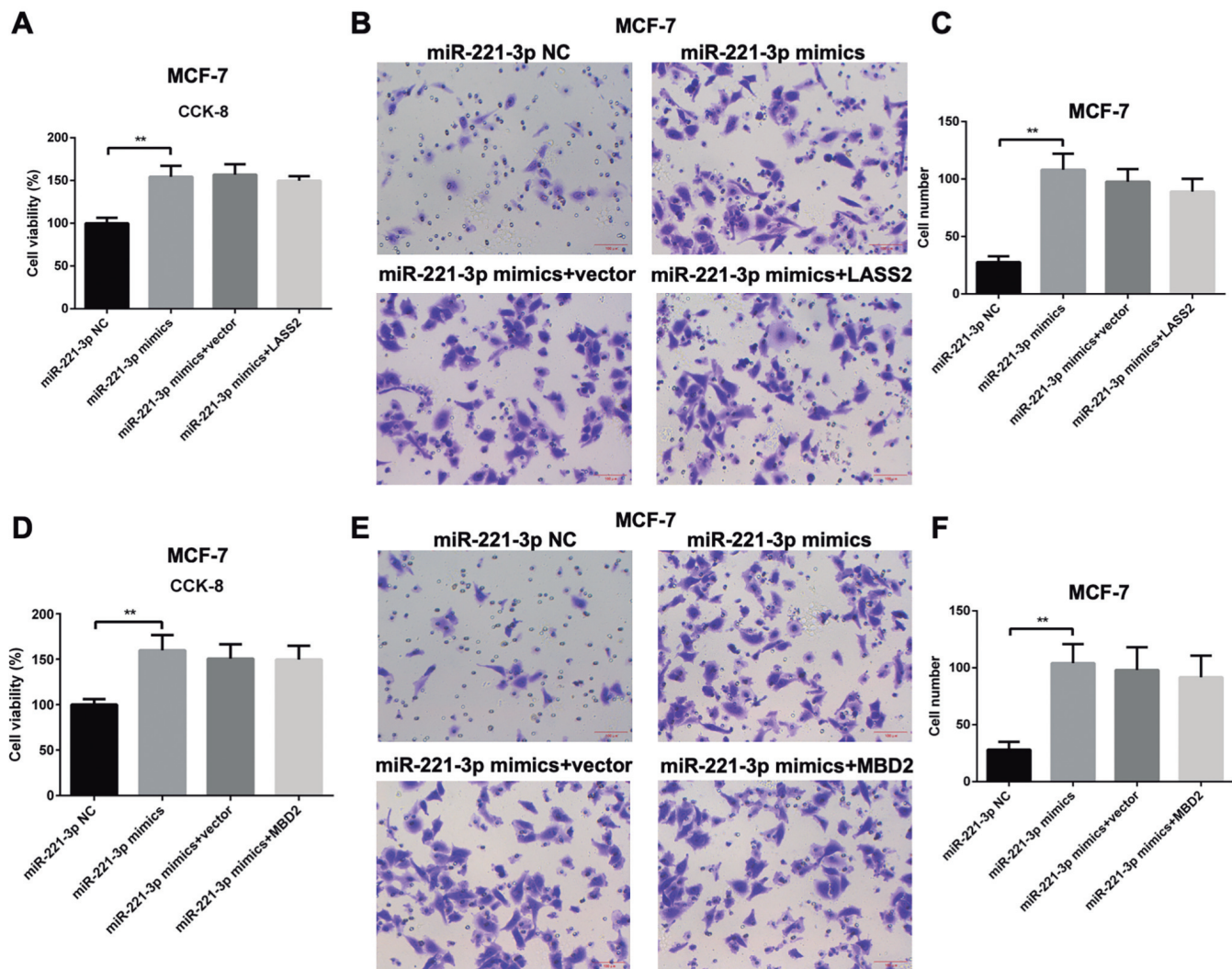
**Fig. 7.** Effects of LASS2 and MBD2 on hsa-miR-221-3p-mediated cell proliferation and migration. **A.** Detection of cell proliferation by using CCK-8 assay in cells transfected with miR-221-3p mimics alone or miR-221-3p mimics combined with LASS2-overexpressed plasmid. **B.** Detection of cell invasion capacity in cells after hsa-miR-221-3p mimics alone and the combination of hsa-miR-221-3p mimics and LASS2-overexpressed plasmid. **C.** Statistics of the number of migration cells. 6 different horizons were chosen in each group. **D.** Measurement of cell viability by CCK-8 assay in cells transfected with miR-221-3p mimics alone or miR-221-3p mimics combined with MBD2-overexpressed plasmid. **E.** Detection of cell migration capacity in cells transfected with hsa-miR-221-3p mimics alone and the combination of hsa-miR-221-3p mimics and MBD2-overexpressed plasmid. **F.** The number of migration cells in panel E. 6 different horizons were chosen in each group. One-way analysis of variance was used for data statistics. \*\* represents  $p < 0.01$ . miR-221-3p NC indicated that AU565 cells were transfected with miR-221-3p NC for 48h. miR-221-3p mimics or mimics NC indicated that AU565 cells were transfected with miR-221-3p mimics or negative control for 48h. The combination of miR-221-3p mimics and vector indicated that AU565 cells were co-transfected with miR-221-3p mimics and empty vector for 48h. miR-221-3p mimics and LASS2 showed that AU565 cells were co-transfected with miR-221-3p mimics and LASS2-overexpressed plasmid for 48h. Scale bars: 100  $\mu$ m.

cell viability and migration in HER2-negative breast cancer cells (Fig. 8D-F). These data suggest that LASS2 and MBD2 downregulation is possibly required for the oncogenic effects of miR-221-3p in HER2-positive but not HER2-negative cells.

*Hsa-miR-221-3p is negatively correlated with LASS2 and MBD2 in HER-2-positive breast cancer patients*

Next, we evaluated the relationship between miR-

221-3p and LASS2 in patient samples. The mRNA levels of *LASS2* in HER2-positive breast tumors from 18 patients were significantly lower than the *LASS2* mRNA levels in non-malignant tissues (Fig. 9A). Moreover, the *LASS2* mRNA levels were negatively correlated with the hsa-miR-221-3p levels in HER2-positive patients ( $R=-0.432$ ,  $P=0.042$ ; Fig. 9B). Similarly, the *MBD2* mRNA levels were lower in HER2-positive tumor tissues than in normal tissues and were negatively associated with the hsa-miR-221-3p levels in patients with HER2-positive



**Fig. 8.** Effects of LASS2 and MBD2 on hsa-miR-221-3p-mediated cell proliferation and migration in MCF-7 cells. **A.** Detection of cell proliferation by using CCK-8 assay in cells transfected with miR-221-3p mimics alone or miR-221-3p mimics combined with LASS2-overexpressed plasmid. **B.** Detection of cell invasion capacity in cells after hsa-miR-221-3p mimics alone and the combination of hsa-miR-221-3p mimics and LASS2-overexpressed plasmid. **C.** Statistics of the number of migration cells. 6 different horizons were chosen in each group. One-way analysis of variance was used for data statistics. **D.** Measurement of cell viability by CCK-8 assay in cells transfected with miR-221-3p mimics alone or miR-221-3p mimics combined with MBD2-overexpressed plasmid. **E.** Detection of cell migration capacity in cells transfected with hsa-miR-221-3p mimics alone and the combination of hsa-miR-221-3p mimics and MBD2-overexpressed plasmid. **F.** The number of migration cells in panel E. 6 different horizons were chosen in each group. \*\* represents  $p<0.01$ . miR-221-3p NC indicated that MCF-7 cells were transfected with miR-221-3p NC for 48h. miR-221-3p mimics or mimics NC indicated that MCF-7 cells were transfected with miR-221-3p mimics or negative control for 48h. The combination of miR-221-3p mimics and vector indicated that MCF-7 cells were co-transfected with miR-221-3p mimics and empty vector for 48h. miR-221-3p mimics and LASS2 showed that MCF-7 cells were co-transfected with miR-221-3p mimics and LASS2-overexpressed plasmid for 48h. Scale bars: 100  $\mu$ m.

breast cancer ( $R=-0.359$ ,  $P=0.002$ ; Fig. 9C-D). Hence, LASS2 and MBD2 are downstream targets of miR-221-3p that regulate the development and progression of HER2-positive breast tumors.

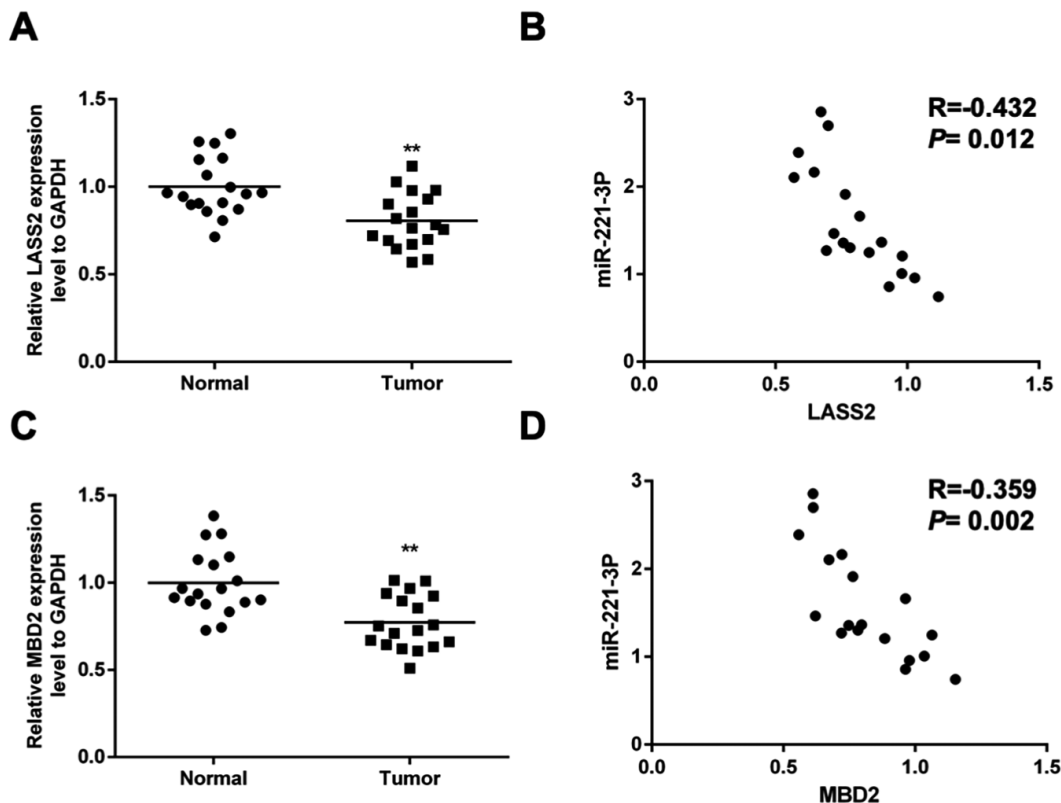
## Discussion

The vast majority of HER2-positive breast cancers eventually progress after treatment with anti-HER2 agents due to *de novo* or acquired resistance (Ahmed et al., 2015; Pernas and Tolane, 2019). Additionally, anti-HER2 therapies offer limited clinical benefit because breast cancer is a multigenic disease (Asif et al., 2016). Although targeted therapies for HER2-positive breast cancer are effective, they cause significant toxicity. Here, we provide compelling evidence that hsa-miR-221-3p may be a promising therapeutic target in patients with HER2-positive breast cancer.

Numerous miRNAs have shown significant diagnostic or prognostic value in HER2-positive breast cancer thus far (Tashkandi et al., 2015; Van Schooneveld et al., 2015). Notably, miR-21 knockdown reduced the proliferation and tumorigenic potential of HER2-positive breast cancer cells (Liu et al., 2018). The expression levels of miR-520d and miR-376b accurately detected the presence of early-stage HER2-positive breast tumors (Lowery et al., 2009). A serum-based miRNA signature predicted the clinical benefit of trastuzumab in patients with metastatic HER2-positive breast cancer (Li et al.,

2018). Dicer, a key component of the miRNA processing machinery, has been implicated in the progression of HER2-positive breast cancer (Kian et al., 2018) and can be used as a predictive marker for metastasis-free survival (Grellet et al., 2009). In this study, we used high-throughput screening to identify hsa-miR-221-3p as a promising miRNA candidate to distinguish HER2-positive breast cancer from other breast cancer subtypes. A previous study showed that in patients with HER2-positive breast cancer, the CA153 levels were reduced after Herceptin therapy, suggesting that CA153 can be used as an indicator of treatment response in patients undergoing Herceptin treatment for HER2-positive breast cancer (Liang et al., 2017). In this study, we found that in addition to CA153, the levels of miR-221-3p gradually decreased during the course of Herceptin treatment. Therefore, miR-221-3p may represent a promising indicator of treatment response in patients with HER2-positive breast cancer treated with Herceptin.

miR-221 regulates numerous oncogenic pathways and has been implicated in the development of various types of cancer (Song et al., 2017). Notably, miR-221 overexpression strongly enhances cell proliferation by directly targeting p27Kip1, ER, and PTEN. Additionally, by binding uPAR isoform 2, miR-221 overexpression promotes cell invasion in breast cancer (Falkenberg et al., 2013). Induced miR-221 transcription by Slug promotes breast cancer progression by targeting the open



**Fig. 9.** The correlation between hsa-miR-221-3p and LASS2 in HER-2-positive breast cancer patients. **A.** LASS2 expression in tumor tissues or paired para-carcinoma tissues of HER-2-positive breast cancer patients were determined by qRT-PCR. **B.** The correlation between miR-221-3p and LASS2 was analyzed by SPSS software. **C.** MBD2 expression in tumor tissues or paired para-carcinoma tissues of HER-2-positive breast cancer patients were detected by qRT-PCR. **D.** The correlation between miR-221-3p and MBD2 was analyzed by SPSS software. \*\* represents  $p < 0.01$ . Normal means paired para-carcinoma tissues and tumor means HER-2 positive breast cancer tissues.

reading frame of E-cadherin, thereby reducing E-cadherin protein levels (Pan et al., 2016). miR-221 upregulated by the basic leucine zipper transcription factor FOSL1 also increases cell migration and invasion by binding to the 3'-UTR of the trichorhinophalangeal 1 syndrome (*TRPS1*) gene and promoting the epithelial-to-mesenchymal transition in basal-like breast cancer (Stinson et al., 2011a,b). Furthermore, miR-221 enhances breast cancer cell growth, migration, invasion, and self-renewal by targeting the PTEN/Akt pathway (Li et al., 2016). In the present study, hsa-miR-221-3p, a splice variant of miR-221, enhanced cell proliferation and migration in HER2-positive breast cancer cell lines. These results suggest that hsa-miR-221-3p plays a critical role in the development and progression of HER2-positive breast tumors; however, the mechanisms underlying the oncogenic roles of hsa-miR-221-3p in HER2-positive breast cancer merit further investigation.

LASS2 inhibits breast cancer cell growth and invasion by regulating vacuolar ATPase activity (Fan et al., 2013; Mei et al., 2015). Additionally, LASS2 regulates breast cancer chemosensitivity by inhibiting the V-ATPase activity of proton pumps and modulating the acidic tumor microenvironment (Fan et al., 2013). LASS2 has also been implicated in the ability of AGPAT9 to inhibit oncogenesis by regulating the KLF4/LASS2/V-ATPase signaling pathway in breast cancer cells (Fan et al., 2015a,b). Notably, the expression of shRNA-resistant MBD2 in breast cancer cells restores cell growth and downregulated tumor suppressor genes (Mian et al., 2011). MBD2 also regulates breast cancer metastasis by affecting the methylation status of various genes. These data indicate that LASS2 and MBD2 are important regulators of breast cancer development and progression. Interestingly, a previous study indicates MBD2 acts as an oncogene in TNBC cells (Bao et al., 2017). Due to the absence of estrogen receptor (ER), progesterone receptor (PR) and Her2/neu in TNBC cells, we speculated that the difference in MBD2 expression between TNBC and HER2-positive tumor tissues is possibly because HER2 accumulation in tumor tissues indirectly inhibits the expression of MBD2.

In this study, we also showed that hsa-miR-221-3p inhibited the expression of LASS2 and MBD2 by binding directly to the 3'-UTR of LASS2 and MBD2 and destabilizing their mRNAs. Interestingly, the ability of hsa-miR-221-3p to regulate MBD2 expression was dependent on the 3'-UTR sequence of the *MBD2* gene. Additionally, overexpression of LASS2 and MBD2 attenuated the pro-tumorigenic effects of miR-221-3p in HER2-positive breast cancer cells. Therefore, we believe that hsa-miR-221-3p enhances the malignancy of HER2-positive breast cancer cells by directly targeting the expression of LASS2 and MBD2 or indirectly by suppressing MBD2 expression via the LASS2-mediated ATPase pathway. Numerous miRNAs, including hsa-miR-107 and miR-4734, have a prognostic potential in HER2-positive breast cancer (Gao et al., 2017;

Sareyeldin et al., 2019). MMP11, CD2, and p95-HER2 can also be used as prognostic biomarkers in patients with HER2-positive breast cancer (Han et al., 2017; Maria et al., 2018). In this study, we found that miR-221-3p levels were strongly associated with response to Herceptin in patients with HER2-positive breast cancer. miR-221-3p expression was elevated in patients with advanced or metastatic HER2-positive breast cancer. Additionally, we observed a negative correlation between the levels of miR-221-3p and those of LASS2 and MBD2 in patients with HER2-positive breast cancer, suggesting that miR-221-3p and its downstream targets LASS2 and MBD2 can be used as prognostic markers in patients with HER2-positive breast cancer. However, the miR-221-3p/LASS2 and miR-221-3p/MBD2 signaling pathways in regulating tumor biology were only observed in HER2-positive breast cancer cells, but not in MCF-7 cells. The difference between the cell lines is the expression status of HER2, with a low level (or no expression) of HER2 in MCF-7 cells (Funakoshi et al., 2020). Thus, the difference between the two cell lines possibly is due to the presence of HER2.

In conclusion, hsa-miR-221-3p overexpression promoted cell growth and migration in HER2-positive breast cancer cell lines by targeting the expression of LASS2 and MBD2. The findings of this study provide strong evidence that hsa-miR-221-3p is a promising target for the treatment of HER2-positive breast tumors.

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**Author's contribution.** ZYB conceived the idea and designed the project; SXY and HY performed the in vitro experiments; LGL and ZWB performed the Transwell assay; SXY performed the qRT-PCR and ELISA assays; LGL determined the cell viability measurement. ZYB and SXY drafted the text; All authors edited and approved the final manuscript.

**Ethics approval and consent to participate.** All animal studies were approved according to institutional guidelines for laboratory animals. All experiments were approved by the local ethics committee of the Chinese Academy of Sciences.

**Conflicts of interest.** The authors declare that they have no competing interests.

**Data Availability.** All data generated or analyzed during this study are included in this published article and its additional files.

**Consent for publication.** Not applicable.

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