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Upregulation of MIIP regulates human breast cancer proliferation, invasion and migration by mediated by IGFBP2

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ABSTRACT

Aims: The migration and invasion inhibitory protein (MIIP) was initially discovered in a yeast two-hybrid screen for proteins that interact and inhibit the migration and invasion-promoting protein insulin-like growth factor binding protein 2 (IGFBP2). This study aims to evaluate the biological effects of MIIP in breast cancer by targeting IGFBP2.

Materials and methods: Reverse transcription quantitative real-time polymerase chain reaction and Western blotting were used to evaluate the abnormal expression of MIIP and IGFBP2 in breast cancer tissue or breast cancer cell lines. Transfection assay was used to overexpress MIIP protein in breast cancer cells. MTT assay and colony formation assay were used to detect cell viability of breast cancer cells after MIIP overexpression. Transwell and wound-healing assays were used to detect cell invasion and migration after MIIP overexpression.

Results: MIIP was significantly decreased and IGFBP2 was significantly increased in breast cancer tissues versus para cancerous. Breast cancer tissues of HER2 overexpression and Basal-like were more significant than Luminal A and Luminal B. MIIP was obviously downregulated and IGFBP2 was upregulated in MDA-MB-231, SKBR3 and MCF-7 versus MCF-10A especially in MDA-MB-231. Cell proliferation, cell migration and cell invasion were significantly inhibited after overexpression of MIIP. IGFBP2 was downregulated after overexpression of MIIP. The effects of MIIP on cell proliferation, cell migration and invasion were significantly reversed by IGFBP2.

Conclusion: The abnormal expression of MIIP in breast cancer affects the cell biological effects. IGFBP2 was regulated via MIIP which may be associated with these biological effects. These results reveal that MIIP can be a potential target for breast cancer treatment.

1. Introduction

The incidence and mortality of Breast cancer is the second most commonly cancer overall, especially in higher income countries [1,2]. The long-term researches demonstrated that the recurrence rate and mortality of breast cancer remains quite unpredictable. Poor differentiation of tumors, ER-negativity in particular, defines poor prognosis even after applying aggressive therapies [3].

The migration and invasion inhibitory protein (MIIP), also called invasion inhibitory protein 45 (IIp45), has emerged as a novel tumor-suppressor gene that regulates cell growth and metastasis [4,5]. MIIP was firstly identified in a yeast two hybrid screen that interact and inhibit insulin-like growth factor binding protein 2 (IGFBP2) [6]. The MIIP is deleted most frequently in a wide spectrum of human cancers, which is located on chromosome 1p36.22 [7–10]. Researches indicate that cell migration and mitosis is related to MIIP expression [5]. Additionally, MIIP has a tumor suppressor activity in a mouse model of glioma [4]. Recent studies has validated that MIIP inhibits many kinds of factors such as IGFBP-2, protein kinase C (PKC) ε and subsequently mediates metastasis in tumor cell [6,11]. Previous investigations have indicated that the occurrence of allelic deletion in MIIP gene and loss of gene expression is possibly associated with progression of breast cancer [12]. However, the mechanism and effects of MIIP in breast cancer has not yet clearly.

In the present study, we first examined the expression level of MIIP and IGFBP2 in different types of breast cancer tissues. To investigate the effects of MIIP and IGFBP2, overexpression of MIIP and inhibition of IGFBP2 to detect the effects on cell migration and invasion in vitro. Furthermore, mechanisms were detected between MIIP and IGFBP2. The results support that downregulation of MIIP promotes proliferation, invasion and migration in breast cancer via targeting IGFBP2.

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2. Methods and materials

2.1. Tissues and cell lines

Cancer tissues from 120 patients were diagnosed and histologically confirmed breast cancer. Para-cancerous from 30 patients were collected. Cancer tissues were divided according to breast cancer types into five groups: para-carcinoma tissue, Luminal A, Luminal B, HER 2 overexpression and basal-like breast cancer. Ages from 18 to 65 y and no previous diagnosis of cancer. All patients had given informed consent before involved. The present study was approved by Ethics Committees of Linyi Cancer Hospital in accordance with Helsinki of Declaration.

MDA-MB-231, MCF-7, MCF-10A were originally from Cell Culture center of institute of basic Medicine of Chinese academy of Sciences (Beijing, China), and MCF-10A was a normal immortalized breast cell. SKBR3 was from China Center for type culture collection (Wuhan, China). MDA-MB-231, SKBR3, MCF-7 were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, ThermoFisher Scientific, Inc., USA) supplemented with 10% fetal bovine serum (FBS; Gibco, ThermoFisher Scientific, Inc., USA). MCF-10A was cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12; Gibco, ThermoFisher Scientific, Inc., USA) supplemented with 5% Horse Serum (HS; Gibco, ThermoFisher Scientific, Inc., USA), 10 μg/ml insulin (91077C, Merck Life Science (Shanghai) Co. Ltd.), 20 ng /ml EGF (PHG0315; Gibco, ThermoFisher Scientific, Inc., USA), and 0.5 μg/ml hydrocortisone (H4001; Merck Life Science (Shanghai) Co. Ltd.). All cells were incubated in 37 °C with complete air, the other cells were all maintained at 37 °C in incubator with 5% CO_2.

2.2. Cell transfection

MIIP, IGFBP2 and control shRNA plasmids, were purchased GenePharma Co. Ltd., China. The precision shuttle vector was pCMV6-AC-HA. Plasmid DNA or siRNA was transfected into MDA-MB-231 cells which were subcultured at a density of 80% by lipofectamine® 2000 transfection reagent (11668019, Invitrogen, USA) according to user manual. Cells were collected 2 days after transfection for preparation of protein lysate for western blotting.

2.3. Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR)

Total RNA of samples from cells and tissues was isolated via Trizol reagent (15596026, Invitrogen, USA) according to user manual. RNA concentration was quantified by Qubit 2.0 Fluorometric Quantitation (Invitrogen, ThermoFisher scientific, USA). Reverse transcription and amplification of cDNA was using 5x Super Mix and 4x gDNA wiper (Vazyme, China) according to user manual. The level of IGFBP2 and MIIP were validated through specific primers and SYBR-Green fluorophore probes (Vazyme, China). β-actin was used as an internal control. The sequence of primers applied in this study was shown in Table 1. Relative expression of mRNA was determined via QuantStudio3 and 5 Real-Time PCR systems (ABI, ThermoFisher scientific, USA) and quantified by $2^{-\triangle\triangle CT}$.

Table 1
Primer list.

Gene	Sense (5′-3′)	Anti-sense (5′-3′)
MIIP	ATCTGCAGCCATCCTGAACC	CAGTGTGTCAGAGGCGTCAA
IGFBP2	GCAAGGGTGGCAAGCATC	GTCTACTGCATCCGCTGGGT
GAPDH	GAAGGTGAAGGTCGGAGTC	GCTGTCACCTTCACCGTTCC

2.4. Western blot

Western Blots was used to detect expression of IGFBP2 and MIIP. Total proteins from the cells or tissues were extracted using cell lysate with protease inhibitors. Protein concentration was quantified via BCA assay (Pierce Biotechnology, USA) according to user manual. Total proteins were separated by SDS-PAGE (10%) for 1 h and then transferred to PVDF membrane for 2 h (Millipore, USA). The PVDF membranes were blotted with specialized primary antibodies of IGFBP2 (dilution 1:1000; Cell signaling technology, USA), MIIP (dilution 1:1000; invitrogen, ThermoFisher scientific, USA) and GAPDH (dilution 1:1000; cell signaling technology, USA) for 12 h at 4 °C. Subsequently, the membranes were incubated with relative secondary antibody for 1 h at room temperature and the target proteins were detected by Immobilon Western Chemiluminescent HRP substrate (Millipore) after incubating. The difference of protein gray scanning analysis was analyzed by Image J.

2.5. Soft agar colony formation assay

Previously prepared 0.7% agar (cool to 40 °C after autoclaving) and 2x DMEM(warm to 40 °C)with 20% FBS medium. 1 ml mixture of 2x DMEM medium and 0.7% agar at a ratio of 1:1 per well was added in 6 cm plates, and then 1 ml mixture with cells were seeded at a concentration of 5000 cells /well. Cells were allowed to grow for about 2 weeks, and colonies were stained with 0.5 ml of 0.005% Crystal Violet for more than 1 h, then observed colonies with light microscope.

2.6. Cell viability

Cells were seeded were seeded into 96-well plate 2500 cells in100 μl DMEM medium per well. Cells was transfected with MIIP mimic and/or treated with human IGFBP2 recombinant protein for 24, 48 and 72 h. Then 20 μl MTT solution per well was added and incubated at 37 °C for 4 h. Cells with 150 μl DMSO per well were subsequently detected via SpectraMax® iD5 (Molecular Devices, Shanghai, China) at 490 nm.

2.7. Wound healing assay

Cells were seeded at 70%~80% confluent for 48 h until grown to confluence in 6-well plate. Wounds were scratched with 200 μl pipette tips. Then cultured in DMEM medium with 20% FBS. The wound area was recorded via microscopy observations at 0 h and 24 h.

2.8. Transwell assay

Transwell chamber assay was performed using 24-well Matrigel invasion chambers(8-μm pore size; Corning, USA). Cells were seeded into upper chambers at a concentration of 10^5/mL in 200 μL of DMEM medium contained 10% FBS, and DMEM medium contained 20% FBS was added into lower chambers. After cultured in 37 °C for 24 h, migrated cells adhered to the bottom of the upper chamber was stained with crystal violet and counted via micrograph (×100).

2.9. Statistical analysis

All data were analyzed via Graphpad 6.0 (GraphPad Software). In short, data were presented as mean ± standard deviation (SD) of at least three independent experiments. One-way analysis of variance (ANOVA) was performed to compare different groups. $P < 0.05$ was considered as a statistically significant difference.

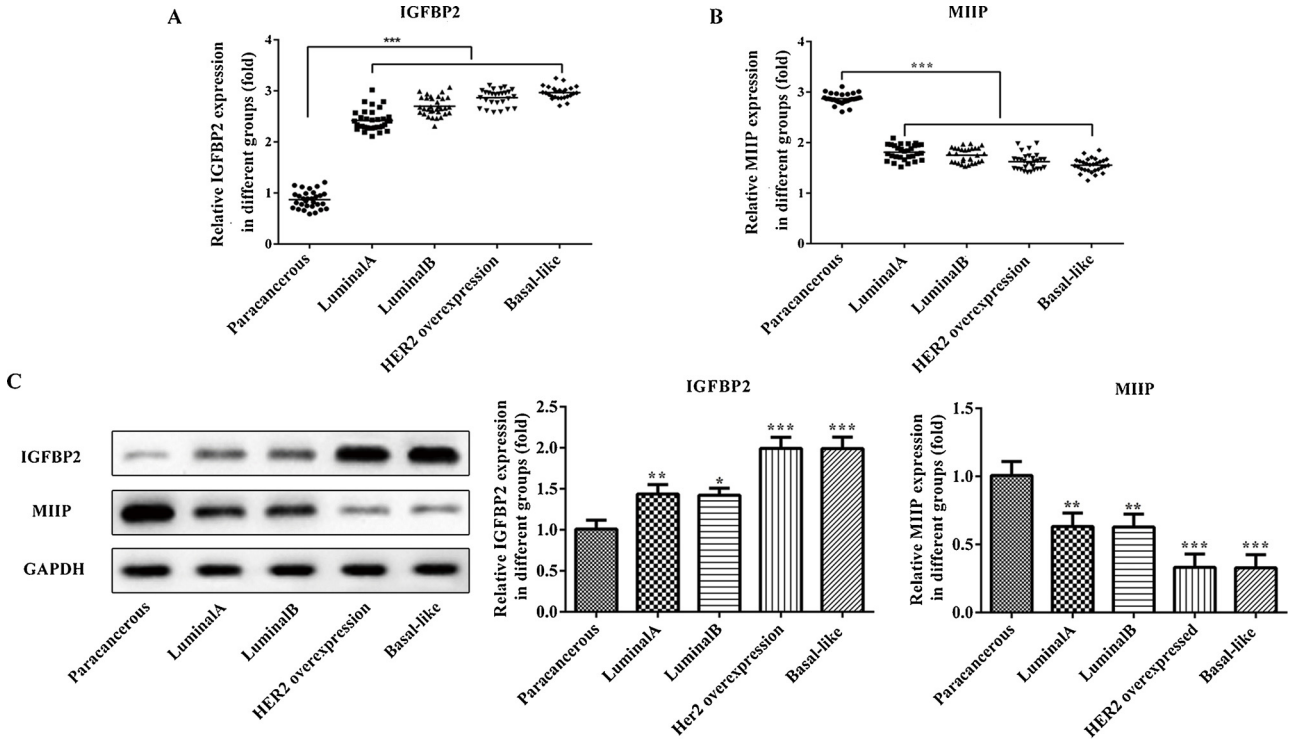

Fig. 1. Tissue expression of MIIP and IGFBP2. (A&B) The mRNA expression level of MIIP and IGFBP2 was detected via RT-qPCR in para-cancerous, Luminal A, Luminal B, HER2 overexpression and Basal like breast cancer tissues. (B) The protein expression level of MIIP and IGFBP2 was validated via Western Blot. All data were expressed as mean ± SD; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, versus para-cancerous.

3. Results

3.1. Expression of MIIP and IGFBP2 in different type of breast cancer tissues

120 patients with primary invasive ductal breast cancer and all cancers had biomarker (ER, PR, or HER2) expression information (Supplementary Table). In 120 cases of breast cancer, MIIP was expressed lower in breast cancer and IGFBP2 level was expressed higher than para-cancerous according to relative expression of mRNA (Fig. 1A &B). Western blot showed the similar expression trend, but MIIP in breast cancer of HER2 overexpression and Basal-like was significantly lower than in Luminal A and Luminal B, whereas IGFBP2 expression was significantly higher compared with para-cancerous (Fig. 1C). Since MIIP and IGFBP2 has been reported to be associated with certain tumors, we evaluated the their role in breast cancer.

3.2. Expression of MIIP and IGFBP2 were differently in different type of breast cancer cells

The relative mRNA expression of MIIP was significantly decreased in MDA-MB-231, SKBR3 and MCF-7 compared to MCF-10A, whereas IGFBP2 was significantly increased (Fig. 2A&B). The protein expression showed the same trends to mRNA expression (Fig. 2C). However, the expression of MIIP and IGFBP2 changed most significantly in MDA-MB-231. This also coincides with the results of the breast cancer tissues. Thus we use MDA-MB-231 to evaluate the further investigation.

3.3. Transfection of MIIP and IGFBP2 and relationship between MIIP and IGFBP2

To investigate the following effects of MIIP and IGFBP2, MIIP, IGFBP2 and vector plasmid were transfected in MDA-MB-231. MIIP was significantly upregulated compared with control by using Western blot analysis (Fig. 3). Meanwhile, IGFBP2 was significantly decreased after MIIP overexpressed (Fig. 3). These results indicate that MIIP was transfected efficiently, and MIIP regulates expression of IGFBP2.

3.4. Biological effects were affected via low expression of MIIP in breast cancer cell

CCK-8 was used to detect the changes of cell viability after MIIP mimic transfected in MDA-MB-231. There were not significant differences between NC and Control both in Fig. 4A and Fig.4C. The results showed that cell viability was significantly decreased after 48 h in MDA-MB-231 overexpressed with MIIP compared with control and NC (Fig. 4A). Colony formation assay further proved the effects of MIIP on cell proliferation (Fig. 4B&C). However, exogenous IGFBP2 can reverse this effects (Fig. 4).

As showed in Fig. 5, cell invasion and migration were determined by wound-healing and transwell assays and were significantly inhibited in MDA-MB-231 cells with MIIP overexpression, whereas IGFBP2 reversed the effects of MIIP. This indicated that MIIP and IGFBP2 played an important role in cell migration and invasion.

In conclusion, upregulation of MIIP inhibits the cell proliferation, migration and invasion by regulating the expression of IGFBP2 in MDA-MB-231 cells.

3.5. Effects of MIIP on biological effects were mediated by IGFBP2

To further investigated whether IGFBP2 exerts effects on cell proliferation, migration and invasion, IGFBP2 was knocked down in MDA-MB-231 cells. The result showed that IGFBP2 was significantly inhibited in MDA-MB-231 cells (Fig. 6A). Subsequently, Colony formation assay was performed to determine the results of cell proliferation and indicated that inhibition of IGFBP2 suppressed cell proliferation (Fig. 6B). The results of wound healing assay and transwell assay showed that cell migration and invasion were inhibited after IGFBP2 knocked-down (Fig. 7). These results indicated that IGFBP2 participated in cell proliferation, migration and invasion.

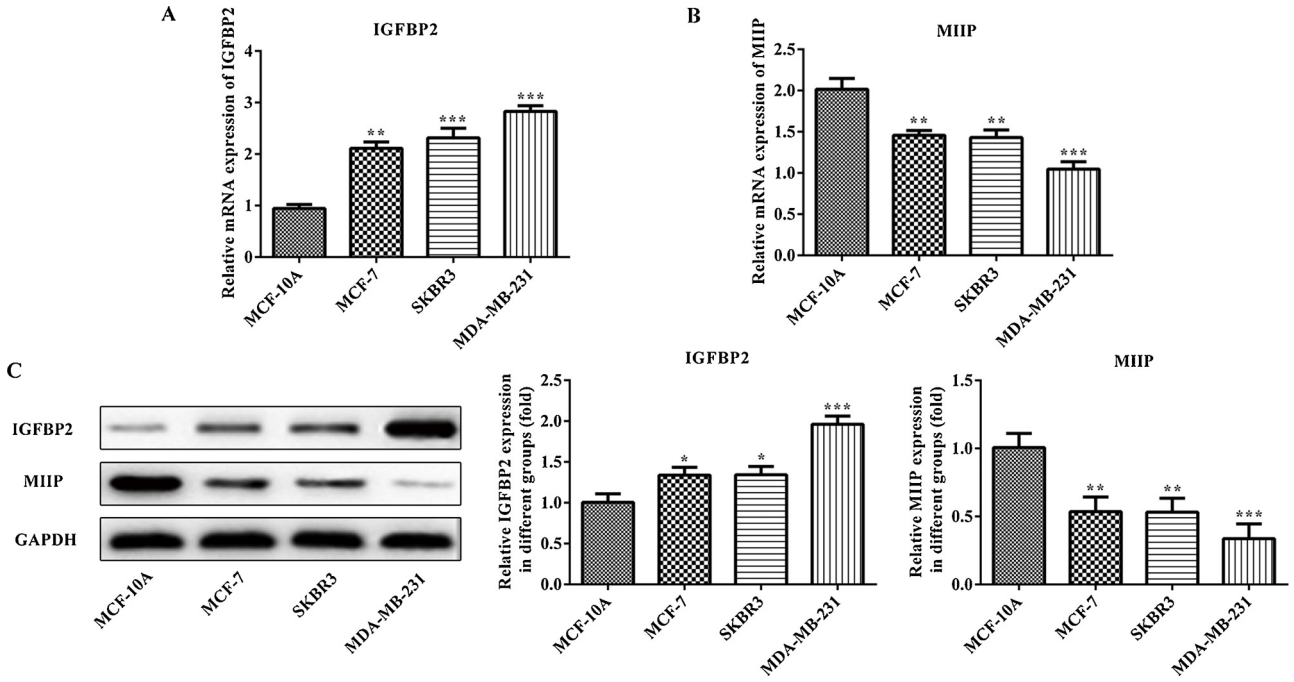

Fig. 2. The expression of MIIP and IGFBP2 in breast cancer cells. (A) mRNA expression of MIIP and IGFBP2 in MCF-10A, MCF-7, SKBR3 and MDA-MB-231. (B) The protein expression level of MIIP and IGFBP2 was evaluated by Western Blot in MCF-10A, MDA-MB-231, SKBR3 and MCF-7. Data were expressed as mean ± SD; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, versus MCF-10A.

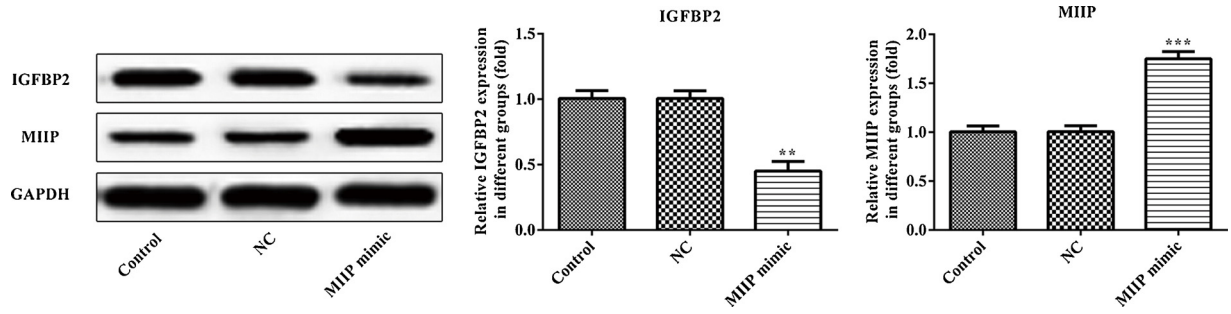

Fig. 3. Effects of MIIP on the expression levels of IGFBP2. MIIP was significantly over expressed after transfected with MIIP mimic. IGFBP2 was significantly down regulated after MDA-MB-231 cells transfected with MIIP mimic. Control, untransfected cells; NC, MDA-MB-231 transfected with mimic negative control; MIIP mimic, overexpression of MIIP. Data were expressed as mean ± SD; $^{**}P < 0.01$, $^{***}P < 0.001$, versus NC.

4. Discussion

Breast cancer as a most common risk for the survival of women has not been completely defeated. In this study we investigate a potential target, MIIP, and its biological effect in breast cancer. Results show that upregulation of MIIP affect cell viability, cell proliferation and cell metastasis inhibited in breast cancer and may work through MIIP.

The MIIP as an inhibitor of cancer cell migration and invasion was first characterized [6]. Subsequently, studies found that MIIP is associated with cell viability, radio sensitivity, tumorigenesis and cancer prediction [13–16]. Recent studies indicated that MIIP is a mitotic checkpoint and suppresses tumor development in breast cancer [12,17]. There are few investigations that study about the roles of MIIP in cancer progression. This article is the first time to evaluate that MIIP affected cell proliferation, migration and invasion by regulating IGFBP2 in breast cancer. MIIP has been investigated as a binding partner of IGFBP2 and cells co-transfected with MIIP and IGFBP2 showed less invasion than cells expressing IGFBP2 only in glioma cells, suggesting that IGFBP2 is a potential target for MIIP in cell invasion [6]. Research indicated that overexpression of IGFBP2 promoted cell metastasis [18,19]. In this study, we have confirmed the effects of IGFBP2 on cell proliferation and metastasis in breast cancer. Furthermore, IGFBP2 from both endogenous and exogenous was participated in these effects. However, whether these two proteins interaction directly or not in breast cancer and their downstream mechanisms still require further investigation.

Estrogen receptor, progesterone receptors and human epidermal growth factor 2 (ER, PR and HER2) are biomarkers in breast cancer and plays important role in treatment and prognosis. Triple-negative tumors, ER-/PR-/HER2-, are also associated with higher grade and advanced stage [20]. In this study, HER2 positive or HER2 negative does not affect the expression changes of IGFBP2 and MIIP in both tissues and cell lines which revealed that existence of HER2 do not influence MIIP and IGFBP2 expression. MIIP was expressed most significantly higher in ER-/PR- cells. This indicates that ER or PR might associated with the expression of MIIP and IGFBP2, and this needs to be further confirmed.

In conclusion, the aberrant expression of MIIP and IGFBP2 in different types of breast cancer patients let us be interested in these two proteins. Results showed that MIIP negatively regulates expression of IGFBP2, and MIIP participates in regulating cell growth, cell migration and cell invasion via IGFBP2 in breast cancer. These results indicate

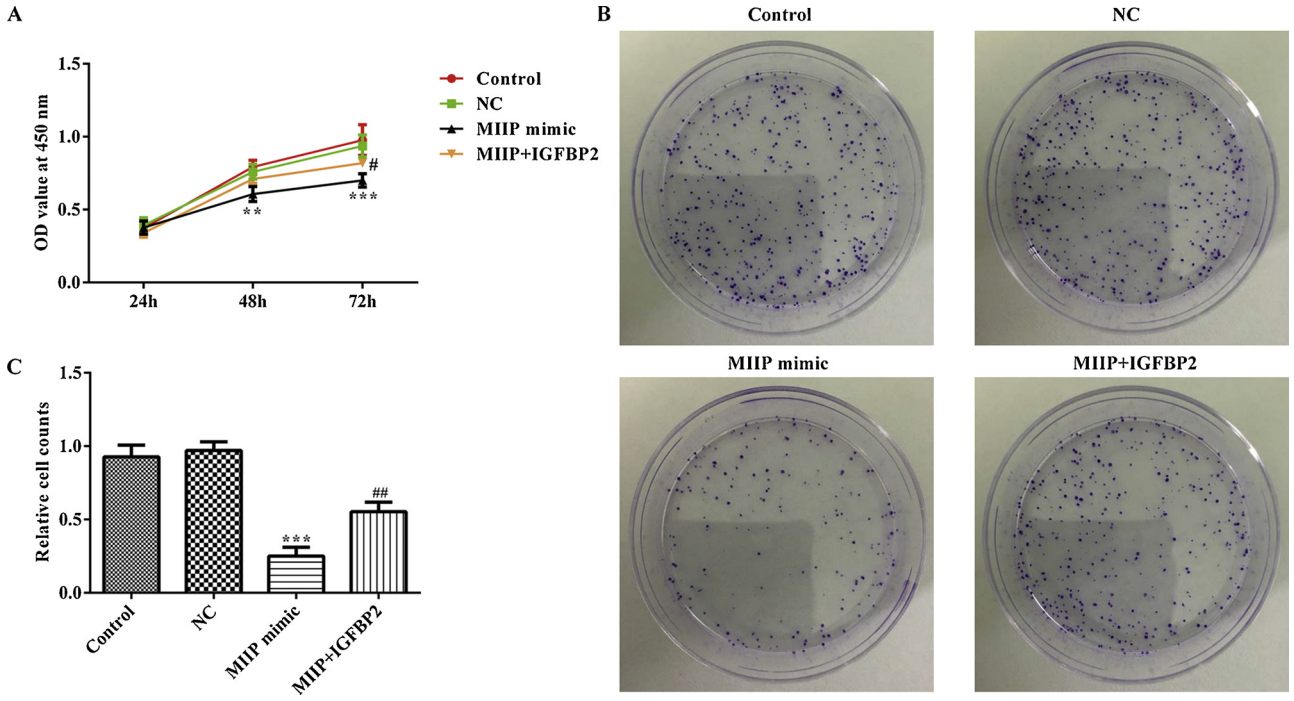

Fig. 4. Cell growth was affected after overexpression of MIIP. (A) Cell viability was decreased after transfecting with MIIP mimic compared to control and NC. (B&C) The colony formation assay was used to detect proliferation ability of MDA-MB-231 cells. Control, untransfected cells; NC, MDA-MB-231 transfected with mimic negative control; MIIP mimic, overexpression of MIIP; MIIP + IGFBP2, cells transfected with MIIP mimic and treated with human recombinant IGFBP2 subsequently. Data were expressed as mean ± SD; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, versus NC.

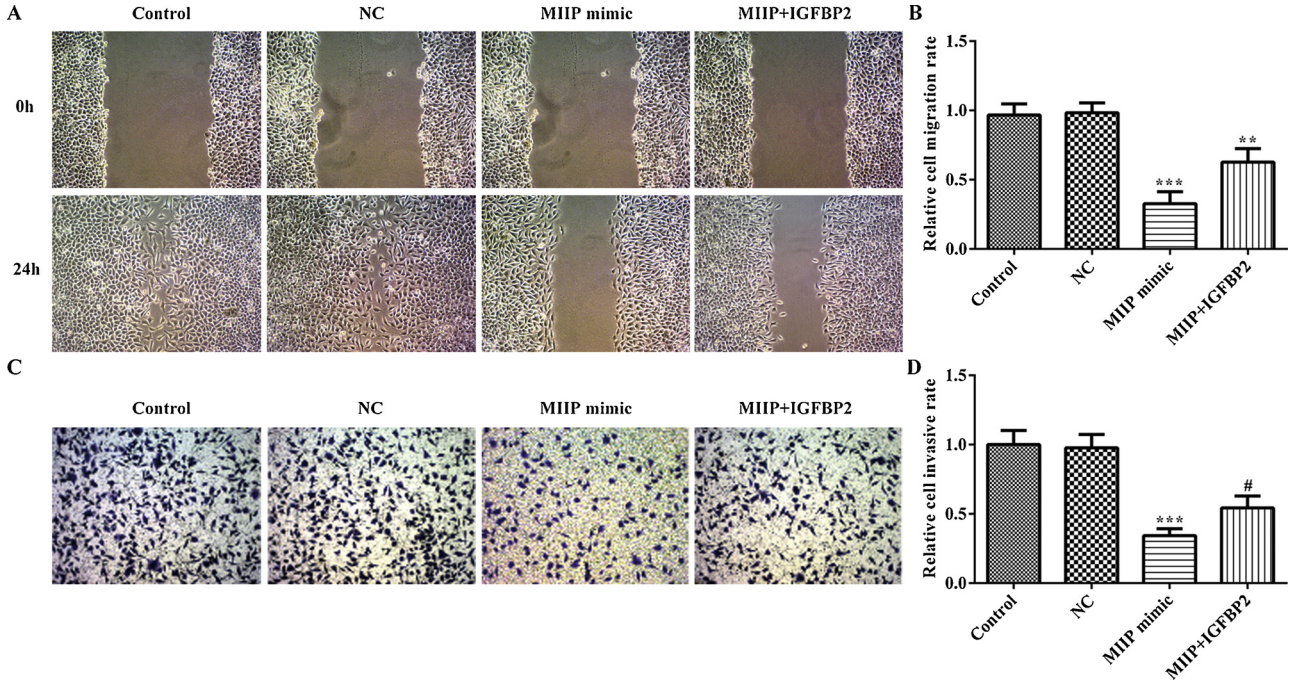

Fig. 5. The effect of MIIP in cell invasion and migration. (A) Wound-healing assay was detected to analyze cell migration after transfecting with MIIP mimic at magnification of 200 × . (B) Transwell assay was used to evaluate cell invasion with MIIP overexpressed at magnification of 100 × . Control, untransfected cells; NC, MDA-MB-231 transfected with mimic negative control; MIIP mimic, overexpression of MIIP; MIIP + IGFBP2, cells transfected with MIIP mimic and treated with human recombinant IGFBP2 subsequently. Data were expressed as mean ± SD; $^{**}P < 0.01$, $^{***}P < 0.001$, versus NC.

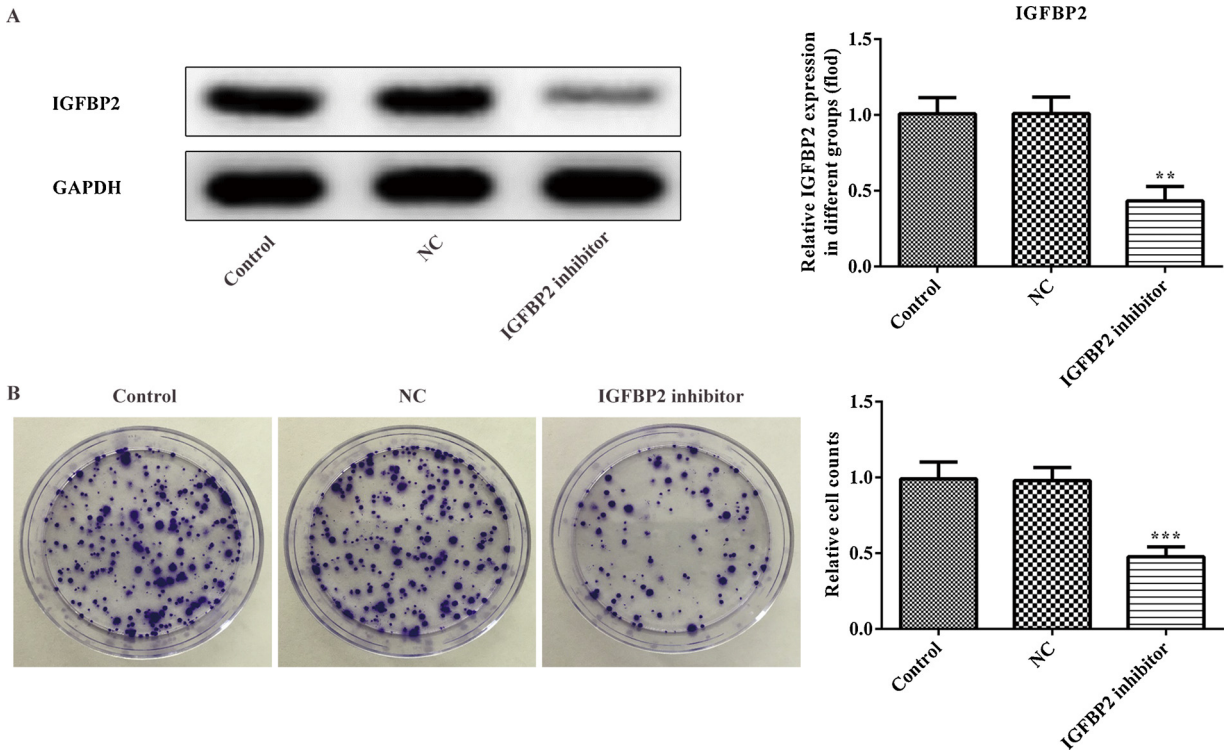

Fig. 6. Effects of IGFBP2 on cell proliferation. (A) Transfection efficiency of IGFBP2 inhibitor. (B) Colony formation assay was used to examine cell proliferation in different groups. NC, cells transfected with inhibitor negative control; IGFBP2-inhibitor, cells transfected with IGFBP2 inhibitor. Data were expressed as mean ± SD; $^{**}P < 0.01$, $^{***}P < 0.001$, versus NC.

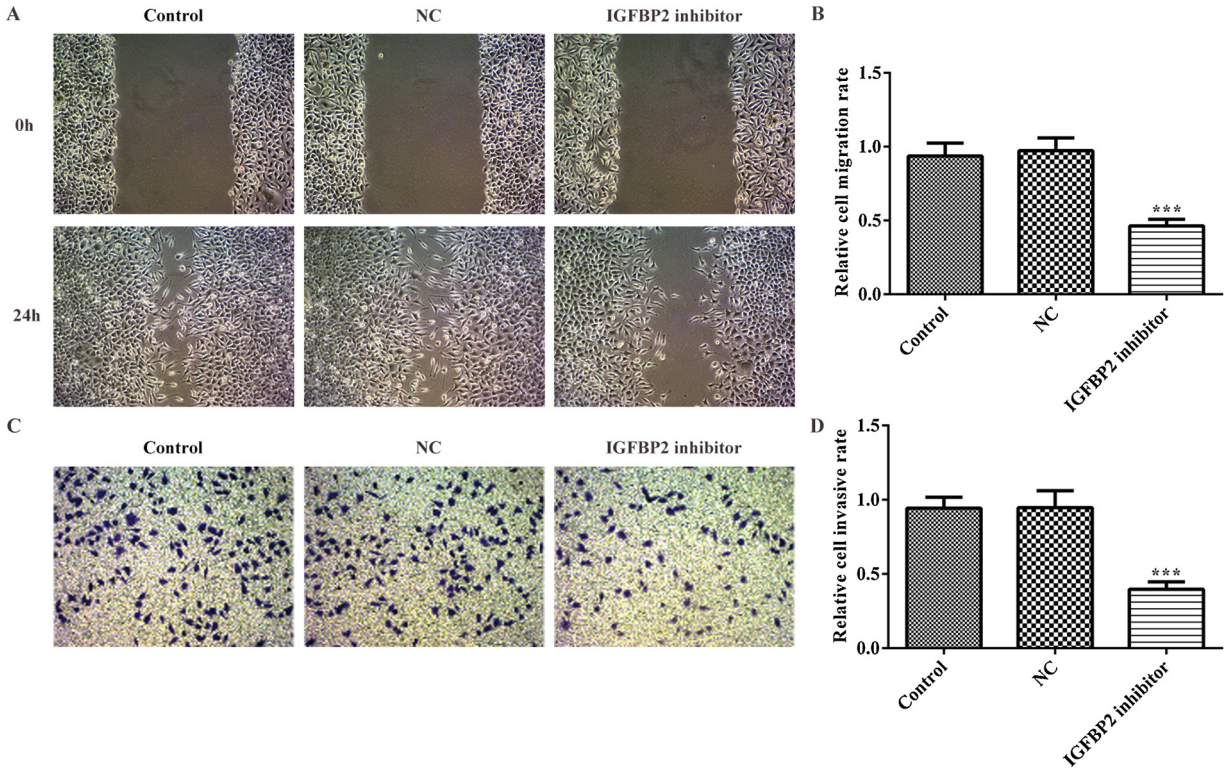

Fig. 7. Effects of IGFBP2 on cell invasion and migration. (A) Wound healing assay was performed to detect cell migration at magnification of 200 × . (B) Transwell assay was used to determine cell invasion at magnification of 100 × . NC, cells transfected with inhibitor negative control; IGFBP2-inhibitor, cells transfected with IGFBP2 inhibitor. Data were expressed as mean ± SD; $^{***}P < 0.001$, versus NC.

that MIIP is a potential target for diagnose and treatment in breast cancer progression.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.prp.2019.152440.

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