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# miR-206 is down-regulated in breast cancer and inhibits cell proliferation through the up-regulation of cyclinD2

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ABSTRACT

MicroRNAs act as important gene regulators in human genomes, and their aberrant expression is linked to many malignancies. Aberrant expression of miR-206 has been frequently reported in cancer studies; however, the role and mechanism of its function in breast cancer remains unclear. Quantitative real-time PCR was performed to detect the relative expression levels of miR-206 in breast cancer and normal breast tissues. Lower expression of miR-206 in breast cancer tissues was associated with larger tumour size and a more advanced clinical stage. Further in vitro observations showed that the enforced expression of miR-206 in MCF-7 breast cancer cells inhibited cell growth by blocking the G1/S transition and suppressed cell proliferation and colony formation, implying that miR-206 functions as a tumour suppressor in the progression of breast cancer. Interestingly, Luciferase assays first revealed that miR-206 inhibited cyclinD2 expression by targeting two binding sites in the 3′-untranslated region of cyclinD2 mRNA. qRT-PCR and Western blot assays verified that miR-206 reduced cyclinD2 expression at both the mRNA and protein levels. A reverse correlation between miR-206 and cyclinD2 expression was noted in breast cancer tissues. Altogether, our results identify a crucial tumour suppressive role of miR-206 in the progression of breast cancer, at least partly via up-regulation of the expression of cyclinD2, and suggest that miR-206 might be a candidate prognostic predictor or an anticancer therapeutic target for breast cancer patients.



## 1. Introduction

Breast cancer is the most common cancer in women and the second leading cause of cancer death among women worldwide [1,2]. Despite well-organised screening and early treatment programs that have been effective in preventing breast cancer, many breast tumours are not eradicated completely due to acquired resistance or relapse following an initial response, thus resulting in metastatic disease at later stages that leads to patient death [3]. Therefore, identification of molecular aberrations that might be helpful in creating novel diagnostic and therapeutic strategies remains an important component in the current management of this malignancy.

In the past decade, much research has been conducted on microRNAs (miRNAs), which were discovered in 1993 [4]. miRNAs are a class of ~ 22 nt non-coding RNAs that regulate gene expression post-transcriptionally [5–7]. It is now well known that miRNAs are key regulators of gene expression and modulate up to one-third of all genes, though they constitute only approximately 1% of the human genome [8–10]. Some miRNAs are oncogenic, whereas others function as tumour suppressors [11]. For instance, miR-21 over-expression occurs frequently in most cancers and is linked to increased tumour cell growth [12,13]. In addition, conditional expression of miR-21 in vivo prompted various clinical signs of haematological malignancies. Meanwhile, the tumours showed rapid regression upon knockdown of miR-21 [14]. Let-7 functions as a tumour suppressor in the lungs because its down-regulation in lung carcinoma allows for increased expression of the Ras protein [15].

Given the importance of miRNAs in cancers, in the present study, we explored the tumour-suppressive significance of miR-206 in breast cancer tissues and showed that miR-206 was down-expressed in tumour samples compared with matched

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normal samples by performing quantitative qRT-PCR. miR-206 down-regulation also correlated with tumour size and advanced pathologic stage. Functional analyses of miR-206 indicated that the enhanced expression of miR-206 in MCF-7 breast cancer cells could inhibit cell growth by blocking the G1/S transition and suppress cell proliferation and colony formation. Luciferase reporter assay and Western blot confirmed that miR-206 might function as an anti-oncogene by directly targeting cyclinD2 (also known as CCND2). The cyclinD2-mediated repression in cell proliferation was reverted by exogenous miR-206 expression. A reverse correlation between miR-206 and cyclinD2 expression in breast cancer tissues was noted by consecutive further analysis.

## 2. Materials and methods

### 2.1. Tissue collection

Breast cancer tissues and normal tissues were obtained from patients who had undergone surgery at the Second Affiliated Hospital of Nanjing Medical University between 2008 and 2011 and who were diagnosed with breast cancer based on histopathological evaluation. No local or systemic treatment had been conducted in these patients before the operation. All the tissue samples were collected, immediately snap frozen in liquid nitrogen, and stored at −80 °C until RNA extraction. The study was approved by the Research Ethics Committee of Nanjing Medical University (Nanjing, Jiangsu, PR China). Informed consent was obtained from all patients.

### 2.2. Cell line and culture conditions

The human breast cancer cell line MCF-7 was provided by Dr. Jianwei Zhou (the Molecular Toxicology Laboratory, Nanjing Medical University). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) supplemented with 10% FBS (Invitrogen) in a 37 °C incubator at 5% $CO_2$.

### 2.3. RNA extraction and qRT-PCR analyses

Total RNA was extracted from tissues or cultured cells with TRIzol reagent (Invitrogen) following the manufacturer's instructions. For qPCR, RNA was reverse transcribed to cDNA from 1 mg of total RNA using a Reverse Transcription Kit (Takara). Real-time PCR (RT-PCR) analyses were conducted with Power SYBR Green (Takara). All protocols were performed according to the manufacturer's instructions. Results were normalised to the expression of U6 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primer sequences were as follows:

miR-206:
Reverse-transcribed primer
5′-GTCAGAAGGAATGATGCACAGCCAACAACA-3′;
Forward: 5′-CGTCAGAAGGAATGATGCACAG-3′;
Reverse: 5′-ACCTGCGTAGGTAGTTTCATGT-3′;
CyclinD2:
Forward: 5′-TGCAACCGACGATTCTTCTACTCAA-3′;
Reverse:5′-CAAGCAGTGATGTATCTGATAAACAAGG-3′;
U6:
Reverse transcribed Primer:
5′-AACGCTTCACGAATTTGCGT-3′;
Forward: 5′-CTCGCTTCGGCAGCACA-3′;
Reverse: 5′-AACGCTTCACGAATTTGCGT-3′;
GAPDH:
Forward: 5′-AGAGGCAGGGATGATGTTCTG-3′;
Reverse: 5′-GACTCATGACCACAGTCCATGC-3′;

qPCR and data collection were performed using an ABI 7500.

### 2.4. miRNA mimic transfection

Breast cancer cells were transfected with miR-206 mimic or miR-NC (Applied Biosystems) with a final concentration 100 pm using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. At 48 h after transfection, cells were harvested for Western blotting or qRT-PCR analyses.

### 2.5. Luciferase reporter assay

HEK293T cells were seeded into 48-well plates, cotransfected with pcDNA-miR-206 or pcDNA (200 ng) and luciferase reporter plasmids (50 ng) containing the wild-type (WT) or mutant (Mut) cyclinD2 3′-UTR using Lipofectamine 2000 (Invitrogen). Firefly and Renilla luciferase activities were measured 48 h after transfection using the Dual-Luciferase Reporter Assay System (Promega; Ref. [18]).

### 2.6. Cell proliferation assays

To evaluate cell proliferation, $3 \times 10^3$ MCF-7 cells per well were plated into wells of 96-well plates and transfected with miRNA mimic or miR-NC. At 24, 48, 72 and 96 h after transfection, cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2, -diphenyltetrazolium bromide (MTT) assay following the manufacturer's protocol. All experiments were performed in quadruplicate.

For the colony formation assay, 500 cells were seeded into each well of a 6-well plate and maintained in media containing 10% FBS for 2 weeks. Colonies were fixed with methanol and stained with 0.1% crystal violet (Sigma) in PBS for 15 min. Colony formation was determined by counting the number of stained colonies in 3 randomly selected fields using an inverted microscope. Triplicate wells were measured in each treatment group.

### 2.7. Flow cytometry

MCF-7 cells transiently transfected with miR-NC and miR-206 mimics were harvested 48 h after transfection by trypsinisation. Cells for cell-cycle analysis were stained with propidium oxide using the Cycle Test Plus DNA Reagent Kit (BD Biosciences) following the protocol and analysed by FACScan. The percentages of the cells in G0–G1, S, and G2–M phases were counted and compared.

### 2.8. Western blot assay and antibodies

Cells were lysed using the mammalian protein extraction reagent RIPA (Beyotime) supplemented with a protease inhibitor cocktail (Roche) and phenylmethylsulfonylfluoride (Roche). Fifty micrograms of protein extracts were separated by 10% SDS–PAGE, transferred to 0.22 mm nitrocellulose (NC) membranes (Sigma) and incubated with specific antibodies. Autoradiograms were quantified by densitometry (Quantity One software; Bio-Rad). The cyclinD2 antibody (1:1000) was purchased from Abcam.

### 2.9. Immunohistochemistry

Breast tumour and non-tumour tissue samples were immunostained for cyclinD2. The signal was amplified and visualised with 3, 3′-diaminobenzidine chromogen, followed by counterstaining with hematoxylin. Expression was considered to be positive when 50% or more cancer cells were stained. The anti-cyclinD2 (1:50) antibody was purchased from BioWorld.

### 2.10. Statistical analysis

The Students *t*-test (two-tailed), one-way ANOVA, and Mann–Whitney *U* test were conducted to analyse the in vitro and in vivo data using SPSS 17.0 software. *P* values less than 0.05 were considered to be statistically significant.

## 3. Results

### 3.1. Expression of miR-206 is frequently down-regulated in breast cancer tissues

Here, we examined miR-206 levels in 30 breast cancer tissues and 30 matched breast normal tissues using quantitative real-time RT-PCR (qRT-PCR). Significantly, the expression of miR-206 was reduced in breast cancer tissues compared with normal tissues (Fig. 1A). Next, we examined miR-206 expression and correlated it with clinical pathology features. miR-206 down-regulation correlated with a larger tumour size ($P = 0.034$; Fig. 1B) and an advanced pathologic stage ($P = 0.038$; Fig. 1C); however, miR-206 expression did not correlate with patient age or the presence of lymph node metastasis. Thus, it was concluded that the decreased expression of miR-206 might play an important role in breast cancer progression and development.

### 3.2. Exogenous over-expression of miR-206 in breast cancer cells

To up-regulate the expression of miR-206, miR-206 mimics were transfected into MCF-7 cells. In addition, to stably sustain the expression of miR-206 in MCF-7 cells, miR-NC was transfected into MCF-7 cells. The cells were collected 48 h after transfection for detection of miR-206 expression. qRT-PCR assays revealed that miR-206 expression was up-regulated by approximately 30-fold after transfection of miR-206 mimics, as compared with miR-NC control cells (Fig. 2A).

### 3.3. Enforced expression of miR-206 functionally suppresses cell proliferation in breast cancer cells

Given that miR-206 was expressed at lower levels in breast cancer tissues compared with normal tissues, we hypothesised that miR-206 might act as a tumour suppressor and that restoration of miR-206 expression could affect cell proliferation in breast cancer cells. To test this effect, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was performed to measure cell viability. miR-206 mimics or miR-NC controls were transfected into human breast carcinoma MCF-7 cells. According to the results of the MTT assay, we found that the proliferation of cells transfected with miR-206 mimics was decreased compared with that of cells transfected with miR-NC controls (Fig. 2B). To further demonstrate the anti-proliferative effect of miR-206 on the growth of breast cancer cells, a colony formation assay was performed. As shown in Fig. 2C, the colony numbers of breast cells transfected with miR-206 mimics were significantly lower than those transfected with miR-NC controls ($P < 0.01$). Thus, the results of the colony formation assay were consistent with those of the MTT assay and further indicated that ectopic miR-206 expression could inhibit in vitro proliferation of MCF-7 breast cancer cells.

### 3.4. Over-expression of miR-206 blocks the G1/S transition in breast cancer cells

To further examine whether the effect of miR-206 on the proliferation of MCF-7 cells reflected a cell-cycle arrest, cell-cycle progression was analysed by flow cytometric analysis. The results revealed that over-expression of miR-206 showed a more significant blocking of MCF-7 cells at the G1–G0 phase and that fewer cells were in the G2–S phase (Fig. 2D). Thus, our findings suggest that miR-206 effectively retards growth by blocking the G1/S transition in breast cancer cells in vitro.

### 3.5. CyclinD2 is a direct target of miR-206 in breast cancer cells

To further explore the mechanism by which miR-206 promotes breast cancer proliferation, we conducted a bioinformatics screen to identify potential downstream target genes that normally have a tumour suppressive effect. Based on this rationale, cyclinD2 was selected. We cloned the wild-type 3′-UTRs of cyclinD2 and inserted them into the region immediately downstream of a luciferase reporter gene (Fig. 3A). Subsequently, the miR-206 vectors were co-transfected with the different luciferase 3′-UTR constructs into HEK293T cells. We found that miR-206 expression decreased the relative activity of the luciferase reporter containing the wild-type 3′-UTR of cyclinD2 mRNA. To test whether cyclinD2 mRNA is a direct target for miR-206, we mutated the predicted binding site of miR-206 in the 3′-UTR. However, luciferase activity did not drop sharply in the UTRs with mutant binding sites when compared with Mut-type counterparts (Fig. 3B).

We next determined whether miR-206 could regulate cyclinD2 at both the mRNA and protein levels. qRT-PCR analysis showed that the expression of cyclinD2 mRNA in MCF-7 cells transfected with miR-206 mimics was down-regulated compared with cells

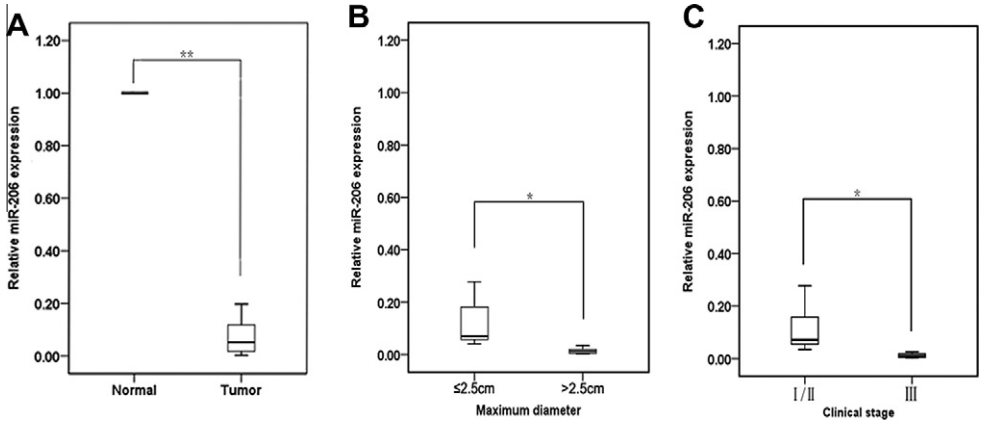


**Fig. 1.** The level of miR-206 expression in breast cancer tissues and its clinical significance. (A) miR-206 was detected in 30 pairs of breast cancer tissues by qRT-PCR. Data are presented as fold change in tumour tissues relative to normal tissues. (B) miR-206 expression was significantly lower in patients with large tumours than in patients with small tumours. (C) miR-206 expression was significantly lower in patients with a higher pathologic stage than in patients with a lower pathologic stage. $^{*}P < 0.05$; $^{**}P < 0.01$.

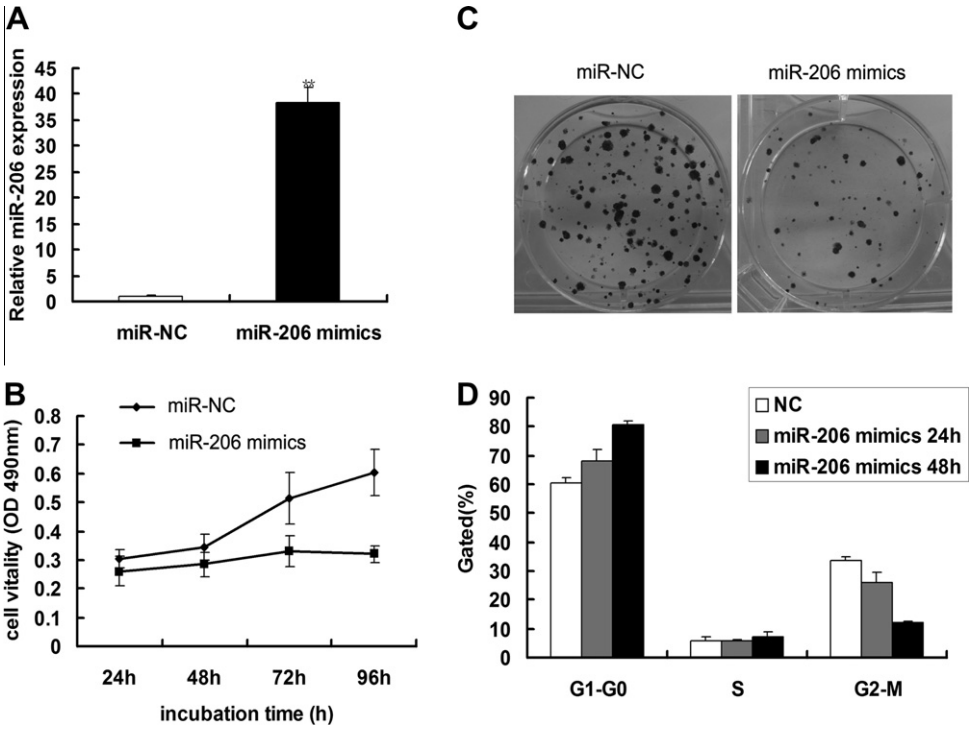


**Fig. 2.** The effects of miR-206 on cell proliferation. (A) The relative expression level of miR-206 in MCF-7 cells transfected with miR-206 mimics or miR-NC was determined by qRT-PCR. (B) Forty-eight hours after transfection, MTT assays were conducted to determine the proliferation rate of MCF-7 cells. (C) Colony-forming growth assays were conducted to determine the proliferation rate of MCF-7 cells. The colonies were counted and imaged. (D) The bar chart represents the percentage of cells in G0, G1, S, or G2–M phase, as indicated. $^{*}P < 0.05$; $^{**}P < 0.01$.

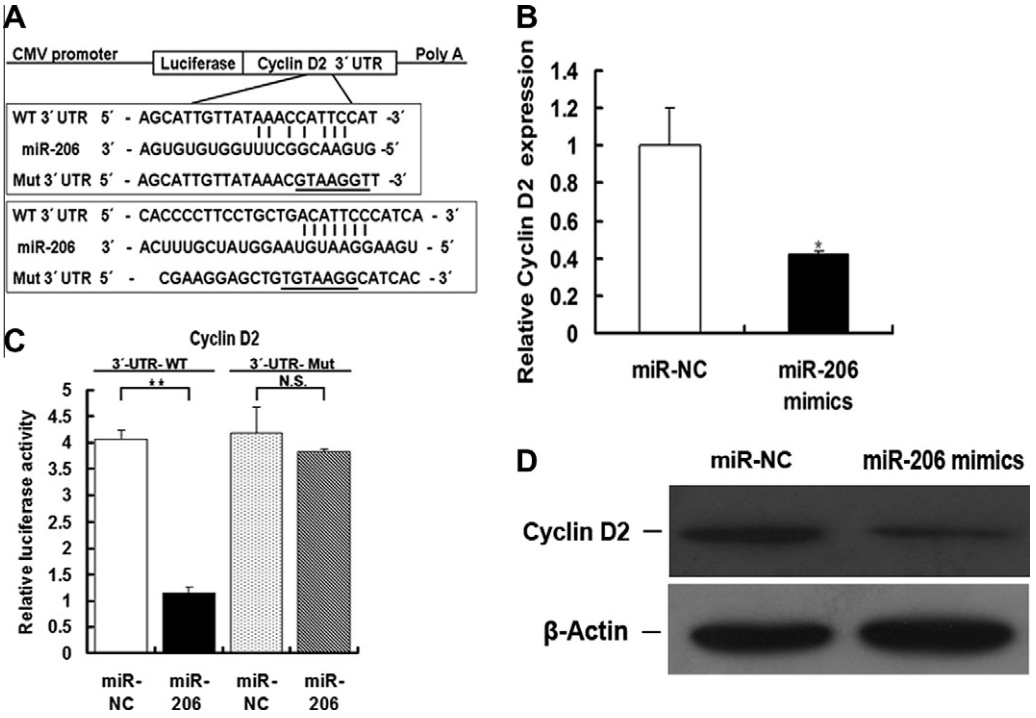


**Fig. 3.** miR-206 directly targets the cyclinD2 gene. (A) A human cyclinD2 3′-UTR fragment containing the wild-type (WT) or mutant (Mut) miR-206 binding sequence was cloned downstream of the luciferase reporter gene. (B) The luciferase reporter plasmid containing wild-type or mutant cyclinD2 3′-UTR was cotransfected into HEK293T cells with miR-206 or miR-NC. Luciferase activity was determined by the dual luciferase assay and shown as the relative firefly activity normalised to Renilla activity. (C) The level of cyclinD2 mRNA was determined by qRT-PCR. (D) The expression of cyclinD2 protein was analysed by Western blotting. β-Actin was used as a control. $^{*}P < 0.05$; $^{**}P < 0.01$; NS, not significant. CMV, cytomegalovirus.

transfected with the control (Fig. 3C). Western blot analysis showed that the expression of cyclinD2 protein in MCF-7 cells transfected with miR-206 mimics was also down-regulated compared with cells transfected with miR-NC controls (Fig. 3D). These data showed that miR-206 could regulate cyclinD2 at both the mRNA and protein levels.

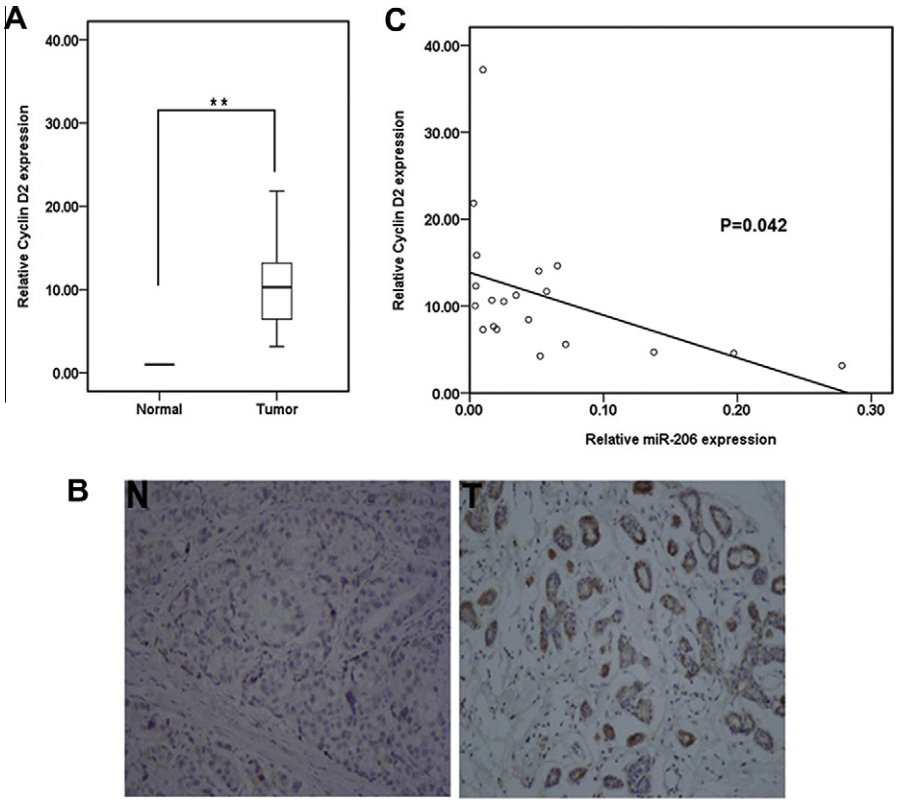


**Fig. 4.** The level of cyclinD2 expression in breast cancer tissues. (A) The level of cyclinD2 mRNA in breast cancer tissues was analysed by qRT-PCR. (B) The level of cyclinD2 protein in breast cancer tissues was analysed by immunohistochemistry. (C) Analysis of the relationship between miR-206 expression and cyclinD2 levels. $^{*}P < 0.05$; $^{**}P < 0.01$.

### *3.6. An inverse relationship between the expression of cyclinD2 and miR-206*

To assess the relationship between cyclinD2 and miR-206 expression in breast cancer, we examined cyclinD2 by qRT-PCR in 20 breast cancer tissues and 20 matched breast normal tissues and by immunohistochemistry in 20 pairs of breast cancer tissues. The results showed that the mRNA levels of cyclinD2 were generally higher in breast cancer tissues when compared with normal tissues (Fig. 4A). We also found that 80% of normal breast tissues showed a cyclinD2 negative signal, whereas most tumour tissues exhibited high levels of cyclinD2 protein compared with the paired normal tissues (Fig. 4B, Table 1). Further analysis revealed that the expression of miR-206 is inversely correlated with cyclinD2 protein levels in breast cancer (Fig. 4C). These data indicated that cyclinD2 levels were mostly opposite to levels of miR-206 expression in breast cancer.

**Table 1**
Expression of cyclinD2 in breast cancer tissues.

| Group | No. | Positive, no. (%) | Negative, no. (%) |
|---|---|---|---|
| Breast cancer | 20 | 17 (85) | 3 (15) |
| Normal | 20 | 4 (20) | 16 (80) |

Breast tumor and non-tumor tissue samples were immunostained for cyclinD2. Expression was considered to be "positive" when 50% or more cancer cells were stained.

## 4. Discussion

Although much remains to be learned, emerging evidence now suggests that in addition to protein-encoding genes, miRNAs play more than a cursory role in the pathogenesis of human tumour development by functioning as agents of the RNA interference pathway [16]. MicroRNAs (miRNAs) are a class of ~22nt non-coding RNAs that regulate gene expression posttranscriptionally [5–7]. miRNAs are known to be key regulators of gene expression that modulate up to one-third of all genes, though they constitute only approximately 1% of the human genome [8–10]. Some miRNAs are oncogenic, whereas others function as tumour suppressors [11]. The miR-206 gene is located at chromosome 6p12.2, and it is conserved noncoding RNAs of 21 nucleotides that regulate the translation and stability of target mRNAs based on sequence complementarity [17]. Previous studies have indicated that miR-206 may function as a metastasis suppressor miRNA in human breast cancer [18], and miR-206 has been proven to have effects on the invasion of lung cancer [19,20]. miR-206 may serve as a key factor that regulates ER-a expression during the development of normal breast epithelium because the expression of miR-206 is under hormonal regulation [21].

In our study, we found that miR-206 was dramatically down-regulated in breast cancer tissues compared with matched normal breast tissues, and lower expression of miR-206 in breast cancer tissues was associated with larger tumour size and a more advanced clinical stage, suggesting that the expression of miR-206 could be used to develop a new independent prognosis or progression marker for breast cancer. Further functional analyses of miR-206 indicated that the enhanced expression of miR-206 in MCF-7 breast cancer cells could inhibit cell growth by blocking the G1/S transition and suppress cell proliferation and colony formation.

In experimental models, a single miRNA can regulate a number of genes. Previous studies have shown that miR-206 is expressed in skeletal and cardiac muscle and promotes muscle differentiation by down-regulating the p180 subunit of DNA polymerase and the myogenic transcription factors Is1–3 and MyoR [22]. Through bioinformatics analysis, we also found that cyclinD2 might be one of the target genes of miR-206.

CyclinD2 is unique among the three D-type cyclins, as it has been shown to be up-regulated many fold under conditions of growth arrest in phenotypically normal human and murine fibroblasts. Furthermore, ectopic over-expression of cyclinD2 effectively blocked cell cycle progression, thus suggesting an alternate role for cyclinD2 in promoting exit from the cell cycle and maintaining a non-proliferative state. The fact that high levels of cyclinD2 are expressed in normal human tissues composed primarily of non-proliferating contact-inhibited cells, such as breast and brain, is consistent with these in vitro observations [23]. Aberrant expression of cyclinD2 has been noted in human ovarian granulosa cell tumours and testicular germ cell tumour cell lines [24]. Recently, evidence was presented suggesting that cyclinD2 is a direct target of Myc and that accumulation of cyclinD2 contributes to sequestration of the cell cycle inhibitor p27 and to cell cycle entry [25].

Our study initially showed that exogenous miR-206 could down-regulate the expression of cyclinD2 protein in breast cancer cells. Moreover, the luciferase assay using a reporter containing the wild type miR-206 binding sequence at the 3′-UTR of cyclinD2 mRNA indicated that the luciferase activity could be significantly reduced or up-regulated by over-expression or down-regulation of miR-206. Furthermore, the cyclinD2 mRNA and protein were over-expressed in breast cancer tissues compared with normal tissues.

In summary, we have shown that miR-206 is dramatically down-regulated in breast cancer tissues compared with normal breast tissues and that lower expression of miR-206 in breast cancer tissues is associated with larger tumour size and a more advanced clinical stage. Moreover, up-regulation of miR-206 suppresses breast cancer cell proliferation and colony formation by blocking the G1/S transition through the targeting of cyclinD2. Our experimental data may provide a strategy for targeting the miR-206/cyclinD2 interaction in a novel therapeutic application to treat breast cancer patients.

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