Pathology - Research and Practice 215 (2019) 152592

Contents lists available at ScienceDirect

Pathology - Research and Practice

journal homepage: www.elsevier.com/locate/prp

CyclinB1 deubiquitination by USP14 regulates cell cycle progression in breast cancer

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ARTICLE INFO

Keywords:
USP14
CyclinB1
Cell cycle
Breast cancer

ABSTRACT

Breast cancer is the most common malignant tumor among women in China, which seriously threatens women's physical and mental health. Tumorigenesis is closely related to the dysregulation of cell cycle. The cell cycle progression includes interphase and mitotic phase (M phase). Cyclin B1 is a key protein in regulating M phase, which is essential for the whole cell cycle progression. CyclinB1 can be degraded through ubiquitination mediated by the anaphase promoting complex/cyclosome (APC/C). However, the mechanism of how CyclinB1 is deubiquitinated in breast cancer still remains unclear. In this study, we discovered that CyclinB1 interacted with ubiquitin-specific peptidase 14 (USP14). Based on the deubiquitinating function of USP14, we detected the effect of USP14 on the ubiquitination of CyclinB1. Inhibiting the activity of USP14 or USP14 knockdown significantly increased the ubiquitination of CyclinB1. In accordance with this, knocking down USP14 arrested cell cycle at G2/M phase. Knocking down USP14 with siRNAs significantly inhibited the proliferation and migration of breast cancer cells. In conclusion, our study demonstrated that USP14 regulated the cell cycle of breast cancer cells by regulating the ubiquitination of CyclinB1, which will provide a solid theoretical basis for the development of anti-cancer drugs targeting USP14.

1. Introduction

Breast cancer is one of the most critical threats to women globally. There are about 1,671,000 new cases of breast cancer in the world, and about 522,000 patients died every year [1–3]. Main treatments for breast cancer are surgery, radiotherapy and chemotherapy [4]. However, because of the existence of chemoresistance, the therapeutic effect of many cancer patients is unsatisfactory. It was reported that multidrug resistance (MDR) has accounted for up to 50% of all cancer-related deaths [5,6]. Molecular targeting therapy is one of the most active research fields in recent years, so it is particularly important to find more effective molecular targets for treatment of breast cancer.

The dysregulation of cell cycle is one of the hallmarks of cancer [7]. CyclinB1 (also known as CCNB1) belongs to the highly conserved cyclin family and is expressed in dozens of cancer types [8,9]. Under normal conditions, Cyclin B1 is expressed at very low levels and accumulates appreciably only at the G2-M cell cycle transition, concurrent with its scheduled translocation to the nucleus [10]. In cancer cells, Cyclin B1 is found overexpressed throughout the cell cycle and resides principally in the cytoplasm [11]. It's also reported that CyclinB1 was a promising biomarker for overall survival in ER + breast cancer [12].

USP14, belonging to the USP family, which binds reversibly to the 26S proteasome [13], limits proteasomal degradation of ubiquitinated proteins [14–16]. As a major member of the ubiquitin-proteasome system, USP14 has received significant attention for its crucial role in various cancers. Specifically, in earlier days, Tomlaki Wada et al constructed the expression library of ovarian cancer cell lines SHIN-3 by reversing transcription virus, then transfected into mouse embryonic fibroblasts in NIH3T3 to conduct lesion formation experiments. They found that USP14 transfected cells could form foci, indicating that USP14 was closely related to the formation of ovarian cancer [17]. Additionally, Huang G. demonstrated that USP14 was up-regulated in tumor tissues of patient with hepatocellular carcinoma (HCC) compared to that in normal liver tissues [18]. Furthermore, in a androgen-responsive prostate cancer cells, it was reported that inhibiting the function of USP14 resulted in the inhibition of cell proliferation and cell

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https://doi.org/10.1016/j.prp.2019.152592
Received 24 April 2019; Received in revised form 18 July 2019; Accepted 9 August 2019

cycle arrest at the G0/G1 phase [19]. However, the mechanism of USP14 in regulating breast cancer is less studied. Our study found that knocking down USP14 could inhibited the proliferation and migration of breast cancer cells. Further studies demonstrated that USP14 knockdown could increased the ubiquitination of CyclinB1 and promote its degradation, resulting in cell cycle arrest at G2/M phase. These results indicated that USP14 could be used as a potential molecular target for the treatment of breast cancer.

2. Materials and methods

2.1. Cell culture

Human breast cancer cell line MDA-MB-231 and MCF-7 were cultured in RPMI 1640 medium supplemented with 10% FBS (GIBICO), at 37 °C with 5% CO2. 293 T cells were cultured in DMEM medium supplemented with 10% FBS (GIBICO), at 37 °C with 5% CO2.

2.2. siRNA knockdown

The knocking down of USP14 was carried out using two distinct Stealth Select RNAi duplexes (Life Technologies Company). Stealth RNAi negative control duplex from Life Technologies Company was used as a control. The RNAi nucleotides were transiently transfected in either MDA-MB231 cells or MCF7 cells using SuperFectin siRNA Transfection Reagent (Pufei, Shanghai, 2103–100) and the relative knock down efficiency was determined by USP14 antibody. The siRNA sequences were: USP14 siRNA1: CCUUAGAGAUUUGUUUGAUUCC AUG; USP14 siRNA2: GGAAGCAAUAGAGGAUGAUUCUGUU.

2.3. Cell proliferation assay

For cell proliferation assay with USP14 siRNAs, cells were seeded in 24-well plates at 3000 cells per well in 0.5 ml medium with 10% FBS. The medium was changed every 2 days. At the indicated time points, cells were fixed in 3.7% formaldehyde and stained with 0.1% crystal violet. Dye was extracted with 10% acetic acid and the relative proliferation was determined by the absorbance at 595 nm.

2.4. USP14 inhibitor (IU1)treatment

Cells were seeded in 24-well plates at 5000 cells per well in 0.5 ml medium with 10% FBS. IU1, a specific inhibitor of USP14, binds to active or proteasome-associated USP14 without showing any effect on other DUBs. After 12–18 hours, different concentrations of diluted IU1 (12.5 μM, 25 μM, and 50 μM) were added to cells. At the indicated time points, cell proliferation was measured as above.

2.5. Cell migration and invasion assays

Cell migration was examined by wound healing and transwell migration assays, and cell invasion was evaluated by using matrigel invasion chambers. These assays were performed as described in the previous study in detail [20]. For phalloidin staining assay, phalloidin can selective bind affinity on filamentous actin (F-actin) to visualize F-actin. MDA-MB-231 cells were seeded into 6-well plates and incubated at a density of 2×10^4 cells/well for 24 h. After knocking down of USP14 for 48 h, cells were treated with TRITC-phalloidin staining assay kit and 200 μl DAPI solution (10 nM), monitored using fluorescence microscope at TRITC Ex/Em = 545/570 nm and DAPI Ex/Em = 364/454 nm.

2.6. Colony formation assay

Colony formation assay was carried out with MDA-MB-231 cells and MCF7 cells that were grown in RPMI1640 with 10% FBS in 60 mm plates and transiently transfected with control siRNA or USP14 siRNAs. After transfection, the cells were trypsinized, counted and 500 cells were seeded in 6-well dishes, and allowed to adhere overnight. On the following day, the media was changed to RPMI1640 + 5% FBS. All cells were then grown for 2 weeks, with medium changed every second day. Plates were fixed with 4% formaldehyde and stained with 2% crystal violet. The images were obtained by a digital camera.

2.7. Quantitative RT-PCR

Total RNA was extracted using Trizol reagent (Invitrogen) and 1 μg total RNA was performed reverse transcription using PrimeScript RT reagent kit with gDNA eraser (TaKaRa), according to the manufacturer's instructions. Quantitative RT-PCR was performed with SYBR Green dye using (Applied Biosystems). The relative amount of cDNA was calculated by the comparative Ct method using GAPDH as a control. PCR reactions were performed in triplicate.

2.8. Cell cycle analysis

Freshly prepared cells were harvested and re-suspended in 0.5 ml PBS. Then the cells were fixed with 70% alcohol on ice for at least 2 h. After centrifugation, the supernatant was discarded and sediment was washed with PBS for once. Cell pellet was re-suspended in 5 ml PBS and then cells were counted. Re-suspended 2×10^5 cells with 400 μl guava cell cycle reagent (Millipore, 4700–0160). After incubating in water bath at 37 °C for 15 min, cell cycle was analyzed by the Millipore GuavaeasyCyte™ flow cytometer (Millipore).

2.9. Immunoprecipitation

After cells were lysed with RIPA buffer (Beyotime, P0013) containing PMSF (DINGGUO, 22WB0181) and cocktail, the cell lysates were precleaned with protein G agarose at 4 ℃ for 1 h, then the supernatant were incubated with indicated antibodies and protein G agarose beads (Roche, 11,243,233,001) at 4 ℃ overnight. On the second day, immunocomplexes combined with beads were washed by lysis buffer, followed by subjected to western blot.

2.10. Protein isolation and western blot

The proteins from tissues and cells were separated by standard 10% SDS-PAGE followed by transferring the proteins to a PVDF membrane. The proteins were detected by the following primary antibodies: USP14 (Proteintech, 14517-1-AP), CyclinB1 (Proteintech, 55004-1-AP), CDC25C (Proteintech, 16485-1-AP), CDK1 (Proteintech, 19532-1-AP), and β-actin (Santa Cruz, sc-4778) and followed by incubation with a secondary antibody. Staining was performed with ECL western blot detection reagent. Antibody to β-actin was served as the endogenous control. All experiments were performed in triplicate.

2.11. Statistical analysis

For each experiment, three independent replicates were performed. All the data were expressed as mean ± SD. Statistical evaluation was conducted using the Student *t*-test The intergroup difference was compared by using one-way analysis of variance followed by Dunnett's test. A p-value of less than 0.05 was considered statistically significant. *, $p < 0.05$ *vs*. control; **, $p < 0.01$ *vs*. control; ***, $p < 0.001$ *vs*. control.

3. Results

3.1. USP14 knockdown inhibits the proliferation of breast cancer cells

To examine the function of USP14 in breast cancer cells, The siRNAs

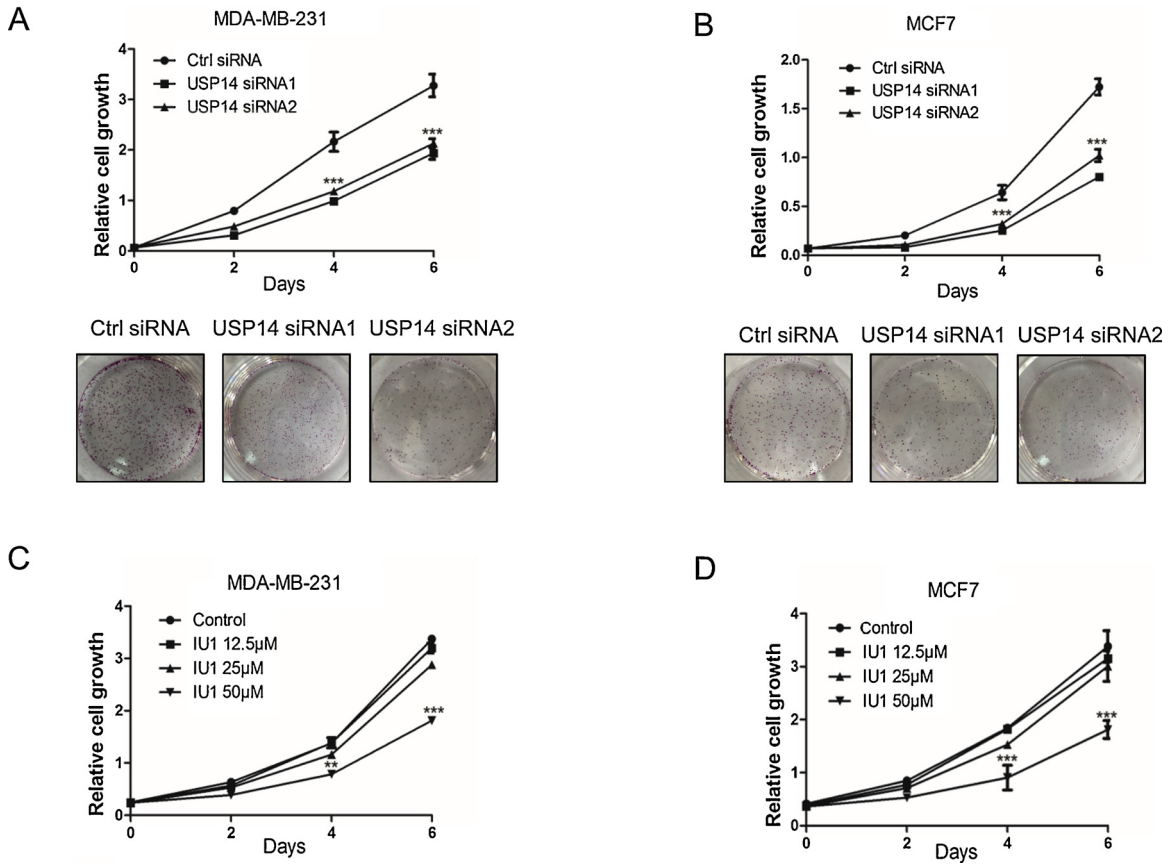

Fig. 1. USP14 knockdown inhibits the proliferation of breast cancer cells. (A and B) MDB-MA-231 and MCF7 cells were cultured in RPMI 1640 with 10% FBS, cells were transfected with either control siRNA (Ctrl siRNA) or USP14 siRNAs. 24 h later, cells were seeded in 24 well plates. At the indicated times, cells were fixed in 3.7% formaldehyde and stained with 0.1% crystal violet. Data represent the average of three independent experiments (mean ± SD). ***P < 0.001. (Upper panels). After transfection with USP14 siRNAs, 500 cells were seeded in 6-well plate. 14 days later, cells were fixed in 3.7% formaldehyde and stained with 0.1% crystal violet. Then the photographs were taken. (Bottom panels). **(C, D)** MDB-MA-231 and MCF7 cell were treated with various concentrations of IU1 (12.5, 25 and 50 μM), At the indicated times, cells were fixed in 3.7% formaldehyde and stained with 0.1% crystal violet. Data represent the average of three independent experiments (mean ± SD). **P < 0.01, ***P < 0.001. Data represent the average of three independent experiments (mean ± SD).

specifically targeting USP14 were transfected into MDB-MA-231 and MCF7 cells. 48 h later, the protein level of USP14 was detected by western blot. As shown in Supplementary Fig. 1A and B, both siRNA1 and siRNA2 efficiently knocked down the expression of USP14 in different cell lines compared with that of control siRNA. Next, we examined the effects of USP14 knockdown on the proliferation of breast cancer cell lines. Knocking down USP14 significantly retarded the proliferation rate and colony formation of breast cancer cells (Fig. 1**A** and **B**). Similarly, IU1, the specific inhibitor of USP14, inhibited the proliferation of cell proliferation in a concentration-dependent manner (Fig. 1**C** and **D**). These data revealed that USP14 was essential for the proliferation of breast cancer cells.

3.2. USP14 knockdown inhibits the migration of breast cancer cells

Given the ability of USP14 to promote breast cancer cell proliferation, we are interested in examining its potential effects on cell migration. To explore these possible effects, wound healing assay was performed. The results showed that the open area created by the "wound" was almost healed after 24 h in control cells. However, the healing of the open area was remarkably attenuated when USP14 was knocked down (Fig.2**A** and **B**). In order to further prove this effect, we also did transwell assay in MDA-MB-231 and MCF-7 cells. As shown in **Supplementary** Fig. 2**A and B**, the number of migrated cells was greatly reduced when USP14 was knocked down. Therefore, USP14 knockdown hindered the migratory ability of breast cancer cells.

3.3. USP14 knockdown arrests cell cycle at G2/M phase

The inhibitory effects of USP14 knockdown on cell proliferation indicated that USP14 might perturb cell cycle-related events. To examine the effects of USP14 knocking down on cell cycle, we transfected USP14 siRNAs or control siRNA into MDA-MB-231 or MCF7 cells, cell cycle progression was analyzed by flow cytometry after propidium iodide staining. As shown in Fig. 3**A** and **B,** knocking down of USP14 significantly increased the proportion of G2/M phase and decreased the proportion of S phase and G0/G1 phase compared with that of control cell. These results indicated that USP14 was indispensable for cell cycle progression in breast cancer cells and knocking down USP14 arrested cell cycle at G2/M phase.

3.4. USP14 knockdown decreases the expression of cell cycle related proteins

To elucidate the mechanism of USP14 in cell cycle regulation, we performed Q-PCR to check the mRNA levels of multiple genes which played essential roles in G2/M phase. As shown in Fig. 4**A** and **B,** the mRNA levels of CCNB1 and CDC25C were not affected in USP14 knockdown cells compared with that in control cells. Furthermore, we examined the protein levels of CyclinB1, CDC25C and CDK1 by western blot. As shown in Fig. 4**C** and **D**, CDK1 and CyclinB1 was significantly decreased in USP14 knockdown cells compared with that of control cells. These findings suggested that USP14 was important for the progression of G2/M phase.

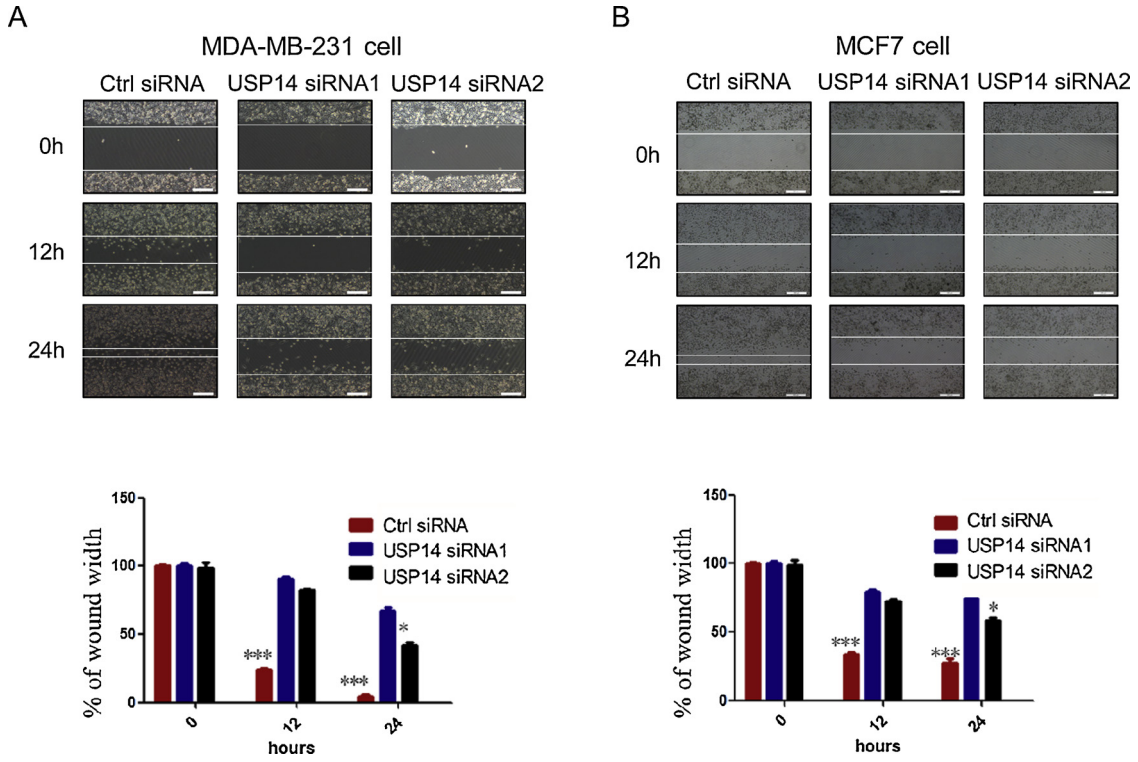

Fig. 2. USP14 knockdown inhibits the migration of breast cancer cells. (A and B) MDB-MA-231 (A) and MCF7 (B) cells transiently transfected with USP14 siRNAs or control siRNA (Ctrl siRNA) were cultured to create a confluent monolayer of 90–100% confluence, then the monolayer was scraped in a straight line to create a "scratch". The extent of cell migration was photographed at the indicated times (Upper panels). The transverse scratch wounds were re-examined and analyzed using image J software at 3 different sites from each wound area of gaps at each time point. Results are presented as mean ± standard error (Bottom panels). *P < 0.05, ***P < 0.001. Scale bar, 200 μm.

3.5. *USP14 regulates the degradation of CyclinB1 by ubiquitination*

We have proved that knocking down USP14 significantly reduced the protein level of CyclinB1, an important question needed to be answered was how USP14 regulated CyclinB1. As a deubiquitinase, we speculated that USP14 decreased the ubiquitination of CyclinB1 to stabilize it. In order to figure out if USP14 could interact with CyclinB1, we performed co-immunoprecipitation experiment and found that USP14 could bind to CyclinB1 (Fig. 5A). We next detected the effect of USP14 on the ubiquitination of CyclinB1. Fig. 5**B** showed that USP14 knockdown increased the ubiquitination level of endogenous CyclinB1. These results demonstrated that USP14 regulated the expression of

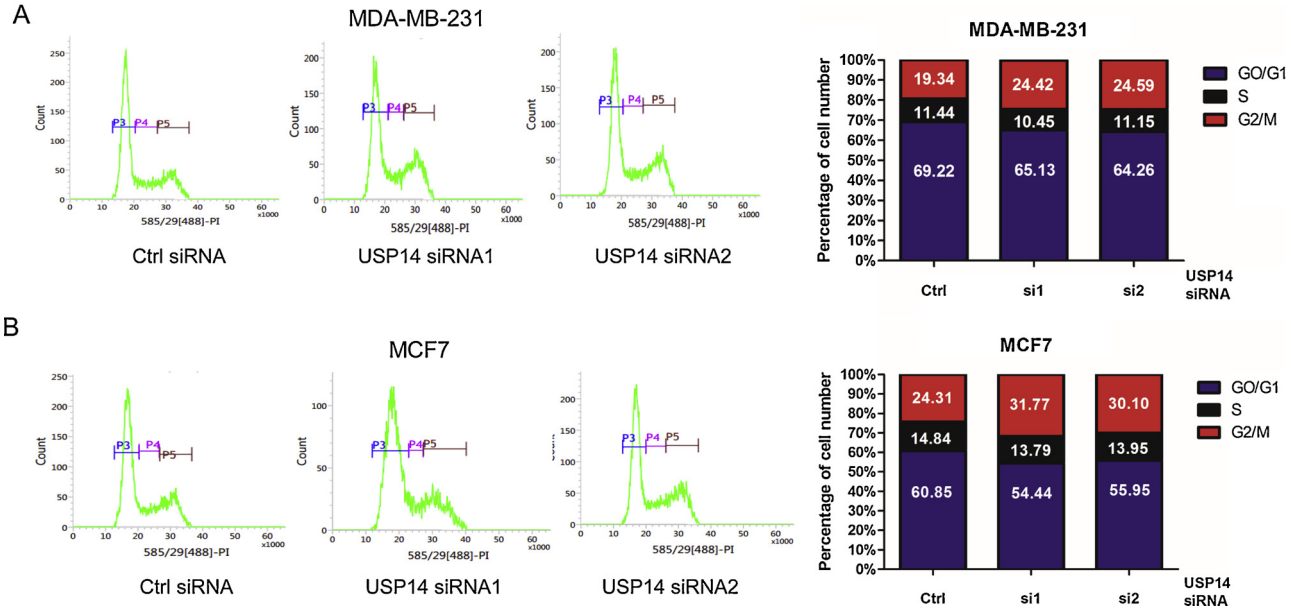

Fig. 3. Knocking down of USP14 arrests cell cycle in G2/M phase. (A and B) MDA-MB-231 (A) and MCF7 (B) cells transiently transfected with USP14 siRNAs or control siRNA (Ctrl siRNA) were cultured in RPMI 1640 with 10% FBS for 48 h. The cells were stained with iodide staining. Adherent cells were collected and cell cycle analysis was done by flow cytometry. The ratio of the cells in each phase was counted.

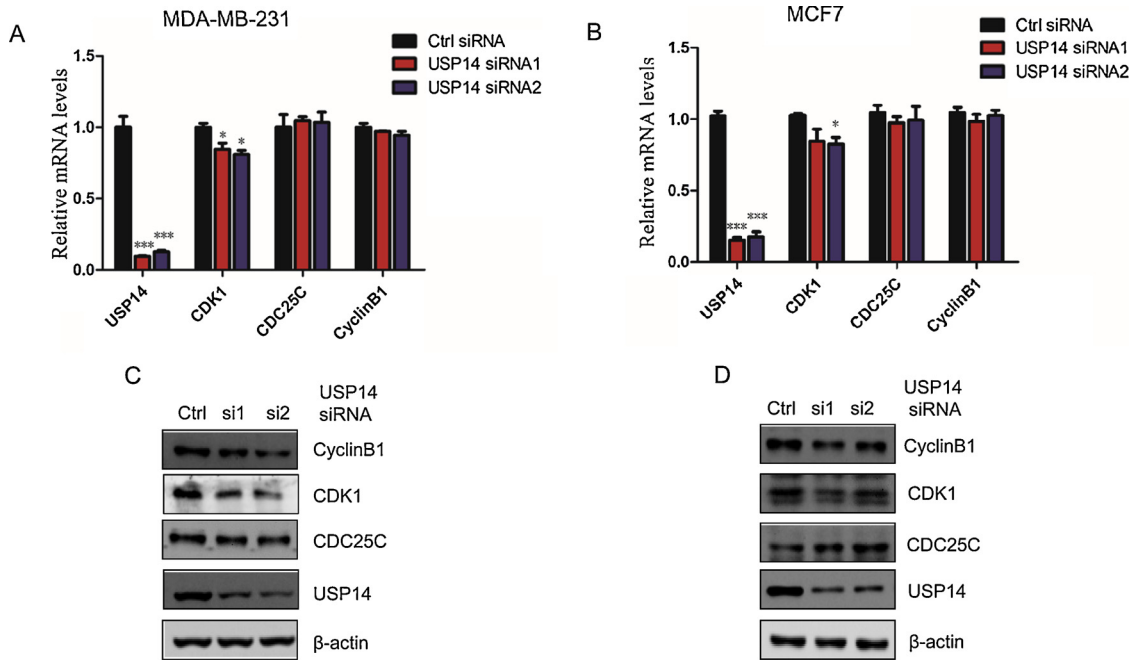

Fig. 4. Knocking down of USP14 decreases the expression of cell cycle related proteins. MDA-MB-231 and MCF7 cells transiently transfected with USP14 siRNAs or control siRNA (Ctrl siRNA) were cultured in RPMI 1640 with 10% FBS for 48 h. **(A and B)** The mRNA expression levels of G2/M phase related genes CyclinB1, CDK1, and CDC25C were tested by quantitative Real-Time PCR. Data represent the average of three independent experiments (mean ± SD). *P < 0.05, ***P < 0.001. **(C and D)** The protein expression levels of CyclinB1, CDK1, and CDC25C were checked by western blot with indicated antibodies.

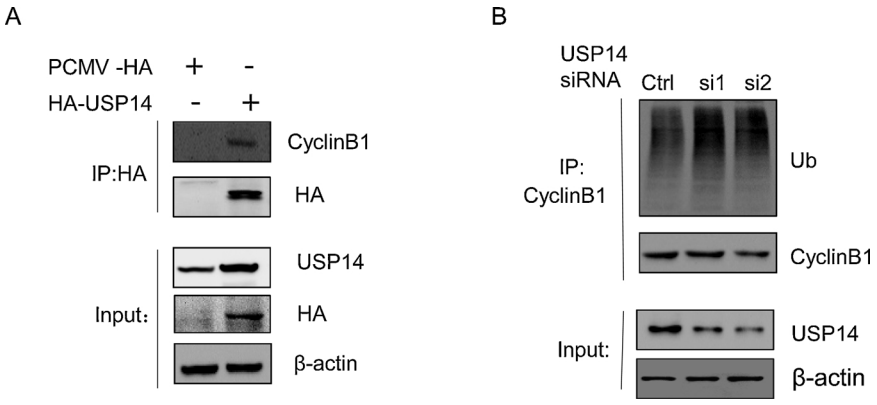

Fig. 5. USP14 regulates the degradation of CyclinB1 by deubiquitination. (A) 293 T cells transfected with or without HA-USP14 were lysed for immunoprecipitation using an anti-HA antibody and blotted with indicated antibodies. **(B)** 293 T cells transfected with or without USP14 siRNAs were lysed for immunoprecipitation using an anti-CyclinB1 antibody and blotted with indicated antibodies.

CyclinB1 through deubiquitination.

3.6. CyclinB1 overexpression recovered the decreased proliferation rate and cell cycle arrest caused by USP14 knockdown

In order to demonstrate that USP14 regulate the proliferation of breast cancer cells through CyclinB1, we co-transfected with HA-CyclinB1 plasmid and USP14 siRNAs followed by cell proliferation assay. Fig. 6**A** and **B** showed that overexpressing CyclinB1 could recover the decreased proliferation of breast cancer cells with USP14 knockdown. Next, we examined the effects of CyclinB1 on cell cycle which was hindered in G2/M phase by USP14 knockdown. CyclinB1 overexpression could partially rescue the cell cycle arrest caused by USP14 knockdown (Fig. 7**A** and **B**). These results demonstrated that USP14 affected the proliferation of breast cancer cells through regulating the ubiquitination of CyclinB1.

4. Discussion and conclusions

USP14, a deubiquitinase reversibly bound to the 26S proteasome, aroused great interest of scientists recently, especially in cancer research. In gastric cancer, silencing of USP14 promoted proteasomal degradation of p-ERK (T202/Y204) and p-Akt (T308/S473), thus inactivating Akt and ERK signaling pathways and sensitizing cells to cisplatin by triggering cisplatin-induced apoptosis [21]. In addition, increased expression of USP14 negatively regulated DNA damage-responsing signaling and promoted radio sensitization in autophagy-deficient cells [22]. Researchers also found that USP14 knockdown dramatically impaired viability of melanoma cells and overcame resistance to MAPK-targeting therapies both in vitro and in human melanoma xenografted mice. Furthermore, they found that USP14 inhibition rapidly triggered accumulation of poly-ubiquitinated proteins and chaperones, mitochondrial dysfunction, ER stress, and ROS production leading to a caspase-independent cell death [23]. In leukemic, USP14 played a vital role in regulating chemotherapy-induced apoptosis by preventing Aurora B to degradate [24]. These results indicated that USP14 was closely related to cancer progression and drug resistance. However, the molecular mechanisms of USP14 in cancer progression were still not so clear. The dysregulation of cell proliferation was one of the hallmarks of cancer [7]. Cell cycle progression was essential for the rapid proliferation of cancer cells. Previous studies also found that USP14 was related to cell cycle progression. Liao Y et al determined

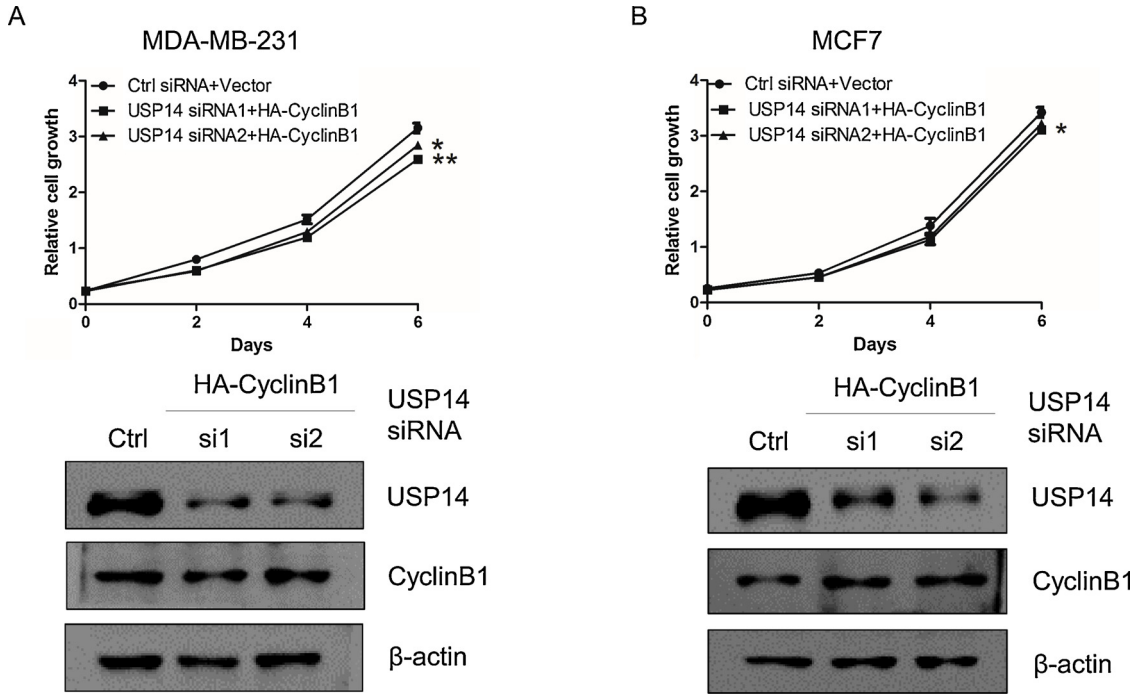

Fig. 6. CyclinB1 overexpression recovered the decreased proliferation rate caused by USP14 knockdown. (A, B) MDB-MA-231 and MCF7 cells were co-transfected with control siRNA and Vector or USP14 siRNAs and HA-CyclinB1. At the indicated times, cells were fixed in 3.7% formaldehyde and stained with 0.1% crystal violet. Data represent the average of three independent experiments (mean ± SD). *P < 0.05, **P < 0.01 (Upper panels). Western blot was used to detect the expression of indicated proteins (Bottom panels).

that inhibition of USP14 accelerated proteasome-mediated degradation of androgen receptor (AR) protein and suppressed cell proliferation by blocking G0/G1 to S phase transition in AR-responsive breast cancer cells [25]. Similarly, downregulation of USP14 expression also arrested the cell cycle by degrading β-catenin [26]. However, little is known about the effects of USP14 in regulating cell cycle related protein.

In our study, we characterized the biological function of USP14 in the proliferation and migration of breast cancer cells. Knocking down USP14 significantly attenuated the growth and migration of breast cancer cells, and arrested cell cycle at G2/M phase. Furthermore, USP14 knockdown affected the expression of a number of cell cycle related proteins including CyclinB1, CDC25C and CDK1. Specially, we

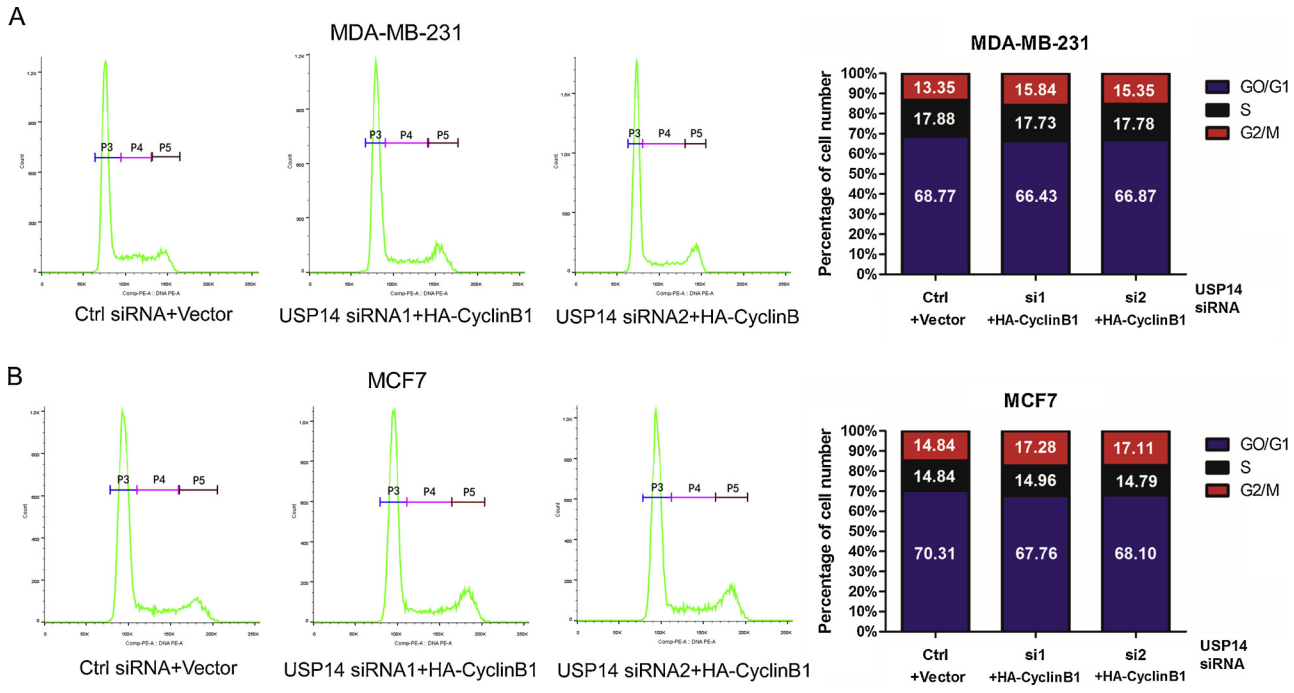

Fig. 7. CyclinB1 overexpression recovered the cell cycle arrest caused by USP14 knockdown. (A, B) MDB-MA-231 and MCF7 cells were co-transfected with control siRNA and Vector or USP14 siRNAs and HA-CyclinB1. Cells were cultured in RPMI 1640 with 10% FBS for 48 h. The cells were stained with iodide staining. Adherent cells were collected and cell cycle analysis was done by flow cytometry. The ratio of the cells in each phase was counted.

found USP14 could bind to CyclinB1 and stabilize CyclinB1 by deubiquitinating. In fact, Lee et al. has done an excellent work to demonstrate that USP14 could regulate the ubiquitination of CyclinB1 [15], however the biological function of this regulation mechanism in cancer was still not known. We demonstrated that USP14 regulated cell cycle progression in breast cancer cell through regulating the ubiquitination of CyclinB1. So our study raised the possibility for targeting USP14 as potential therapeutic strategy to inhibit the progression of breast cancer cells. In conclusion, our study demonstrated that USP14 could regulate cell cycle progression through deubiquitinating and stabilizing CyclinB1, thus affected the proliferation and migration of breast cancer cells.

Declaration of Competing Interest

The authors have no conflicts of interest to disclose.

Acknowledgment

This work is supported by Natural Science Foundation of Jiangxi Province, China (20192BAB215038).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.prp.2019.152592.

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