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ORIGINAL RESEARCH

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CDCA2 promotes breast cancer progression by downregulating KISS1 expression

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Abstract

Breast cancer is a females' prevalent malignancy, resulting in higher mortality rates than other cancers. Therefore, exploring effective therapeutic targets and diagnostic biomarkers is critical for improving drugs' curative effect in treating breast cancer patients. The MDA-MB-231 and Michigan Cancer Foundation-7 (MCF-7) cells were transfected by pc-CDCA2, Small interfering RNA Negative Control (siNC), siCDCA2, and pc-KISS1 to investigate the effects of cell division cycle associated 2 (CDCA2) and kisspeptin 1 (KISS1) protein on breast cancer cell's viability, migration and invasion. Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR) was used to examine the CDCA2 mRNA expression. Impact of CDCA2 and KISS1 on invasion ability, motility and viability of breast cancer cells were detected *via* colony-forming unit, Cell Counting Kit-8 (CCK-8) and transwell assay. The Annexin V-Fluorescein Isothiocyanate (annexin V-FITC) staining was employed to determine the cellular apoptosis ratio based on flow cytometry. Findings indicated that CDCA2 expression got increased in breast cancer tissues, which greatly enhanced the cell proliferation, migration and invasion ability. Moreover, the regulation of CDCA2 may suppress the activation of KISS1 in breast cancer cells. In summary, the findings depicted that in breast cancer cells, the CDCA2 expression was enhanced to promote cells motility, invasiveness and viability through inhibition of KISS1 expression. Therefore, targeted gene therapy to suppress the expression levels of CDCA2 and upregulation of the KISS1 have the potential to be promising therapeutic strategies for treating breast cancer metastasis.

Keywords

CDCA2; KISS1; Breast cancer; Apoptosis; Metastasis

1. Introduction

One of malignant tumors is the breast cancer in women globally, and its incidence has continued to rise [1, 2]. Approximately 1.7 million per annum new breast cancer cases are reported worldwide and more than 30% patients die [3]. Overall 5-year survival rate of breast cancer patients is still less despite advances in the treatment and early diagnosis [4, 5]. Exploring effective therapeutic targets and diagnostic biomarkers is thus critical for improving drugs' curative effect in treating breast cancer patients. There is growing evidence indicating that the cell division cycle-associated proteins (CDCA) has vital function in tumor progression [6]. CDCA1 is a potential prognosis biomarker in oral cancer [7]. In oral squamous cell carcinoma (OSCC), expressions of CDCA2 and CDCA3 were remarkably enhanced [8]. The upregulation of CDCA2 and CDCA3 prevented G1 phase arrest by inhibiting cell cycle protein-dependent kinase inhibitors and enhanced apoptosis [9]. The expression of CDCA4 suppressed apoptosis rate and enhanced proliferation of human breast cancer MCF-7/Acinar-to-Ductal Metaplasia cell (ADM cells) [10]. Moreover, CDCA5 is an oncogene that is overexpressed in bladder, gastric and hepatocellular cancers and is involved in functional regulation of cancer cells [11]. A recent study indicated that CDCA2 enhanced cell proliferation and reduced apoptosis *via* Hypoxia-Inducible Factor 1-alpha (HIF-1α) pathway modulation in prostate cancer [12]. KISS1 is a multifunctional protein involved in modulating cancer cells migration and invasion, induction of autophagy and apoptosis progression [13]. Pervious study reported that KISS1 overexpression in cells of the breast cancer developed to more aggressive tumor and mortality [14]. In addition, numerous research suggested that KISS1 was involved in the tumor angiogenesis, autophagy and apoptosis regulation [15, 16], and may have critical function in enhancing cancer cell invasion. According to The Cancer Genome Atlas (TCGA) data analysis, CDCA2 expression levels were obviously increased in breast tumor samples, but CDCA2 function is yet not clear. Present work aimed to evaluate whether CDCA2 promotes breast cancer cells metastasis and viability by controlling their KISS1 expression levels.

2. Methods

2.1 Cell culture

The MCF10A, MCF-7 and MDA-MB-231 cells were procured from American Type Culture Collection cell bank (ATCC, USA). Short Tandem Repeat (STR) profiling was employed to identify the cell lines. Cell culturing was carried out in Dulbecco's modified Eagle's medium (DMEM) or DMEM/Ham's F12 (Thermo Fisher, USA) containing 10% fetal bovine serum (FBS) (F4135, Thermo Fisher, USA). Upon attaining the cell confluence of 80%–90%, transfection of breast cancer cells was made by empty pcDNA3 vector, pcDNA3 vector having full cDNA sequence encoding human CDCA2 or KISS1, negative control siRNA (siNC) (5′-GTCGAACGTCGTGAACCTACCATG-3′), or CDCA2 siRNA (siCDCA2) (5′-GGGCAAAGGAUCAAGUGAUTT-3′) with Lipofectamine® 3000 reagent as per the company's provided procedure.

2.2 Reverse transcription quantitative polymerase chain reaction (qRT-PCR)

To investigate mRNA expression of CDCA2 in cell lines, the MCF10A, MCF-7 and MDA-MB-231 cells were harvested, and Trizol (Invitrogen) digestion was employed to extract the total RNA by following manufacturer's protocol. The nanodrop quantified the purity and concentration of extracted RNA. SuperScript III Reverse Transcriptase (Thermo Fisher, U.S.) and SYBR Green Master Mix (Bio-Rad) were used to reversely transcribe RNA into cDNA. β-actin acted as internal control. The comparative $2^{-\Delta\Delta Ct}$ method calculated the RNA expression. Primers of the current work were as follows:

CDCA2:
Forward (5′-3′): TGCCGAATTACCTCCTAATCCT;
Reverse (5′-3′): TGCTCTACGGTTACTGTGGAAA;
β-actin:
Forward (5′-3′): GAGCGCGGCTACAGCTT;
Reverse (5′-3′): TCCTTAATGTCACGCACGATTT.

2.3 Western blot

Total protein from MCF10A, MDA-MB-231 and MCF-7 cells were harvested and separated by 10 % Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). Next, proteins were moved onto nitrocellulose membranes and treated overnight at 4 °C with primary antibody against CDCA2 (1:3000, ab214289, Abcam), KISS1 (1:3000, NBP2-34010, Novus Biologicals), and β-actin (1:10,000, ab8227, Abcam). The incubation was then made at 37 °C for 60 min with horseradish peroxidase-labeled secondary antibodies. The proteins on membrane were quantified by enhanced chemiluminescence (Bio-Rad). The internal control was β-actin.

2.4 CCK-8

The cell counting kit-8 (96992, Sigma-Aldrich, USA) evaluated the effects of pc-CDCA2, pc-KISS1, siNC or siCDCA2 on the viability of MCF-7 and MDA-MB-231 cells. Seeding density of MDA-MB-231 and MCF-7 cells was 3×10^3 cells per well of 96-well plate. The 10 μM of CCK-8 solution was then added, and incubated at 37 °C for 4 hours to determine cell viability. The absorbance at the wavelength of 450 nm was recorded *via* microplate reader.

2.5 Transwell assay

The 8 μm pore Boyden chamber (Corning) as the upper chamber in 24-well was used to perform the migration assay of transfected MCF-7 and MDA-MB-231 cells. 5×10^4 cells were placed in the medium with no serum and seeded into upper chamber. In the meantime, 10% FBS medium was added into the lower chamber was added with. The Matrigel (Corning) was used to pre-coat the upper chamber in invasion assay, while 10% FBS medium was added into the lower chamber. Cells in upper chamber were removed after 24 hours. Cells were fixed for 30 min with 4% paraformaldehyde present on bottom membrane. The fixed MDA-MB-231 and MCF-7 cells were stained by haematoxylin solution. Number of invaded cells were counted under an upright microscope and images were taken from 4 distinctive fields.

2.6 Colony-forming unit assays

They were conducted by incubating MDA-MB-231 and MCF-7 cells (300 cells/well) transfected with siCDCA2, siNC or pc-CDCA2. Then, the cell culturing was done in 24-well plates using growth medium with 10% FBS. Staining of colonies was made by crystal violet solution (0.5% crystal violet/10% ethanol) after the incubation of 14 days at 37 °C. The stained colonies were calculated under microscope and the image was analyzed by ImageJ software (1.8.0., National Institute of Health, USA).

2.7 Cell apoptosis analysis

The cellular apoptosis ratio was determined by using annexin V-fluorescein isothiocyanate (FITC) staining. After transfection with pc-CDCA2, pc-KISS1, siNC or siCDCA2, the MCF-7 and MDA-MB-231 cells were collected, and annexin V-FITC apoptosis detection kit (APOAF, Sigma-Aldrich, USA) was employed to analyze the number of apoptotic positive cells by following the provided protocol. MDA-MB-231 and MCF-7 cells apoptosis was assessed *via* the flow cytometry (BD Biosciences) by employing the detector for Annexin V-PI at 488 nm.

2.8 Statistical analysis

GraphPad Prism Software 7 (GraphPad Software Inc., La Jolla, CA, USA) was used to analyze the data obtained from the experiments conducted in triplicate. Data were shown as mean $\pm$ Standard Error of the Mean (SEM). The *t*-tests and One-way Analysis of Variance (ANOVA) were employed for the comparison. The statistical analysis was made by Tukey-Kramer post hoc test. $p < 0.05$ was considered as the statistically significant difference.

3. Results

3.1 CDCA2 was overexpressed in breast cancer cells

To explore expression levels of CDCAs in breast cancer tissue and normal tissue, this study analyzed the Gene Expression Profiling Interactive Analysis (GEPIA). The present study analyzed 291 normal and 1085 breast cancer tissues. CDCA2 was upregulated in breast cancer tissues as shown in Fig. 1a. The results of other subtypes of CDCAs were shown in **Supplementary Fig. 1**. To determine whether CDCA2 expression changes in breast cancer cells, the study herein compared CDCA2 expression in normal breast cell line, and MDA-MB-231 and MCF-7 by western blotting and qRT-PCR analysis, respectively. CDCA2 mRNA expression was significantly increased in MDA-MB-231 and MCF-7 cells (Fig. 1b,c). The representative western blotting analyses of MDA-MB-231, MCF-7 and MCF10A cells were depicted in Fig. 1c. CDCA2 protein expression level was obviously enhanced for MDA-MB-231, and MCF-7 cells (Fig. 1c). Together, the data reveal that CDCA2 expression level was enhanced in the breast cancer tissue and cells.

3.2 CDCA2 promoted cell proliferation in breast cancer cells

To analyze impact of CDCA2 on breast cancer cells' biological functions, the MDA-MB-231 and MCF-7 cell lines were transfected with pc-CDCA2, siNC and siCDCA2. Densitometric analysis of the bands from the western blots exhibited that CDCA2 expression level was increased for both breast cancer cell lines in the pc-CDCA2 transfected group and obviously suppressed in siCDCA2 transfected group than NC and siNC (Fig. 2a). Biological functions of cells with different transfection were evaluated by employing colony-forming unit assay and CCK-8 assay. The CCK-8 assay results revealed that pc-CDCA2 remarkably enhanced the cell proliferation ratio, whereas transfection with siCDCA2 evidently inhibited the ability of proliferation of MDA-MB-231 and MCF-7 cells (Fig. 2b). Similarly, MDA-MB-231 and MCF-7 cells' colony number was obviously decreased in siCDCA2-transfected group compared to siNC-transfected group (Fig. 2c). Conversely, the proliferation of cells was significantly improved in pc-CDCA2 transfected group. Cell apoptosis was determined *via* Annexin V-FITC/PI double staining to investigate the action mechanism of CDCA2 protective impact on breast cancer. Flow cytometry data disclosed that the apoptosis rates of early and late apoptosis touched to ~15 to 20% in siCDCA2 transfected breast cancer cells compared with those in other groups (Fig. 2d). Meanwhile, pc-CDCA2 transfection significantly decreased the cell apoptosis ratio of breast cancer cells. Together, the outcomes suggested that CDCA2 might greatly increase proliferation of MCF-7 and MDA-MB-231 cells.

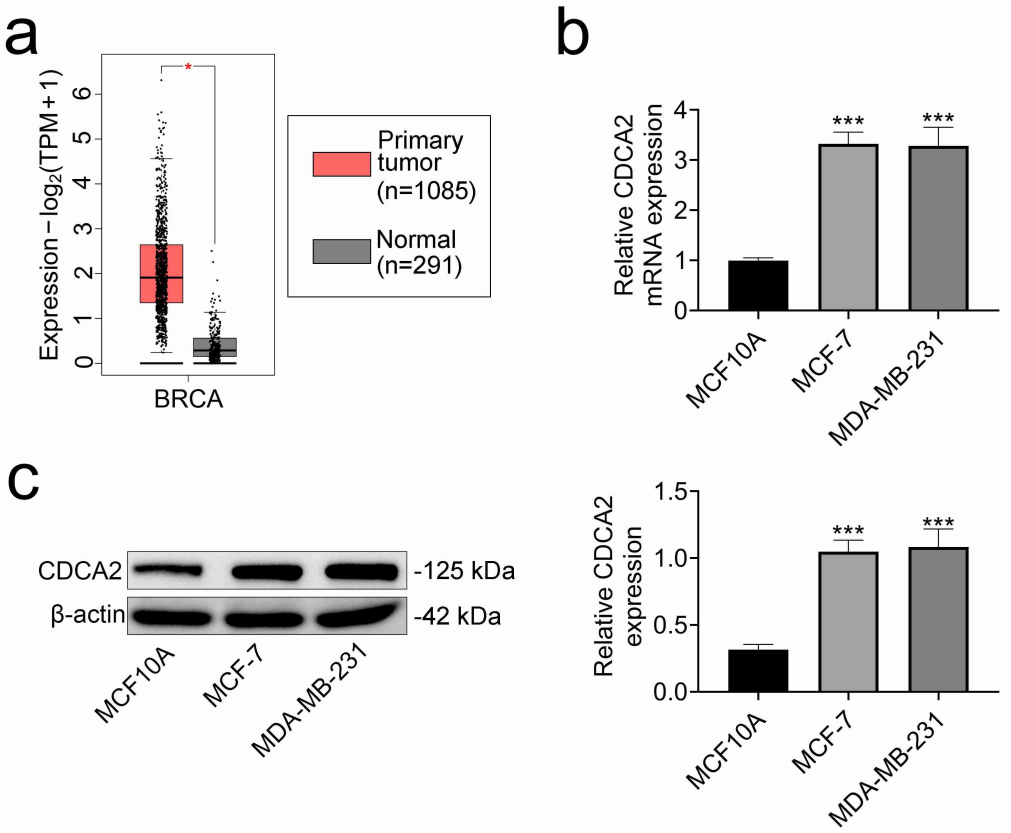

FIGURE 1. The transcriptional levels of CDCA2 in breast cancers. (a) The transcriptional levels of CDCA2 in breast cancer tissue (GEPIA). BRCA (BReast CAncer), T (Tumor tissue), N (Normal tissue). (b) CDCA2 mRNA expression levels were measured by RT-qPCR in MCF10A, MCF-7 and MDA-MB-231 cells. (c) CDCA2 protein expression level was explored by Western blot. *$p < 0.05$. ***$p < 0.005$ *vs.* MCF10A. The data were presented as mean ± SEM. CDCA2: cell division cycle associated 2; TPM: Transcripts Per Kilobase of exon model per Million mapped reads; MCF: Michigan Cancer Foundation.

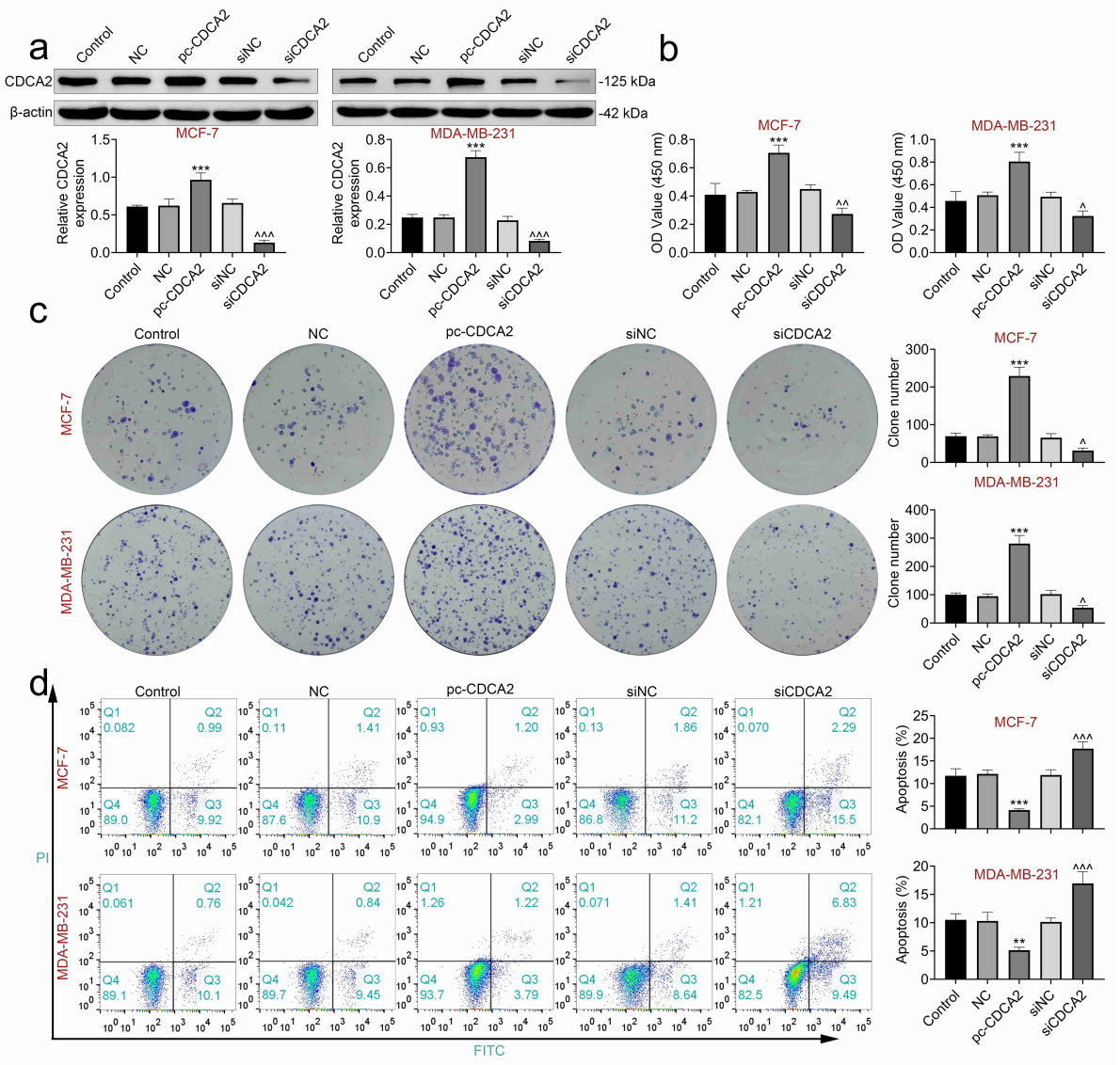

FIGURE 2. The impact of CDCA2 on breast cancer cells proliferation. (a) After transfection of pc-CDCA2, siNC, and siCDCA2, western blotting was employed to determine the expression levels of CDCA2 in MDA-MB-231 and MCF-7 cells. β-actin was used as an internal control. (b) CCK-8 assay measured the breast cancer cells viability in various groups. (c) The impact of CDCA2 expression on cell viability was measured by colony-forming unit assay. (d) The apoptotic rate in CDCA2 transfected MDA-MB-231 and MCF-7 cells was shown by flow cytometry. siNC: empty siRNA, ***$p < 0.005$ *vs*. Control. **$p < 0.01$ *vs*. Control. ^$p < 0.05$ *vs*. siNC. ^^$p < 0.01$ *vs*. siNC. ^^^$p < 0.005$ *vs*. siNC. The data were presented as mean ± SEM. CDCA2: cell division cycle associated 2; NC: Negative Control; OD: Optical Density; PI: Propidium Iodide; MCF: Michigan Cancer Foundation.

3.3 CDCA2 enhanced invasion and cell motility of breast cancer cells

Motility and invasion of breast cancer cells was quantified *via* transwell assays to explore whether CDCA2 was involved in regulating invasion ability and cell motility of breast cancer cells. After pc-CDCA2, siNC and siCDCA2 transfection, the data from the transwell migration assay disclosed that the motility of MDA-MB-231 and MCF-7 cells was obviously enhanced by transfecting with pc-CDCA2 compared with that in NC (Fig. 3a). Moreover, the pc-CDCA2 induced effect was reversed by the siCDCA2 transfection. Likewise, the invasion assay results revealed that invasive cells transfected with siCDCA2 were significantly less than cells transfected with pc-CDCA2 in MDA-MB-231 and MCF-7 cells (Fig. 3b). Therefore, the findings suggest that CDCA2 could modulate breast cancer cells' invasion ability and motility.

3.4 CDCA2 suppressed KISS1 expression in breast cancer cells

Next, we assessed correlation between the level of CDCA2 and KISS1 activation. As shown in Fig. 4, immunoblotting analysis demonstrated that KISS1 expression was enhanced for breast cancer cells transfected by siCDCA2. In contrast, overexpression of CDCA2 inhibited the activation of KISS1. These results indicate that CDCA2 may be able to inhibit the expression of KISS1 in breast cancer cells.

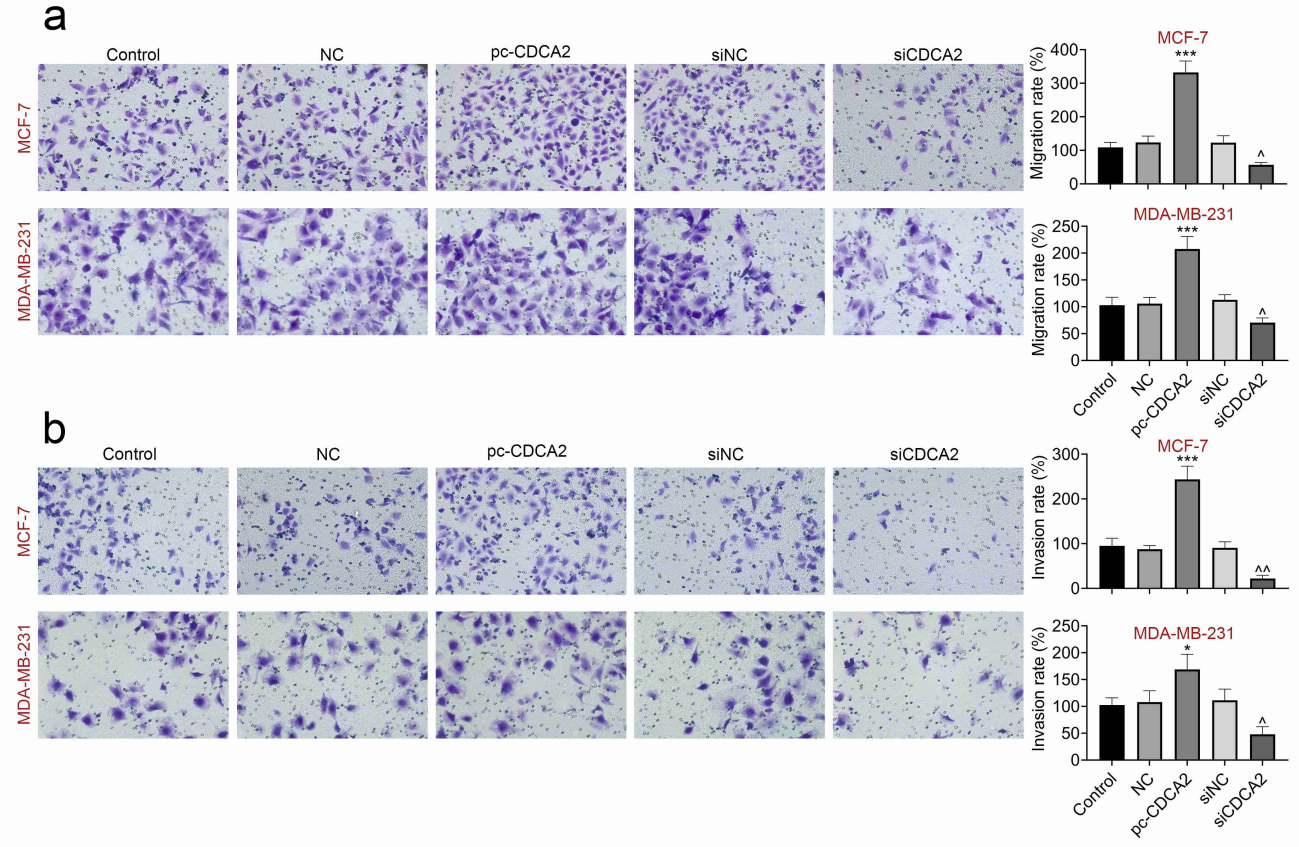

FIGURE 3. CDCA2 effects on breast cancer cells motility and invasion. (a) Migration assay was performed for breast cancer cells transfected with pc-CDCA2, siNC and siCDCA2. (b) Invasion rate of MDA-MB-23 and MCF-7 cells transfected with pc-CDCA2, siNC and siCDCA2 was measured by invasion assay. siNC: empty siRNA, $*p < 0.05$ *vs.* Control. $***p < 0.005$ *vs.* Control. $\hat{}p < 0.05$ *vs.* siNC. $\hat{}\hat{}p < 0.01$ *vs.* siNC. The data were presented as mean ± SEM. CDCA2: cell division cycle associated 2; NC: Negative Control; MCF: Michigan Cancer Foundation.

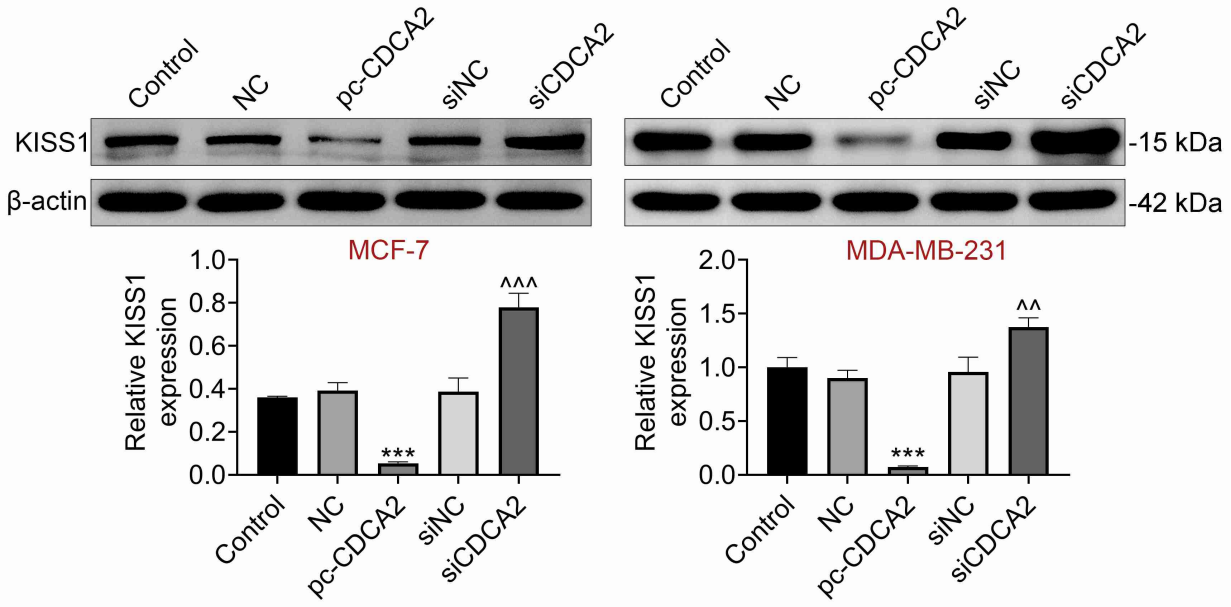

FIGURE 4. CDCA2 impact on KISS1 expression in breast cancer cells. KISS1 protein expression levels in MDA-MB-231, and MCF-7 cells were explored by western blot. β-actin was acted as an internal control. siNC: empty siRNA, $***p < 0.005$ *vs.* Control. $\hat{}\hat{}p < 0.01$ *vs.* siNC. $\hat{}\hat{}\hat{}p < 0.005$ *vs.* siNC. The data were presented as mean ± SEM. CDCA2: cell division cycle associated 2; KISS1: kisspeptin 1; NC: Negative Control; MCF: Michigan Cancer Foundation.

3.5 CDCA2 promoted cellular proliferation, motility and invasion through downregulation of KISS1

To further evaluate the role of CDCA2 in regulating KISS1 expression, the cell viability, motility, and invasion ability was monitored *via* migration and invasion assay, and CCK-8 assay. The immunoblotting results revealed that KISS1 protein expression was obviously reduced after cells transfected with pc-CDCA2, while co-transfection with pc-KISS1 could reverse this effect (Fig. 5a). The biological functions of cells with different transfections were investigated by employing annexin V-FITC/PI double staining and CCK-8 assay. CCK-8 assay revealed that pc-CDCA2 remarkably enhanced cell proliferation ratio, but pc-KISS1 co-transfection obviously suppressed the MCF-7 cells proliferation ability (Fig. 5b). Cells were analyzed by flow cytometry and stained *via* annexin V-FITC/PI to understand the effects of KISS1 on cellular apoptosis. The outcomes reflected that the expression of KISS1 enhanced the apoptotic cell numbers, while co-transfection of pc-CDCA2 reversed this effect (Fig. 5c). The invasive and migrate ability of transfected MCF-7 cells was measured by counting the cells number that digested matrigel and migrated through the 8 μm pores in the transwell. Findings illustrated in above assay depicted that CDCA2 induced motility and invasion of MCF-7 cells was remarkably inhibited by co-transfection with pc-KISS1 (Fig. 5d). These results disclosed that upregulation of CDCA2 boosted the migration, invasion and proliferation of breast cancer cells through suppressing KISS1 expression.

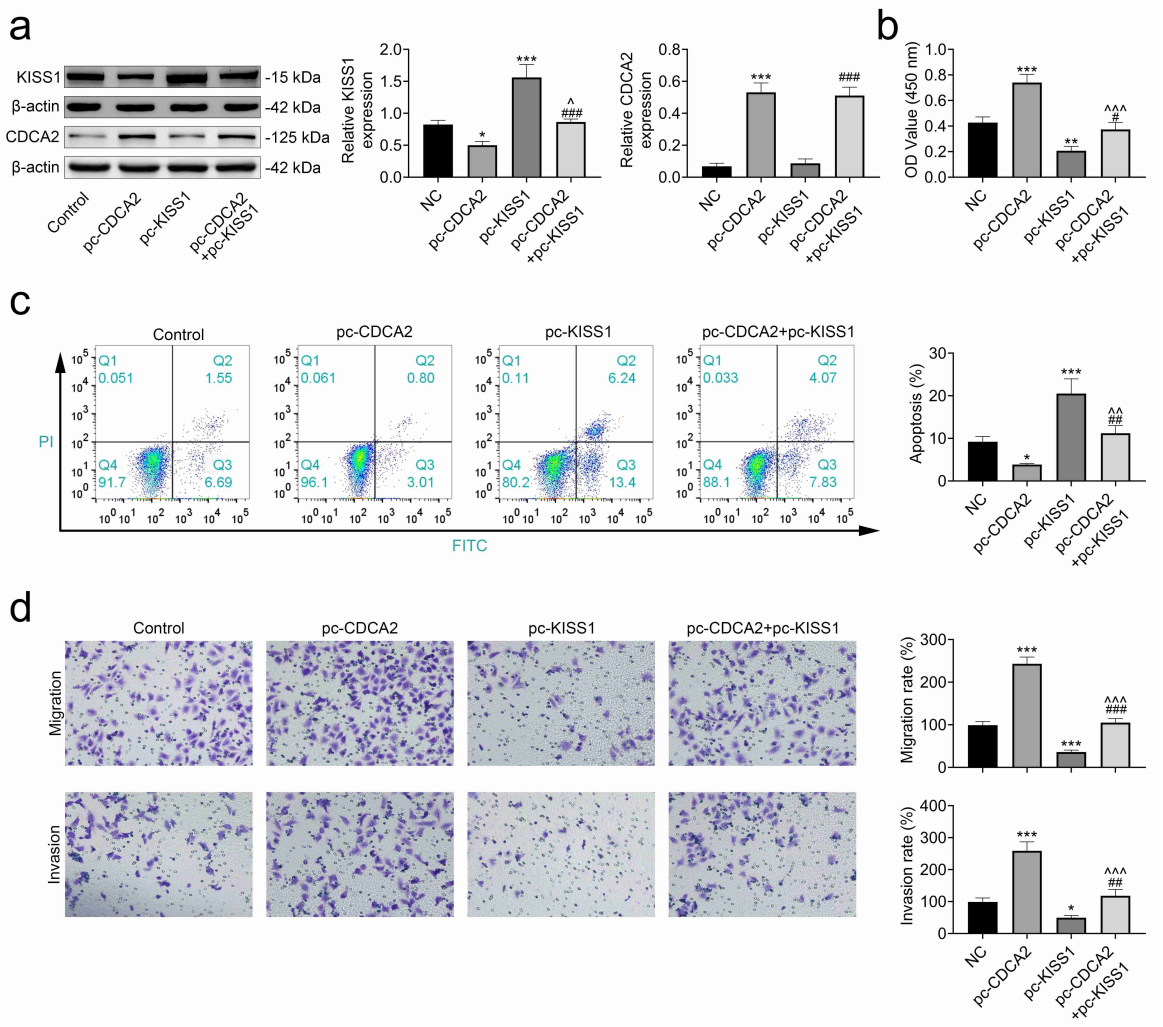

FIGURE 5. KISS1 inhibition by CDCA2 contributed to breast cell proliferation, motility and invasion. (a) Protein expressions of KISS1 and CDCA2 in MCF-7 cells transfected with pc-CDCA2, pc-KISS1, and pc-CDCA2 + pc-KISS1 were explored by western blot. β-actin was used as internal control. (b) CCK-8 assay was employed to measure the breast cancer cells viability in various groups. (c) Representative images and quantification by flow cytometry depicted MCF-7 cells apoptotic rate. (d) Distinctive images and quantification of MCF-7 cells by transwell assay that were transfected with pc-CDCA2, pc-KISS1, and pc-CDCA2 + pc-KISS1. NC: empty pcDNA3 vector, $^{*}p < 0.05$ *vs*. NC. $^{**}p < 0.01$ *vs*. NC. $^{***}p < 0.005$ *vs*. NC. $^{\wedge}p < 0.05$ *vs*. pc-CDCA2. $^{\wedge\wedge}p < 0.01$ *vs*. pc-CDCA2. $^{\wedge\wedge\wedge}p < 0.005$ *vs*. pc-CDCA2. $^{\#}p < 0.05$ *vs*. pc-KISS1. $^{\#\#}p < 0.01$ *vs*. pc-KISS1. $^{\#\#\#}p < 0.005$ *vs*. pc-KISS1. The data were represented as mean ± SEM. CDCA2: cell division cycle associated 2; KISS1: kisspeptin 1; FITC: fluorescein isothiocyanate; OD: Optical Density; PI: Propidium Iodide.

4. Discussion

In the current study, it has been exhibited that overexpression of CDCA2 occurred in the breast cancer tissues and greatly enhanced cell invasion, migration and proliferation ability. Moreover, CDCA2 regulation may suppress the expression of KISS1 in breast cancer cells.

Breast cancer is one of the prevalent malignant tumor in females with higher mortality rates than other cancers. Several treatment methods have been exploited for the therapy and prevention of this disease. The mortality of breast cancer is related to the cancer cells metastasis to multiple organs including lungs, bones and brain [17]. The mechanism of breast cancer cells metastasis is intricate and involves various genes as a regulator or a mediator. Therefore, the expression of target genes in specific subtypes of breast cancer play an important role in tumor development and metastasis. This study investigated the CDCA2 expression and its effects on regulation of the KISS1. Our results showed that modulation of CDCA2 expression was critical for the metastasis of breast cancer cells. The work herein provided first evidence of upregulated CDCA2 in breast cancer cells and promoted their invasion, motility and proliferation ability. Results from these experiments were consistent with previous research that CDCA2 was overexpressed in both oral squamous cell carcinoma and lung adenocarcinoma, and it stimulated the proliferation of certain types of tumors [18, 19]. Recent studies indicated that CDCA2 could regulate the activation of DNA damage checkpoint and was involve in the chromatin remodeling [20]. Numerous studies have also illustrated that CDCA2 was related to the occurrence, progression and proliferation of tumor in various type of cancers, such as squamous cell carcinoma, colorectal cancer and neuroblastoma [19, 21]. Moreover, the inhibition of CDCA2 expression can cause the G1 arrest of oral squamous carcinoma cells and lung adenocarcinoma cells [18]. In the present study, CDCA2 showed a modulatory effect on breast cancer cells proliferation. The cell apoptosis rate was obviously enhanced when the CDCA2 expression in breast cancer cells was significantly inhibited. The data in this study also suggested that the expression of CDCA2 facilitates the invasion and motility of breast cancer cells. This results was consistent with a previous study that knockdown of CDCA2 markedly suppressed migratory abilities of melanoma cells [22]. However, it is not clear how CDCA2 contributes to cancer cells migration and invasion. KISS1 is one of the metastasis suppressor genes in tumorigenesis. Therefore, the work herein evaluated the association between the expressions of KISS1 and CDCA2. Results of this study suggested that overexpression of CDCA2 suppressed KISS1 expression in the breast cancer cells. Furthermore, CDCA2 can promote cell invasion, motility and proliferation *via* KISS1 expression downregulation. This finding was similar to the recent research which showed that overexpression of KISS1 could suppress invasion and migration of breast cancer MDA-MB-435 cells [23]. Moreover, KISS1 reduced breast cancer cell invasion and motility *via* the inhibition of Tumor Necrosis Factor-α (TNF-α) induced activation of Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) [24]. Anti-metastatic characteristics of KISS1/KISS1 receptor (KISS1R) signaling are demonstrated in several cancers, being obtained *via* KISS1/KISS1R-induced suppression of activity by matrix metalloproteinase 9 [25]. This evidence revealed that high KISS1R expression in Human Epidermal Growth Factor Receptor (HER) 2-negative carcinoma could show alternate pathway to stimulate the tumor cells proliferation upon low HER 2 expression. The recent study also reported that KISS1 can be a potential therapeutic target for treating metastasized cancers *via* the elimination of quiescent cells or by the long-term induction of dormancy in tumor cells [26]. The kisspeptins family is generated from proteolytic cleavage by KISS1 and could affect the metastatic ability of ovarian cancer cells. Some preclinical data implied that the KISS1 expression gene induction and therapeutic usage of kisspeptins might block metastasis of breast cancer [27]. Thus, kisspeptins could be used as a novel potential anticancer molecular agent. Previous study indicated that KISS1 could trigger apoptosis and prevent breast cancer cells mobility and also suppressor controls angiogenesis of brain metastases [28]. In clinical research, also found that the levels of KISS1 were raised in breast cancer. The lesser elevation of KISS1 expression was negative prognosis for overall survival, metastatic propensity, axillary lymph node status, advancing grade, and advancing tumor stage of breast cancer patient [29]. For the breast cancer, KISS1 might act as prognosis biomarker.

The current work had shortcoming that effect of CDCA2 expression was only analyzed in *in-vitro* study, which needs to be studied *in vivo*. We will confirm these results with animal experiments in the future. The potential implication of CDCA2 in breast cancer cells remains to be investigated.

5. Conclusions

In summary, this research demonstrated that CDCA2 was overexpressed in breast cancer cells that can ameliorate cells motility, invasiveness and viability through inhibition of KISS1. Therefore, targeted gene therapies to suppress the expression levels of CDCA2 and upregulation of the KISS1 expression may be novel therapeutic strategies for breast cancer metastasis treatment.

AVAILABILITY OF DATA AND MATERIALS

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

AUTHOR CONTRIBUTIONS

YL—designed the research study. ZH—performed the research. GLF and DGX—analyzed the data. JC—wrote the manuscript. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

ACKNOWLEDGMENT

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found, in the online version, at https://oss.ejgo.net/files/article/1779756757534294016/attachment/Supplementary%20material.docx.

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