

Original Article

USP43 promotes tumorigenesis through regulating cell cycle and EMT in breast cancer

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Abstract: USP is one protease family of deubiquitylating enzyme (DUB). It removes ubiquitin from substrate and stabilizes the substrate. Here, we scanned several novel deubiquitinase enzymes in breast cancer tissue samples, and found that USP43 was up-regulated in breast cancer and closely relationship with metastasis, tumor size and a poor prognosis. Consequently, CCK-8 assay and colony formation assay were used to assess the function of USP43 in cell proliferation. Moreover, cell apoptosis assay and cell cycle assay revealed that USP43 facilitated cells G₁/S phase transition, but had slightly influence in cell apoptosis. EMT is a current event in breast cancer; the cells which happened have strong metastasis ability. We next detected whether USP43 regulated EMT, our results demonstrated that USP43 promoted EMT through regulated snail, thereby facilitated breast cancer cells metastasis. In brief, our works showed a novel mechanism of USP43 in facilitating breast cancer cells proliferation and metastasis. In addition, USP43 might be a novel therapeutic target for breast carcinoma.

Keywords: USP43, EMT, Snail, cell cycle, proliferation, breast cancer

Introduction

Breast carcinoma has become the second most common female carcinoma. There are almost 1.67 million new patients diagnosed in 2012 [1]. Several treatment options, such as radiation therapy, surgery and systemic therapy have successfully increased the survival e of breast cancer patients [2].

USP is one protease family of deubiquitylating enzyme (DUB). It has complex structural and a cysteine box [3-6]. Multiple USP family proteins have found that play crucial role in tumor development. Such as USP11 [7], USP28 [8], USP36 [9] and USP42 [10]. A previous report revealed 22 novel human USP proteins [11], including USP41, USP43, USP50. The function of those protein in cancer were still unknown.

Epithelial mesenchymal transition (EMT) is a crucial tumor event, it promotes cancer cells metastasis [12]. One characterize of EMT is lost epithelial cell phenomenon and gain mesenchymal cell phenomenon. In molecular level,

the epithelial marker, such as α -catenin and E-cadherin were decreased, whereas the mesenchymal marker, such as fibronectin and N-cadherin were obviously increased. Multiple transcription factors promote EMT.

Here we found that USP43 was up-regulated in breast carcinoma. And the expression of USP43 closely associated with tumor size, metastasis and poor prognosis. Moreover, USP43 facilitated breast cancer cells proliferation through promoted cell cycle. In addition, our work revealed that USP43 facilitated EMT process through regulated snail. But the detail mechanism of USP43 regulating snail was still unknown.

Materials and methods

Patients and tissue samples

Retrieved 89 breast cancer specimens from The Third Affiliated Hospital of Kunming Medical University during 2013 and 2015. We have informed to all patients that their tissues were used for research purposes, and our work was

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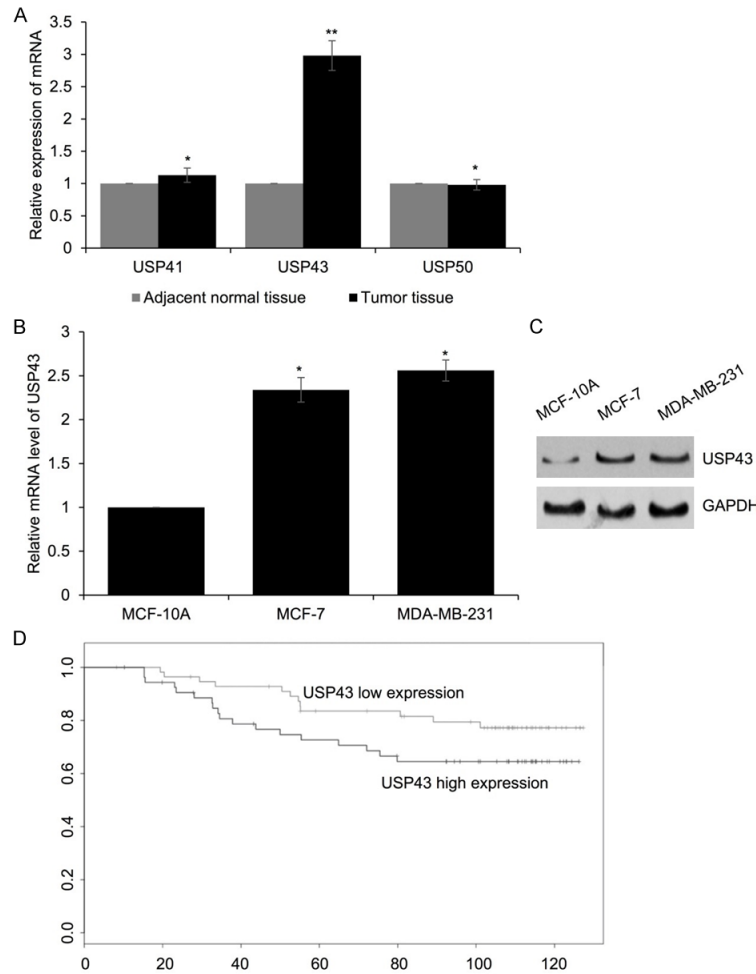


Figure 1. USP43 is up-regulated in breast cancer and indicated a poorly prognosis. A. Collected patients tissue samples and extracted RNA. Performed qRT-PCR to assess the relative mRNA level of USP41, USP43 and USP50. B. QRT-PCR was used to measure the mRNA level of USP43 in breast cancer cells MCF-7 and MDA-MB-231, compared with human normal breast carcinoma cells MCF-10A. C. Western blot was used to measure the protein level of USP43 in breast cancer cells MCF-7 and MDA-MB-231, compared with human normal breast carcinoma cells MCF-10A. D. Survival curve was drawn by Kaplan-Meier method. The result revealed that the patients who have high USP43 expression have shorter survival time than the patients who have low USP43 expression.

approved by The Ethics Committee of The Third Affiliated Hospital of Kunming Medical University. The breast cancer tissues and adjacent normal tissues were from patients who were diagnosed to have breast cancer.

Cell culture and antibodies

Purchased human normal breast cells, MCF-10A and human breast cancer cells, MDA-MB-231 and MCF-7 from ATCC (USA). MCF-7 and MDA-MB-231 cells were cultured in DMEM

medium which supplement with 10% fetal bovine serum (FBS) and 2 mg/mL streptomycin, 2 U/mL penicillin G. MCF-10A cells were cultured in DMEM/F12 medium supplemented with EGF (10 ng/ml), cholera toxin (1 µg/ml), insulin (10 µg/ml), heat-inactivated horse serum (5%) and hydrocortisone (1 µg/ml). Cells were incubated at 37°C with 5% CO₂.

Anti-USP43 antibody was purchased from Abcam (UK). EMT Antibody Kit was purchased from Cell Signaling Technology. GAPDH was used as internal control, and anti-GAPDH antibody was purchased from Sigma-Aldrich (USA).

Cell invasion assay

Matrigel coated transwell chamber filters (Chemicon Incorporation) were used to cell invasion assay. After over expressed or silenced USP43 in MDA-MB-231 for 48 h, starved cell 24 h with serum-free medium, next placed 3×10⁵ cells/well into the upper chamber with serum-free medium. Transferred the chamber to six-well plate which contained DMEM supplement with 10% FBS. Incubated cells for 8-12 h at 37°C. Removed the cells in the upper membrane by cotton swabs, used 0.1% crystal

violet to stain cells for 15 min at room temperature. Finally, counted cells under microscope. Each independent experiment was performed at least three times.

Western blotting

Utilized RIPA buffer to lyse the whole proteins from cell and measured the protein concentration by BCA Protein Assay Kit (TIANGEN). Mixed protein sample with 5× SDS/PAGE loading buffer at boiled for 15 min. The equal

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Table 1. Clinicopathologic variables in 89 breast cancer patients

Variables	No. (n=89)	USP43 protein expression		P value
		Low (n=33)	High (n=56)	
Age				
<40	45	15	30	0.459
≥40	44	18	26	
Tumor size (diameter)				
Small (≤3 cm)	44	24	20	<0.001
Large (≥3 cm)	45	9	36	
Pathological grade				
I-II	46	23	23	0.009
III-IV	43	10	33	
pT status				
pT1	41	25	16	<0.001
pT2-4	48	8	40	
pN status				
pN0	47	23	24	0.014
pN1-2	42	10	32	
Metastasis				
Yes	46	11	35	0.008
No	43	22	21	
Smoker status				
Never smoke	41	16	25	0.725
Smoker	48	17	31	
Differentiation				
Well/moderate	41	15	26	0.929
Poor	48	18	30	

amount protein were resolved in 8% SDS/PAGE gels and next transferred protein onto nitrocellulose (NC) filter membrane. Blocked NC membranes with 5% skimmed milk at room temperature for 1 h, consequently incubated membranes with indicated antibody at 4°C overnight. Washed membranes with PBST solution three times, followed by incubation with indicated secondary antibody. Visualized the blots on X-ray films by ECL reagent (Millipore) followed to the manufacturer's protocol. GAPDH was used as internal control.

qRT-PCR assay

Extracted the whole RNA from cells by TRizol reagent according to the manufacturer's method, and then synthesis cDNA by Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific). Next performed qRT-PCR to analyze relative mRNA expression of indicated gene. Each inde-

pendent experiment was performed at least three times. The primers sequences as follow: GAPDH: 5'-CCTCAAGATCATCAGCAATGC-3' and 5'-ATGGACTGTGGTCATGAGTC-3'; a-catenin: 5'-AAACCACAGAGTAACTGGC-3' and 5'-AATGTGATTCTCTGAGACAGC-3'; E-cadherin-5'-ACCTGGTTCAGATCAAATCC-3' and 5'-TCATTCTGATCGTTACCGT-3'; Fibronectin: 5'-CAATGTGAACGACACATTCCA-3' and 5'-ACC-ACTTGAGCTTGGATAGG-3'; N-cadherin: 5'-CAGAGTTTACTGCCATGACG-3' and 5'-AAAGTC-GATTGGTTTGACCA-3'. Each independent experiment was performed at least three times.

CCK-8 assay

The CCK-8 assay was used to assess the cell proliferation ability. Transfected MCF-7 cells with USP43 siRNA or FLAG-USP43 or control, after 48 h, cells were placed in a 96-well plate at 3×10^3 each well with 200 μ l DMEM medium. Incubated cells at 37°C with 5% CO₂. Added 20 μ l CCK-8 solution to each well, next incubated for 1 h. Finally, measured absorbance at 450 nm wavelength. Each independent experiment was performed at least three times.

Colony formation assay

According to previous described [13, 14], we performed colony formation assay to detected cells proliferation ability under indicated experiment condition. In brief, MCF-7 cells which over expressed or silenced USP43 were placed in a 6-well plate at 5×10^3 each well, then incubated at 37°C with 5% CO₂ for 12 days. Fixed colonies with 70% ethanol and stained with 0.5% crystal violet for 15 min at room temperature. Counted the clones number under microscope. Each independent experiment was performed at least three times.

Apoptosis and cell cycle analysis

Flow cytometer (FACS) was used to perform cell apoptosis assay and cell cycle assay. Silenced or over expressed USP43 in MCF-7 cells, after transfection 48 h, collected and washed cells with PBS for three times, then fixed cells in 70% ethanol, stained cells with PI solution or AnnexinV-PI solution in cell cycle analysis or apoptosis analysis, respectively. Finally, detected apoptosis and cell cycle by flow cytometer

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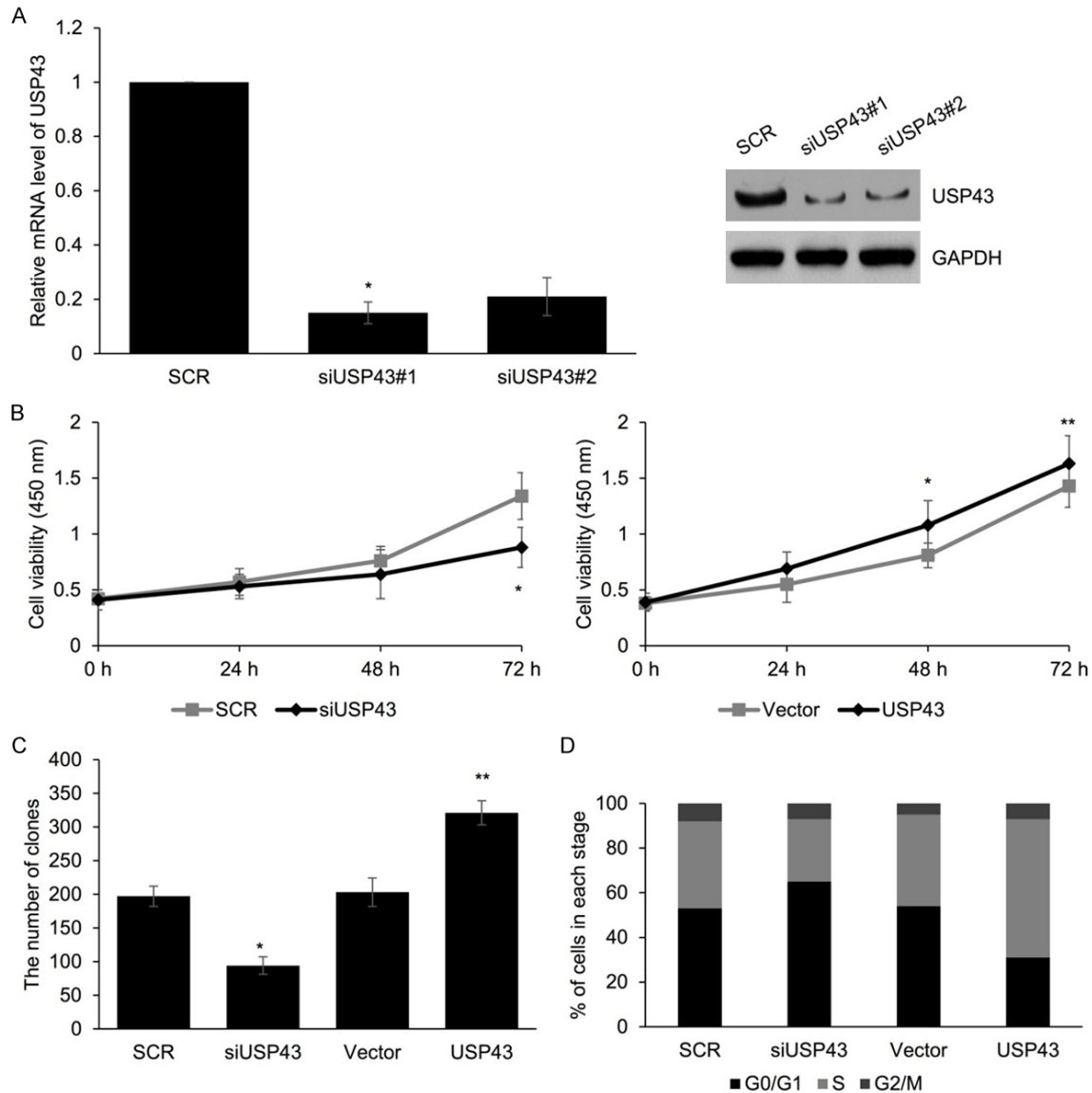


Figure 2. USP43 promotes breast cancer cell growth. A. Transfected with SCR or USP43 siRNA in MCF-7 cells, after transfection 48 h, extracted total RNA and whole protein, qRT-PCR and western blot were used to verify the efficiency of USP43 siRNA. B. Silenced or over expressed USP43 in MCF-7 cells, after transfection 48 h, performed CCK-8 assay, the growth curves was drew by CCK-8 assay. C. Silenced or over expressed USP43 in MCF-7 cells, after transfection 48 h, placed 5×10^3 cells/well in a 6-well plate, then incubated at 37°C with $5\% \text{CO}_2$ for 12 days. Fixed and stained clones. Counted the clone number under microscope. D. Silenced or over expressed USP43 in MCF-7 cells, after transfection 48 h, serum starvation for 24 h, and released cells 12 h. Cell cycle was measured by flow cytometry (FACS).

(FACS). Each independent experiment was performed at least three times.

Statistical analysis

All results were analyzed with SPSS V.17.0. The values were analyzed by one-way ANOVA followed by Tukey's test and reported as mean \pm S.D. The considered statistically significant was $P < 0.05$ (* $P < 0.05$, ** $P < 0.01$). Each inde-

pendent experiment was performed at least three times.

Result

USP43 is up-regulated in breast cancer and indicated a poorly prognosis

USP41, USP43 and USP50 as novel USP protein, their function remain unknown. Here,

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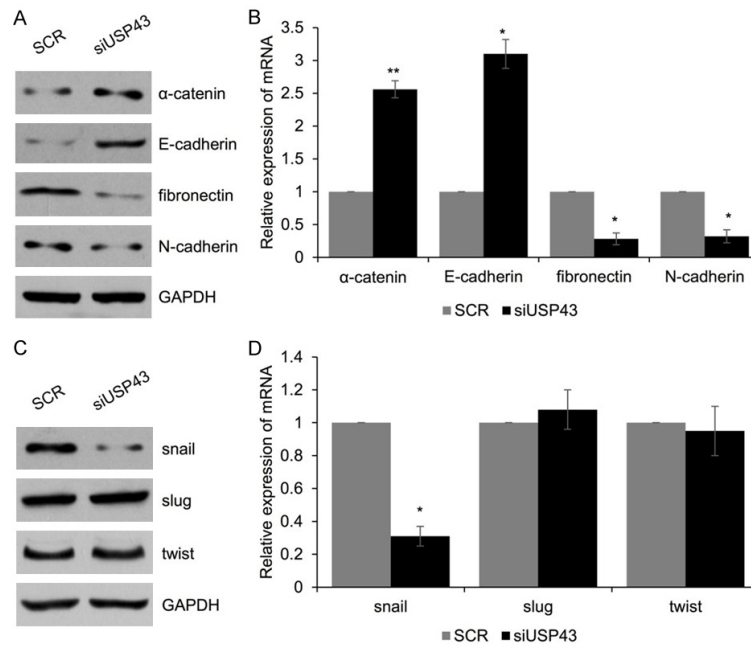


Figure 3. USP43 facilitates EMT and regulated snail. A. Transfected with SCR or USP43 siRNA in MCF-7 cells, after transfection 48 h, extracted whole protein, Western blot was used to detect the protein level of EMT associated protein. B. Transfected with SCR or USP43 siRNA in MCF-7 cells, after transfection 48 h, extracted total RNA, qRT-PCR was used to assess the mRNA level of EMT associated gene. C. Transfected with SCR or USP43 siRNA in MCF-7 cells, after transfection 48 h, extracted whole protein, Western blot was used to detect the protein level of indicated transcription factors. D. Transfected with SCR or USP43 siRNA in MCF-7 cells, after transfection 48 h, extracted total RNA, qRT-PCR was used to assess the mRNA level of indicated transcription factors.

we investigated whether those proteins take part in breast cancer development, we first collected 89 breast cancer patients tissue and adjacent normal tissue samples, extracted RNA from those samples and performed reverse transcription and qRT-PCR to assess the relative mRNA level of USP41, USP43 and USP50. The results showed that USP43 was significantly up-regulated in human breast cancer tissue samples compared with normal tissue samples. USP41 and USP50 have slightly changed in tumor samples (**Figure 1A**). So, we next focus on USP43. Consequently, MCF-10A, a human normal breast cell lines and MDA-MB-231, MCF-7, breast carcinoma cell lines were used to confirm the USP43 expression condition. As shown in **Figure 1B** and **1C**, both mRNA level and protein level of USP43 were dramatically up-regulated in MDA-MB-231 and MCF-7 cell lines compared with MCF-10A cell lines. Moreover, according analysis the correlation between USP43 expression and patients'

clinical information, we found that USP43 expression also positively correlated with tumor size, metastasis and pathological grade (**Table 1**). Moreover, survival curve revealed that the patients who have high USP43 expression have shorter survival time than the patients who have low USP43 expression (Hazard Ratio=1.31, P=0.034, **Figure 1D**).

USP43 promotes breast cancer cell growth

USP43 expression was positive correlation with tumor size, so we assumed that whether USP43 regulated cell proliferation or cell apoptosis. To further explore the cellular function of USP43 in breast cancer, we utilized two different USP43 siRNA to knockdown USP43 in MCF-7 cell, and qRT-PCR and Western blotting were used to determine the efficiency of siRNA. As shown in **Figure 2A**, USP43 were knockdown almost 85%

in MCF-7 cells which transfected USP43 siRNA#1, compared with control groups which transfected scramble siRNA (SCR). The USP43 siRNA#1 was more efficiency than USP43 siRNA#2, so USP43 siRNA#1 was used for further experiments. Next, CCK-8 assay and colony formation assay were used to explore whether USP43 regulated cell proliferation. The results demonstrated that USP43-depleted effectively suppressed cell proliferation and over expression of USP43 facilitated cell proliferation, including cell growth rate assay and foci formation frequency assay in vitro (**Figure 2B** and **2C**). Subsequently, to further investigate the proliferation of cells is due to apoptosis or cell cycle, flow cytometry (FACS) was used. We found there are no significantly changed while silenced USP43 in MCF-7 cells (data not shown). In addition, we detected whether USP43 take part in cell cycle. We synchronized MCF-7 cells at G_0/G_1 via serum starvation for 24 h, and released cells 12 h through

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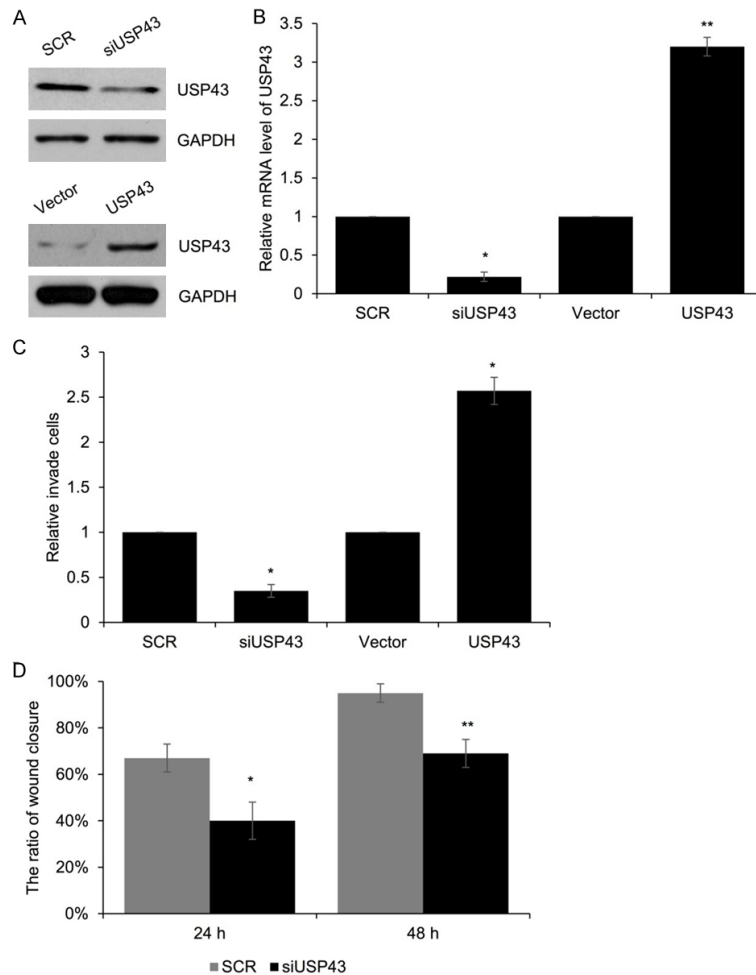


Figure 4. USP43 promotes breast cancer cell metastasis. A. Silenced or over expressed USP43 in MDA-MB-231 cells, after transfection 48 h, extracted whole protein, western blot was used to verify the expression of USP43 in MDA-MB-231 cells. B. Silenced or over expressed USP43 in MDA-MB-231 cells, after transfection 48 h, extracted total RNA, qRT-PCR was used to verify the mRNA level of USP43 in MDA-MB-231 cells. C. Silenced or over expressed USP43 in MDA-MB-231 cells, after transfection 48 h, and cultured in transwell chamber for 8-12 h. Each independent experiment was performed at least three times. D. Silenced or over expressed USP43 in MDA-MB-231 cells, after transfection 48 h, utilized 100 μ l pipettes to scratch cells. Measured the ratio wound closure at 24 h and 48 h compared with 0 h.

cultured cells with DMEM medium which supplement 10% FBS. Then we assessed cell cycle profiling. To our surprised, we found USP43 facilitated G_1 phases to S phases transition (**Figure 2D**). Together, USP43 promoted breast cancer cell growth by facilitated cells G_1/S phase transition.

USP43 facilitates EMT and regulated snail

Cancer cells metastasis is one reason which poorly prognosis of breast cancer. Our previous

work revealed that USP43 expression was correlation with metastasis. EMT regulated multiple cell program, such as cell metastasis. So we next detected whether USP regulated EMT process. We silenced USP43 in MCF-7 cells and detected EMT marker. As shown in **Figure 3A**, while USP43-depleted obviously increased the expression of epithelial marker, such as α -catenin and E-cadherin, meanwhile, the mesenchymal marker, fibronectin and N-cadherin were decreased (**Figure 3A** and **3B**). In order to further decipher the molecular mechanisms that USP43 facilitated EMT, we next identified whether USP43 regulated several transcription factors which involved in EMT process, such as slug, snail and twist. As shown in **Figure 3C** and **3D**, qRT-PCR and western blot demonstrated that snail was significantly down-regulated in MCF-7 cells which USP43 was depleted, but slug and twist have no significantly changed, it suggested that snail was regulated by USP43.

USP43 promotes breast cancer cell metastasis

Consequently, we investigated the function of USP in cancer cell metastasis in vivo. We performed transwell assays in

the MDA-MB-231, which a human highly invasive breast carcinoma cell line. We first over expressed and silenced USP43 in MDA-MB-231 cells and detected USP43 expression (**Figure 4A** and **4B**). Next, we performed transwell assay to explore the function of USP43 in breast cancer cells metastasis. The results revealed that the number of invade cells through matrigel were decreased while USP43 was depletion. However, ectopic expression of USP43 resulted in the number of invade cell through matrigel increased (**Figure 4C**). Above works

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revealed that USP43 enhanced the invasion capability of MDA-MB-231 cells. Moreover, wound healing assay also confirmed that USP43 facilitated cell metastasis. To sum up, USP43 plays a crucial function in EMT and cell invasion.

Discussion

Post-translational modification is a pivotal event in cancer development. E3 ubiquitin ligases catalyzed proteins ubiquitination, thereby regulate protein degradation and transcription [15]. Deubiquitylating enzyme (DUB) reversed the function of E3 ubiquitin ligases [16, 17]. Multiple DUB proteins play a crucial role in cancer development, such as USP14 [18], UCH-L1 [19], OTUB1 [20]. Recently, a report revealed 22 novel human USP proteins [11], including USP41, USP43, USP50. The function of those protein in cancer were still unknown.

In order to investigate the function of novel USP proteins in breast carcinoma. We first detected the expression level of those proteins in breast cancer tissue samples. We found that USP43 was high expression, but USP41 and USP50 had similar expression level in tumor and normal tissue samples. Moreover, we found high expression of USP43 was closely associated with pathological grade, metastasis, and tumor size. Survival curve was drew by Kaplan-Meier method and it revealed that the patients who have high expression level of USP43 had worse survival than the patients who have low expression level of USP43. To further explore the molecular mechanism of USP43 in cancer development, we utilized two siRNA to knock-down USP43 and chose the more efficiency siRNA for consequent experiments. Colony formation assay and CCK-8 assay all showed that USP43 promoted breast cancer cell proliferation. To further investigate the proliferation of cells is due to apoptosis or cell cycle, flow cytometry (FACS) was used and apoptosis assay demonstrated that USP43 had slightly function in cell apoptosis. However, cell cycle assay revealed that USP43 promoted cell G₁/S phase transition.

EMT as an important event in breast carcinoma, it plays complex function, including facilitates cancer cell metastasis. Meanwhile, several reports indicated that DUB regulated EMT [19, 20]. So, we next deciphered the function of

USP43 in EMT. To our surprised, we found USP43 promoted EMT. In addition, snail, a key transcription factor which facilitates EMT, was also regulated by USP43. However, the detail mechanism of USP43 regulation of snail was still unknown. Moreover, while silenced USP43 in human highly invasive breast cancer cells, MDA-MB-231, the cell invasiveness ability was obviously suppressed. These results demonstrated that USP43 regulated EMT, and facilitated the invasion ability of breast cancer cells. In conclusion, our work showed that USP43 was up-regulation in breast cancer, and USP43 expression level was correlation with tumor size, metastasis and pathological grade in breast cancer. Moreover, we found that USP43 promoted EMT through regulated snail. In addition, USP43 might be a novel therapeutic target for breast carcinoma.

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Disclosure of conflict of interest

None.

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