Overexpression of the Ubiquitin-Specific Peptidase 9 X-Linked (USP9X) Gene is Associated with Upregulation of Cyclin D1 (CCND1) and Downregulation of Cyclin-Dependent Inhibitor Kinase 1A (CDKN1A) in Breast Cancer Tissue and Cell Lines

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Background: The role of the ubiquitin-specific peptidase 9 X-linked (USP9X) gene in breast cancer remains poorly understood. This study aimed to investigate the role of USP9X in breast cancer tissue and cell lines.

Material/Methods: Immunohistochemistry was used to examine the expression levels of USP9X in 102 breast cancer tissue samples and 41 normal breast tissue samples. Overexpression of USP9X in MCF-7 and MDA-MB-231 breast cancer cell lines were studied by USP9X lentivirus vector transfection. Clustered regularly interspaced short palindromic repeats (CRISPR)/caspase-9 USP9X gene knockout was performed. Cell proliferation, growth, and survival were examined using the cell counting kit-8 (CCK-8) assay, the colony formation assay, flow cytometry assays, and a tumor xenograft study.

Results: Immunohistochemistry showed that USP9X was significantly overexpressed in 93 of 102 (91.1%) breast cancer tissue samples compared with 41 normal breast tissue samples and was associated with tumor size ≥5.0 cm (P<0.05). USP9X overexpression in MCF-7 and MDA-MB-231 breast cancer cell lines were increased cell proliferation and survival, significantly reduced the number of cells in the G1-phase cells and increased the number of cells in the S-phase cells, which were reversed by CRISPR/caspase-9 USP9X gene knockout. Overexpression of USP9X upregulated the CCND1 gene encoding cyclin D1 and downregulated cyclin-dependent inhibitor kinase 1A (CDKN1A) gene in breast cancer cells, which were reversed by USP9X knockout.

Conclusions: Overexpression of USP9X was associated with upregulation of the CCND1 gene and downregulation of the CDKN1A gene in breast cancer tissue and cell lines.

MeSH Keywords: Breast Neoplasms • Cell Cycle • Cell Proliferation • Cell Survival • Cyclin-Dependent Kinase Inhibitor p21

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Background

Worldwide, breast cancer is the most common cancer in women [1]. Several environmental and genetic factors influence the onset of breast cancer [2]. Genetic traits, age, and hormone status are considered the most important risk factors for susceptibility to breast cancer [3]. Breast cancer includes a variety of characteristics that differ morphologically, biologically, and molecularly, leading to differences in clinical outcome and survival [4]. The main clinical treatments are surgery, chemotherapy, radiotherapy, targeted therapy, and endocrine therapy. Although these treatments can kill most tumor cells in a short time and have a therapeutic effect, most patients develop tumor resistance and recurrence, which leads to disease progression [5].

Currently, the molecular mechanisms involved in the development of breast cancer incidence are poorly understood. While several studies have shown that aberrant oncogene expression and expression of their protein products or key signaling molecules may have essential roles in cancer development [6], the pathways involved in breast cancer remain to be determined. Investigation of the molecular mechanisms associated with the occurrence, development, invasion, metastasis, and survival in patients with breast cancer would be of diagnostic or prognostic value for providing a foundation for clinical treatment and for studying the prognosis of patients with breast cancer.

Recently, several studies have shown that abnormal expression of X-linked ubiquitin-specific peptidase 9 (USP9X) is closely associated with tumor progression [7–12], but whether USP9X is associated with breast cancer remains unclear. Therefore, the aims of the present study were to investigate USP9X expression in breast cancer tissues and its effect on breast cancer cell proliferation, growth, and survival. This study included the investigation of the effects of overexpression and loss of expression of USP9X following the knockout of the USP9X gene.

Material and Methods

Ethics statement

The Affiliated Hospital of Hangzhou Normal University Ethics Committee approved this study. All patients consented to the use of their tissue samples for research.

Tissue samples

We obtained 102 paraffin-embedded breast cancer tissue samples and 41 paraffin-embedded normal breast tissue samples from patients who had undergone surgical resection between January 2008 and December 2015 at the Affiliated Hospital of Hangzhou Normal University (Zhejiang, China). All samples had been diagnosed pathologically and classified by hospital pathologists.

Immunohistochemistry (IHC) for USP9X

The anti-USP9X antibody was from Cell Signaling Technology (Danvers, MA, USA). The other IHC reagents were immunohistochemical staining reagent, citric acid antigen repair solution, concentrated diaminobenzidine (DAB) color reagent kit, and hematoxylin staining reagent (all from Beyotime Institute of Biotechnology, Shanghai, China). IHC detection of USP9X expression in the tissues was performed according to the agent instructions. Pathologists evaluated the results using a semi-quantitative method. Based on the degree of tissue staining, the results were divided into four grades: –, no staining; +, weak staining; ++, moderate staining; and ++++, strong staining. Staining intensity ≥ + was considered positive. USP9X expression was determined by ratios, where USP9X expression levels in the cancer tissue were divided by that in the normal breast tissue. Ratios >1 indicated USP9X overexpression in the cancer tissues; ratios <1 indicated no significant difference between USP9X expression levels in the tissue containing breast cancer and the normal breast tissue. Ratios <1 indicated downregulated USP9X in the cancer tissues.

Cells and mice tumors

The human breast cancer cell lines MCF-7 (HTB-22™) and MDA-MB-231 (HTB-26™) were from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Eagle’s minimum essential medium with 10% fetal bovine serum (FBS) at 37°C in a humidified incubator with 5% CO₂ according to the supplier’s instructions. Nude BALB/c mice (5-week-old) were purchased from Shanghai Slaccas Laboratory Animals Co., Ltd. (Shanghai, China), and maintained under standard conventional conditions. The Animal Care and Use Committee of the Zhejiang Academy of Medical Sciences approved the animal studies.

Stable overexpression of USP9X

USP9X complementary cDNA was inserted into a pLXV-Neo vector to construct the USP9X overexpression vector pLXV-Neo-USP9X. After sequencing, the target lentiviruses were constructed by transfecting either pLXV-Neo-USP9X or pLXV-Neo empty vector with lentivirus packaging plasmds into 293T cells according to the manufacturer’s protocol. The MCF-7 and MDA-MB-231 cells were transfected with the resulting lentiviruses, and G418 was used to screen for cell lines stably overexpressing USP9X. The cellular expression of USP9X was confirmed by Western blot.
USP9X knockout in MCF-7 and MDA-MB-231 cells

The CRISPR/Cas9 (Clustered regularly interspaced short palindromic repeats/caspase-9) system was used to knock out the USP9X gene. A single guide RNA (sgRNA: CCATTATCCGGGCACGTACA) targeting the USP9X gene was designed using an online CRISPR design tool (crispr.mit.edu). The sgRNA oligonucleotide was cloned into the lentivirus vector (Addgene #52962) and co-transfected into MCF-7 and MDA-MB-231 cells using lentiviruses (Addgene #52963). Following puromycin screening, the efficiency of the USP9X-deficient cells was identified by sequencing.

Western blot

USP9X, cyclin D1 (CCND1), and cyclin-dependent inhibitor kinase 1A (CDKN1A) expression in the MCF-7 and MDA-MB-231 cells were detected by Western blot. Pierce™ ECL Western Blotting Substrate was used, and the results were detected using a ChemiDoc™ Touch Imaging System (Bio-Rad, Hercules, CA, USA). The anti-USP9X antibody (1: 1000) used was the same as that used for IHC. The anti-CCND1 (1: 1000) and anti-CDKN1A (1: 1000) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody (1: 4000) serving as the control was purchased from Cell Signaling Technology.

Cell counting kit-8 (CCK-8) assay

The MCF-7 and MDA-MB-231 cells were divided into five groups: non-transfected or wild-type, transfected with empty vector lentivirus particles, transfected with USP9X lentivirus particles, transfected with negative CRISPR/Cas9 vector, and USP9X-deficient. The cells were seeded in 96-well plates (4000 cells/well; culture medium, 100 µL/well). Seeding per group was repeated in six different wells. After overnight culture in a 37°C incubator with 5% CO₂, the cells were grown in culture medium containing 5% FBS for a total of 72 h. During this period, CCK-8 assays were performed at 6, 24, 48, and 72 h by adding CCK-8 reagent (10 µL, 5 mg/mL; Beyotime Institute of Biotechnology) to each well, and culturing the cells for another 1 h. The blank contained culture medium and CCK-8. The absorbance at 450 nm (OD450) against that of the blank was measured using a microplate reader (Bio-Tek, Winooski, VT, USA).

Colony formation assay

The colony formation assay was performed using MCF-7 and MDA-MB-231 cells (density=500 cells/well) in 6-well plates. After 2 weeks, the formed cell clones were checked. The clones were fixed in methanol and stained with 0.1% crystal violet at room temperature. Clones containing ≥50 cells were deemed positive clones when observed under a microscope.

Flow cytometry assay

Annexin V-fluorescein isothiocyanate and propidium iodide (FITC/PI) kits for detecting apoptosis were from Beyotime Institute of Biotechnology. MCF-7 and MDA-MB-231 cell apoptosis were examined with flow cytometry by staining with the kits according to the manufacturer’s instructions. The PI staining kit for cell cycle analysis was also from Beyotime Institute of Biotechnology. The cell cycle was analyzed by flow cytometry after the MCF-7 and MDA-MB-231 cells had been stained using the kit, according to the manufacturer’s instruction.

Xenograft tumor assay

In the xenograft tumor experiment, 1×10⁷ cells from individually constructed MCF-7 cell lines were resuspended in phosphate-buffered saline and injected into the left axilla of the BALB/c nude mice in each group. The mice were sacrificed after 6 weeks, and the weight and volume of the xenograft tumors were calculated.

Statistical analysis

Statistical differences in USP9X expression levels between the breast cancer tissues and normal breast tissue samples were determined by the Wilcoxon signed rank test. The chi-squared (χ²) test and Fisher’s exact test were used to analyze the clinical significance between USP9X expression levels and patient clinicopathological features. Differences in cell proliferation, colony formation, apoptosis, and stages of the cell cycle of the MCF-7 and MDA-MB-231 cells were analyzed using the Kruskal-Wallis single-factor analysis of variance (ANOVA) and ANOVA independent t-test. All statistical analyses were performed using GraphPad PRISM version 6.0 (GraphPad Software, La Jolla, CA, USA). Data were presented as the mean ± standard deviation (SD). P-values <0.05 were considered to be significant.

Results

USP9X was overexpressed in breast cancer tissues

Immunohistochemistry (IHC) showed that in the 102 breast cancer tissues, 93 (91.1%) had positive USP9X expression and two of the 41 normal breast tissues showed positive USP9X expression. The USP9X expression levels between the breast cancer tissues and normal breast tissues were significantly different (P<0.001) (Figure 1). Subsequently, USP9X expression in the cancer tissues was divided into low expression (− or + USP9X expression) and high expression (++ or +++ USP9X expression) groups. USP9X expression was not related with patient age, sex, tumor node metastasis (TNM) stage, or metastasis (all, P>0.05), but was related with tumor size (≥5.0 cm vs.
<5.0 cm, P=0.032). These results suggest that USP9X overexpression may be related to breast cancer development and growth.

**USP9X overexpression increased MCF-7 and MDA-MB-231 cell proliferation**

The CCK-8 assay showed that USP9X overexpression increased MCF-7 cell and MDA-MB-231 cell proliferation significantly, with the highest increased peak at 72 h compared with the empty vector cells or wild-type cells (P<0.05), after the cells had been grown for 48 h. The proliferation of the empty vector cells and wild-type cells was not significantly different (Figure 2A, 2B). USP9X knockout inhibited MCF-7 and MDA-MB-231 cell proliferation compared with that in the negative CRISPR/Cas9 vector-transfected cells (both, P<0.05) after the cells had been grown for 48 h (Figure 2A, 2B). The results indicate that USP9X overexpression can increase breast cancer cell proliferation, whereas USP9X gene knockout can decrease breast cancer cell proliferation.

**USP9X overexpression increased MCF-7 and MDA-MB-231 cell growth**

The colony formation assay showed that USP9X overexpression significantly increased MCF-7 and MDA-MB-231 cell growth compared with that of the empty vector cells (both, P<0.05) (Figure 3A, 3B). Similar to the cell proliferation assay results, the cell growth of the empty vector cells and wild-type cells
was not significantly different (Figure 3A, 3B). USP9X gene knockout significantly inhibited MCF-7 and MDA-MB-231 cell growth compared with that of cells transfected with negative CRISPR/Cas9 vector (both, P<0.05) (Figure 3A, 3B). The results indicate that USP9X overexpression can increase breast cancer cell growth, whereas USP9X gene knockout can decrease breast cancer cell growth.

**USP9X overexpression decreased MCF-7 and MDA-MB-231 cell apoptosis**

Annexin V-FITC and PI staining combined with flow cytometry showed that USP9X overexpression decreased MCF-7 and MDA-MB-231 cell apoptosis compared with that of the empty vector cells and wild-type cells (both, P<0.05) (Figure 4A–4D). However, the apoptosis of the empty vector cells and wild-type cells was not significantly different (Figure 4A–4D). USP9X gene knockout significantly increased MCF-7 and MDA-MB-231 cell apoptosis compared with cells transfected with negative CRISPR/Cas9 vector (both, P<0.05). The results indicate that USP9X overexpression can decrease breast cancer cell apoptosis, whereas USP9X gene knockout can increase breast cancer cell apoptosis.
USP9X stimulated tumorigenesis

We explored the biological significance of USP9X in MCF-7 cells. USP9X overexpression significantly increased xenograft tumor growth in the BALB/c mice compared with the control group (P<0.01) (Figure 5A, 5B). However, the xenograft tumor growth of the empty vector cells and wild-type cells was not significantly different. USP9X gene knockout in the MCF-7 cells reduced the xenograft tumor growth compared with that of cells transfected with negative CRISPR/Cas9 vector (P<0.01). These results indicate that USP9X is required for breast cancer xenograft tumor growth.

**Figure 4.** Flow cytometry assay for the detection of apoptosis in the breast cancer cell lines, MCF-7 and MDA-MB-231. USP9X transfection decreased apoptosis in MCF-7 breast cancer cells (A, B) and MDA-MB-231 breast cancer cells (C, D) compared with the empty vector cells or wild-type cells (P<0.05). Apoptosis was unchanged in the empty vector cells when compared with the non-transfected cells (P>0.05). USP9X gene knockout increased apoptosis compared with cells transfected with negative CRISPR/Cas9 vector (P<0.05). * P<0.05; ** P<0.01. a) Non-transfected cells; b) Cells transfected with empty vector lentivirus particles; c) Cells transfected with USP9X lentivirus particles; d) Cells transfected with negative CRISPR/Cas9 vector; e) USP9X-deficient cells.
USP9X overexpression increased breast cancer cell proliferation, growth, and survival by increasing the S-phase fraction.

PI staining combined with flow cytometry showed that, compared with the empty vector cells and wild-type cells, USP9X overexpression significantly increased MCF-7 and MDA-MB-231 cells in S-phase (both, P<0.05) (Figure 6A–6D). The empty vector cells and wild-type cells in S-phase were not significantly different (Figure 6A–6D). USP9X knockout significantly decreased MCF-7 and MDA-MB-231 cells in the S-phase compared with cells transfected with negative CRISPR/Cas9 vector (both, P<0.05) (Figure 6A–6D). USP9X overexpression also decreased G1-phase cells, and USP9X knockout increased MCF-7 and MDA-MB-231 cells in G1 (Figure 6A–6D). The results indicate that USP9X overexpression can increase breast cancer cell development by regulating cell cycle progression.

USP9X overexpression upregulated CCND1 and downregulated CDKN1A in MCF-7 and MDA-MB-231 cells

The roles of CCND1 and CDKN1A in mediating cell cycle progression have been widely documented [13]. Many studies have confirmed that CCND1 mainly has an oncogenic effect, whereas CDKN1A mainly acts as a cancer suppressor; both are closely linked to the development of various human cancers [14]. However, their roles in USP9X promotion of breast cancer cell cycle progression have not been determined. Western blot showed that CCND1 was upregulated in USP9X overexpression MCF-7 and MDA-MB-231 cells and was downregulated in their USP9X non-expressing counterparts (Figure 7); the opposite was true for CDKN1A. The results indicate that USP9X overexpression leading to increased breast cancer cell development by regulating cell cycle progression may be associated with the regulation of CCND1 and CDKN1A expression.

Discussion

Protein ubiquitination is a versatile process of covalent modification of cellular proteins at the post-translational level. Ubiquitination and de-ubiquitination involve a wide range of biological processes, including cell growth, differentiation, and apoptosis. The ubiquitin-proteasome pathway is an important protein control system in the cell [15]. Most cellular proteins require degradation by this system, especially abnormal and short-acting regulatory proteins (such as transcriptional activator c-fos, p53, platelet-derived growth factor receptor beta [PDGFR-β], and fibroblast growth factor receptor 1 (FGFR1)). These short-acting regulatory proteins are involved in many biological processes, such as apoptosis, the cell cycle, and intracellular signal transduction. They are of great significance in maintaining cell stability, and their abnormalities at intracellular levels often occur with tumors. De-ubiquitinases (DUBs) are ubiquitin-specific proteases that can separate ubiquitin from proteins [13]. USP9X encodes a ubiquitin-specific protease that can affect a variety of biological pathways by targeting various substrates. USP9X can separate ubiquitin from the target protein, preventing ubiquitin from accumulating on the substrate protein and acting as a de-ubiquitinase. The USP9X is widely expressed in tissues, regulating various functions of the proteins by its DUB action [16].

Currently, it has been confirmed that USP9X plays an important role in tumor development as an important regulator of the reverse regulation of protein ubiquitination [14]. Increased USP9X protein expression has been observed in various human
Figure 6. Flow cytometry to determine the cell cycle of MCF-7 and MDA-MB-231 cells. USP9X transfection increased the S-phase fraction and decreased the G1-phase fraction in the MCF-7 (A, B) and MDA-MB-231 (C, D) cells compared with the empty vector cells or wild-type cells (P<0.05). The cell cycle stages were unchanged in the empty vector cells compared with the non-transfected cells (P>0.05). USP9X gene knockout decreased S-phase cells and increased G1-phase cells compared with cells transfected with negative CRISPR/Cas9 vector (P<0.05). * P<0.05. a) Non-transfected cells; b) Cells transfected with empty vector lentivirus particles; c) Cells transfected with USP9X lentivirus particles; d) Cells transfected with negative CRISPR/Cas9 vector; e) USP9X-deficient cells.
cancers, including non-small cell carcinoma, breast cancer, leukemia, cervical cancer, follicular lymphoma, colon cancer, and esophageal squamous cell carcinoma [17–22]. In pancreatic cell studies, USP9X played a role in the pro-survival pathway by regulating autophagy [23]. Elevated USP9X expression is associated with poor prognosis in patients with multiple myeloma and esophageal squamous cell carcinoma [24–26]. High USP9X protein levels can affect tumor growth, invasion, and metastasis by regulating genes such as those for transforming growth factor beta (TGF-β), myeloid leukemia factor-1 (MCL1), and β-catenin.

Perez-Mancera et al. found that more than 50% of pancreatic cancer USP9X gene mutations are silenced, confirming that its expression is closely related to pancreatic cancer prognosis [27]. Dupont et al. found that USP9X can depolymerize the monomeric ubiquitin bound to Co-SMAD and SMAD4 in breast cancer cells, so that it can form a complex with the acidified SMAD2 and activate the downstream pathway of TGF-β through Ras mitogen-activated protein kinase (MAPK), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PI3K)-AKT, Rho-associated coiled-coil-containing protein kinase 1 (Rho-ROCK1), Jagged-Notch, nuclear factor kappa B (NF-κB), Wnt-β-catenin, and other signal transduction pathways to transmit signals into cells; activate different nuclear transcription factors; regulate target gene transcription; and control tumor cell growth, differentiation, and apoptosis [28]. Sun et al. have shown that chronic myeloid leukemia cells were significantly more sensitive to imatinib through inhibition of USP9X activity by the chemical drug WP1130 or by USP9X knockout by RNA interference (RNAi) [29]. In a colorectal cancer study, Harris et al. reported that tumor sensitivity to the conventional chemotherapy drug 5-FU (5-fluorouracil) increased after USP9X expression had been inhibited [30]. Peddaboina et al. also found that USP9X inhibition downregulated MCL1, an anti-apoptotic factor in the BCL2 (BCL2, apoptosis regulator) gene family, thereby increasing the sensitivity of solid tumor cells to various chemotherapeutic agents, including Bcl-2/Bcl-XL inhibitors. USP9X promotes tumor cell survival and inhibits apoptosis by de-ubiquitinating and stabilizing MCL1. Downregulating USP9X increased tumor sensitivity to chemotherapy by degrading the MCL1 protein [31]. Furthermore, RNAi of USP9X reduced breast cancer cell sensitivity to tamoxifen [32].

However, the role of USP9X in breast cancer remains poorly understood. To date, few investigations have focused on the relationship between USP9X and breast cancer. Accordingly, we investigated its role in breast cancer in the present study. We found that USP9X was overexpressed in breast cancer tissues and that its upregulation was related to tumor size. Further research showed that USP9X overexpression resulted in elevated breast cancer cell proliferation, growth, and survival, which were related to the overexpression contributing to increased cell cycle progression. Further data showed that the molecular mechanism of USP9X function may be linked to its ability to alter the expression levels of CCND1 and CDKN1A, two of the most widely demonstrated cell cycle regulators.

Conclusions

USP9X was overexpressed in breast cancer tissue, and increased expression of USP9X was associated with increased tumor size and breast cancer cell proliferation. Overexpression of USP9X was associated with upregulation of the CCND1 gene and downregulation of the CDKN1A gene in breast cancer tissues and cell lines. Controlled clinical studies should be undertaken to determine whether the USP9X gene may represent a potential target for the treatment of breast cancer.

Conflict of interest

None.
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