

Ubiquitin specific protease 21 upregulation in breast cancer promotes cell tumorigenic capability and is associated with the NOD-like receptor signaling pathway

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Abstract. Ubiquitination and deubiquitination have emerged as critical regulators in cancer. In the present study, the expression pattern of 50 ubiquitin-specific proteases (USPs) was summarized in breast cancer using a bioinformatics approach, and USP21 was identified as the most altered gene in breast cancer. In particular, expression of USP21 in triple negative breast cancer (TNBC) cell lines was greater compared with other subtypes of breast cancer. Knockdown of USP21 in TNBC cells inhibited cell proliferation, migration and invasion. Microarray profiling of the USP21 knockdown cells revealed significant downregulation of multiple genes associated with the NOD-like receptor signaling pathway. The results of the present study suggest that USP21 has a significant role in TNBC progression, and therefore may represent a novel therapeutic target.

Introduction

Breast cancer is the most common carcinoma in women, with low survival rates in patients due to metastatic lesions (1,2). Triple negative breast cancer (TNBC) is an aggressive breast cancer subtype in which the tumor cells lack expression of the estrogen, progesterone and human epidermal growth factor 2 receptors. TNBC has a high rate of relapse and metastasis, and accounts for approximately 12-17% of all breast cancer

cases (1,3). Due to poor prognosis and a lack of treatment options, TNBC patients have a disproportionately high mortality rate: No more than 30% of patients with metastatic TNBC survive for 5 years (4). Therefore, understanding the mechanism regulating TNBC progression may assist with the development of accurate prognosticators and more effective treatments.

Cancer comprises a collection of complicated genetic and epigenetic alterations that arise via multistep processes (5-7). Ubiquitin-specific proteases (USPs) are frequently involved in cancer regulation, as oncogenic mutations in USP genes are able to disrupt deubiquitination of proteins that control cell growth and apoptosis (8-10). To date, 50 USP family members have been identified in humans (11). USP21 is able to facilitate initiation of transcriptional activity via catalyzing the hydrolysis of the ubiquitylation of histone H2A (12). USP21 is also able to regulate the stability of proteins through deubiquitination. For example, USP21 can mediate deubiquitination of GATA3 and maintain GATA3 expression in regulatory T cells (13). Thus, USP proteins may serve as a good point of intervention for the prevention of cancer and other mutation-associated diseases (14).

Nucleotide oligomerization domain (NOD)-like receptor (NLR) signaling pathways have a significant role in numerous human diseases, including bacterial infections, autoimmune and inflammatory disorders, and cancer (15). Stimulation of NLRs results in the activation of nuclear factor (NF)- κ B and mitogen-activated protein kinases (MAPKs), which drive the transcription of numerous genes involved in both innate and adaptive immune responses (16). Previously, USPs have been shown to participate in the regulation of the NF- κ B signaling pathway (17,18). For example, USP4 promotes stimulation of NF- κ B mediated by tumor necrosis factor α (TNF- α) through deubiquitination-dependent downregulation of TGF β -activated kinase 1 (19). USP11 is able to modulate TNF- α -induced NF- κ B activation through regulation of nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor α (I κ B α) stability (20). By contrast, USP21 inhibits TNF- α -induced NF- κ B signaling by promoting the deubiquitination of receptor-interacting protein 1 (RIP1) in HeLa cells (21). However, the role of USP21 in breast cancer remains to be elucidated.

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In the present study, bioinformatics tools were used to study data online and characterize the gene alteration status of USP family members in breast cancer. Subsequently, the expression of the most altered member, USP21, was validated *in vitro*. The expression of USP21 was then knocked down using small interfering RNA (siRNA) in TNBC cell lines, and cellular experiments were performed to investigate its biological function, in the hope that the results may provide useful insights into the prognosis and treatment of TNBC.

Materials and methods

Cell culture and siRNA transfection. All breast cancer cells were obtained from American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% PenStrep (100 U/ml penicillin and 100 μ g/ml streptomycin; Invitrogen; Thermo Fisher Scientific, Inc.), in an incubator at 37°C with 5% CO₂. MDA-MB-231 or MDA-MB-157 cells were plated onto tissue culture plates 24 h prior to transfection. Transient transfection of siUSP21 (siUSP21-1: 5'-GCUAGAAGAACCUGAGUUA-3'; siUSP21-2: 5'-GAGCUGUCUCCAGAAAUA-3') or siControl (5'-GUACUUGUACUCAGCUUUGUG-3') (Invitrogen; Thermo Fisher Scientific, Inc.) at a final concentration of 50 nM was accomplished with Lipofectmine[®] 2000 reagent according to the manufacturer's protocol (Invitrogen; Thermo Fisher Scientific, Inc.).

Cell migration assay. For scratch wound-healing assays, 48 h following siRNA transfection, 1.5x10⁵ cells were seeded into six-well plates and serum starved for 24 h. Cells were wounded by scratching with a pipette tip and cultured in medium containing 0.5 g/ml mitomycin-C (Sigma-Aldrich; EMD Millipore, Billerica, MA, USA) to block cell division. Cells were photographed at 0, 5 and 10 h timepoints. The distance of migration was calculated using ImageJ software (version 1.46; imagej.nih.gov/ij/). Matrigel invasion assay was performed using a Corning[®] BioCoat[™] Matrigel Invasion Chamber (Corning Incorporated, Corning, NY, USA). Tumor cells (1x10⁵) treated with control or USP21 siRNA in 200 μ l serum-free DMEM were placed in the upper chamber. The lower chamber was filled with 600 μ l conditioned medium (DMEM medium containing 1% FBS for 24 h) as chemoattractant. After 24 h of incubation at 37°C, the cells on the upper surface of the filter were removed with a cotton swab. The cells that had invaded the Matrigel and reached the lower surface of the filter were fixed in methanol, stained with hematoxylin and eosin, and counted under magnification, x400. A total of five fields were randomly selected and the number of invasive cells was counted.

Protein isolation, western blot analysis and co-immunoprecipitation (Co-IP). Cells were lysed with radioimmunoprecipitation assay buffer (Sigma-Aldrich; EMD Millipore) as previously described (22-24). Cells were centrifuged at 4°C for 10 min at 16,000 x g. Protein concentrations were determined by the Bradford assay (25). Aliquots containing 20 μ g of total protein were separated by 10% sodium dodecyl-polyacrylamide gel

electrophoresis and transferred to nitrocellulose membranes. Blots were probed with primary antibodies against USP21 (1:1,000; goat polyclonal; sc-79305; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and β -actin (1:5,000, mouse monoclonal; A5316; Sigma-Aldrich; EMD Millipore). Appropriate secondary antibodies (1:3,000; rabbit anti-goat; ab6741; 1:5,000; rabbit anti-mouse; ab97046; Abcam, Cambridge, MA, USA) conjugated to horseradish peroxidase and enhanced chemiluminescence (GE Healthcare Life Sciences, Chalfont, UK) were used to detect the bound primary antibodies. Co-IP was performed with cell lysate (500 μ g) incubated with USP21 (1:1,000; goat polyclonal; sc-79305; Santa Cruz Biotechnology, Inc.), relA (1:1,000; rabbit polyclonal; sc-372; Santa Cruz Biotechnology, Inc.) or non-specific-IgG antibodies (1:1,000; rabbit IgG, monoclonal; ab172730; Abcam) using μ MACS[™] Protein A/G MicroBeads and MACS[®] Separation Columns according to the manufacturer's protocol (Miltenyi Biotec, Auburn, USA).

RNA isolation, cDNA preparation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). As previously described (26-28), total RNA was isolated from cells using the RNeasy Mini kit (Qiagen, Inc., Valencia, CA, USA). cDNA was prepared from 1 μ g of RNA using the iScript cDNA Synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). RT-qPCR was performed using a GeneAmp Gold RNA PCR Core kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) and an iCycler iQ[™] Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.), where each 20 μ l reaction included 1% cDNA preparation, 0.5 μ M primers and 10 μ l SYBR Green (Bio-Rad Laboratories, Inc.). Primer sequences are presented in Table I. Expression of glyceraldehyde-3-phosphate dehydrogenase was used to normalize the gene expression level. The relative difference in the expression level was calculated using the $\Delta\Delta$ Cq method (29). The data presented are representative of three independent biological repeats each assayed in triplicate and show the relative expression levels.

Microarray hybridization data analysis and The Cancer Genome Atlas (TCGA) analysis. MDA-MB-231 cells were plated onto a 6-well plate at 70% confluence and transfected with siControl or siUSP21. A total of 48 h subsequent to transfection, the cells were washed with phosphate-buffered saline, and total RNA was obtained using the RNeasy Mini kit (Qiagen, Inc.) and treated with 1 unit of DNase I (Qiagen, Inc.). Expression profiles were generated by hybridizing 10 μ g of total RNA to GeneChip[®] Human Genome U133 Plus2.0 Gene Chips (Affymetrix, Inc., Santa Clara, CA, USA) according to the Affymetrix Eukaryote One-cycle protocol (30). Briefly, 5-10 μ g of total RNA were used to generate biotinylated cDNA, which was fragmented and hybridized to a chip for 16 h at 45°C in a GeneChip Hybridization Oven 640 (Affymetrix, Inc.). Arrays were then washed and stained on a GeneChip Fluidics Station 450 (Affymetrix, Inc.) and subsequently scanned on a GeneChip Scanner 3000 7G (Affymetrix, Inc.) to obtain fluorescence intensities. To eliminate data with low reliability, genes whose expression was regarded as absent in these cell lines as a result of software analysis were excluded. Following identification of the differentially expressed genes, DAVID

Table I. Reverse transcription-qPCR primer sequences.

Gene name	F/R	Sequences of the qPCR primer pairs (5'-3' direction)
GAPDH	F	GGTGAAGGTCGGAGTCAACGG
GAPDH	R	GAGGTCAATGAAGGGGTCATTG
USP21	F	ATCTCGGACCAACTTAGCCC
USP21	R	GTGCCCTCCCAAGGCAATC
IL8	F	TTTTGCCAAGGAGTGCTAAAGA
IL8	R	AACCCTCTGCACCCAGTTTTC
CARD8	F	GAAGCGAAACTGCATATTCTGGT
CARD8	R	GGGTTGGAAGAGGCATGGC
CCL2	F	CAGCCAGATGCAATCAATGCC
CCL2	R	TGGAATCCTGAACCCACTTCT
IL6	F	ACTCACCTCTTCAGAACGAATTG
IL6	R	CCAICTTTGGAAGGTTCAAGTTG
CXCL1	F	GCGGAAAGCTTGCCCTCAA
CXCL1	R	TCAGCATCTTTTCGATGATTTTCTT
NLRP3	F	GATCTTCGCTGCGATCAACAG
NLRP3	R	CGTGCATTATCTGAACCCAC
NFKB1A	F	AACAGAGAGGATTTCTGTTTCCG
NFKB1A	R	TTTGACCTGAGGGTAAGACTTCT

qPCR, quantitative polymerase chain reaction; F, forward; R, reverse; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; USP21, ubiquitin specific protease 21; IL, interleukin; CARD8, caspase recruitment domain family member 8; CCL2, chemokine (C-C motif) ligand 2; CXCL1, chemokine (C-X-C motif) ligand 1; NFKB1A, nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor α .

online software (david.ncifcrf.gov/) was used to perform Kyoto Encyclopedia of Genes and Genomes pathway significant enrichment analysis of 1,007 differentially expressed genes associated with USP21, and the six signaling pathways with the smallest P-values were selected. For TCGA analysis, data including 1,105 breast invasive carcinoma samples from 1,098 patients were obtained from the TCGA website using the cBioPortal tool (www.cbioportal.org/).

Statistical analysis. Data are presented as the mean \pm standard deviation. Statistical analyses were performed using SPSS version 19.0 software (IBM SPSS, Armonk, NY, USA). Statistically significant differences were determined by Student's t-test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

USP21 is the most upregulated USP family member in breast cancer. USP family proteins have a significant role in multiple signaling and cell regulatory networks in breast cancer (9). To characterize the extent of changes in all 50 USP genes in breast cancer, the present study generated an alteration-summary for these genes in breast cancer using the cBioPortal tool for Cancer Genomics (31,32) (Fig. 1A). The results of the

present study demonstrated that USP21 was altered in 39% (375/971 samples) of patients with breast invasive carcinomas, indicating it is the most altered gene among all the USP gene family members. In all the deregulation situations, including gene copy number amplification and mRNA expression alteration, of USP21, 13.6% (132/971) of the patients displayed copy number amplification, while 37.8% (367/971) of the patients showed mRNA upregulation (Fig. 1B), suggesting a role for USP21 in breast cancer.

To investigate the expression of USP21 in breast cancer cell lines *in vitro*, the present study analyzed a panel of 10 breast cancer cell lines including luminal, basal and non-cancerous breast epithelial lines. Notably, it was observed that the expression of USP21 was enriched in triple negative cell lines, MDA-MB-231 and MDA-MB-157 (Fig. 1C). Therefore, it appears that USP21 is required for the cancerous ability of TNBC cells.

USP21 affects TNBC cell proliferation, migration and invasion. To directly investigate the contribution of USP21 to breast tumorigenesis, the present study knocked down USP21 protein using two specific siRNAs in MDA-MB-231 and MDA-MB-157 cells. Western blot analysis confirmed the knockdown of USP21 under these conditions (Fig. 2A). It was initially investigated whether USP21 is crucial to the proliferation of these cells. Cells transfected with control or USP21-siRNAs were cultured for up to 7 days. The difference in cell proliferation was measured by viable cell counting from day 1 to day 7. Knockdown of USP21 reduced the proliferation of MDA-MB-157 and MDA-MB-231 cells 5 days after siRNA transfection (Fig. 2B and C).

To determine the effects of USP21 on cell migration, a wound-healing assay was performed following USP21 silencing. The scratch wounds were almost identical sizes in each experimental group at 0 h; however, knockdown of USP21 using two different siRNAs markedly decreased the migration ability of MDA-MB-231 and MDA-MB-157 cells at 5 and 10 h (Fig. 3A). The present study measured the distance between the migrating frontlines and calculated the rate of wound closure. It was observed that the USP21 silenced groups were less effective than the control group in terms of the healing process (Fig. 3A).

To investigate the role of USP21 in TNBC cell invasion, the present study measured the invasive ability using invasion assays. Consistent with the migration assay results, downregulation of USP21 in MDA-MB-231 and MDA-MB-157 cells by siRNA knockdown resulted in a marked reduction in the cell invasive capability compared to the control group (Fig. 3B). These results suggested that USP21 is involved in TNBC cancer growth, migration and invasion.

Microarray data analysis indicates that USP21 expression is associated with NOD-like receptor signaling pathway. To further investigate USP21-mediated gene expression changes, the cDNA from USP21-knockdown and control MDA-MB-231 cells was subjected to microarray analyses. A total of 1,007 genes in USP21 siRNA treated cells exhibited a differential expression more than double that of the control sample. A total of 309 genes were upregulated in the USP21 knockdown sample compared with the control, while

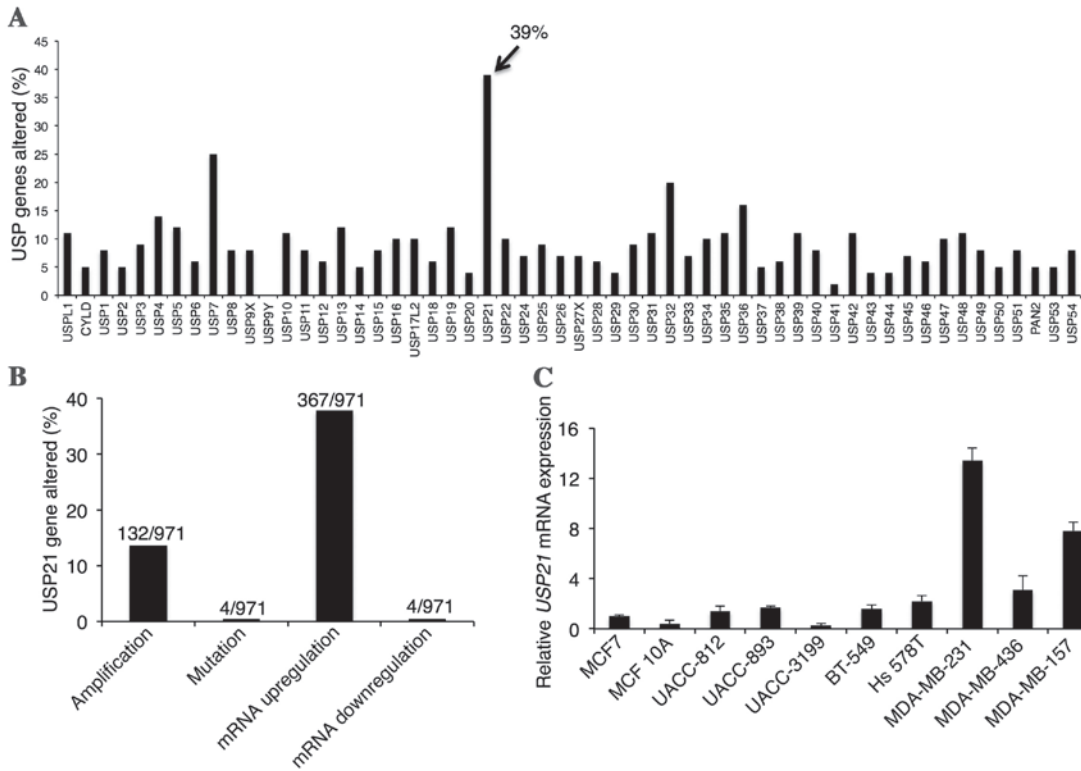


Figure 1. Alterations of the human USP21 gene in breast invasive carcinoma (TCGA). (A) Alterations of the USP gene family in breast invasive carcinoma (TCGA). (B) Amplification, mutation, mRNA upregulation and mRNA downregulation of the USP21 gene in breast invasive carcinoma (TCGA). Data was obtained using c-BioPortal. (C) The relative mRNA expression level of USP21 across ten breast cancer cell lines. USP21, ubiquitin specific protease 21; TGCA, The Cancer Genome Atlas.

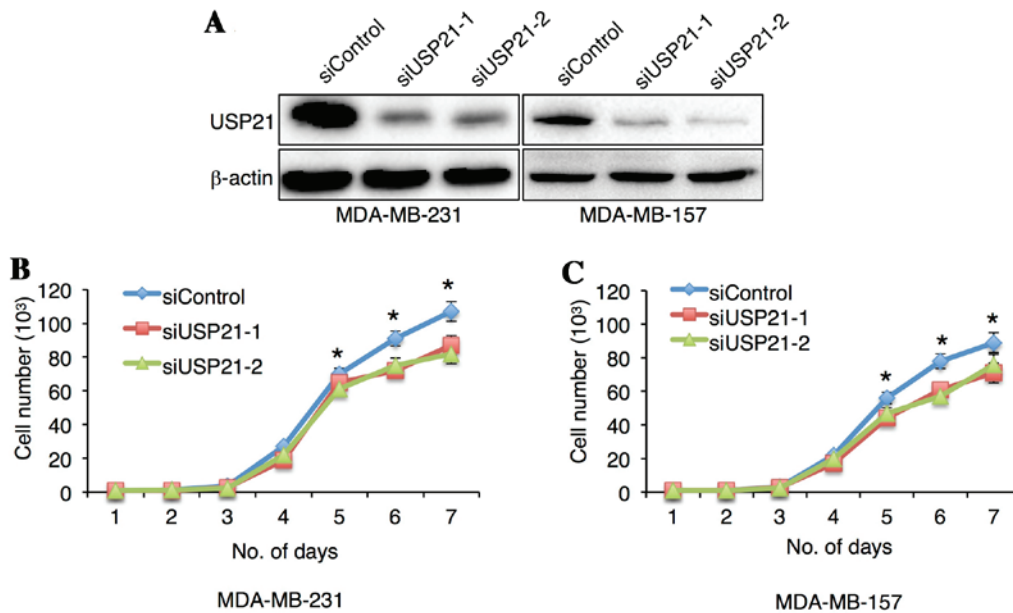


Figure 2. Effect of USP21 on triple negative breast cancer cell proliferation. (A) MDA-MB-231 and MDA-MB-157 cells were transfected with scrambled siRNA (control) and two specific USP21 siRNAs, followed by western blot analysis using anti-USP21 antibody (upper panel). β-actin was used as loading control (lower panel). (B and C) Proliferation of MDA-MB-231 and MDA-MB-157 cells transfected with USP21 siRNA or control siRNA. *P<0.05. siRNA, small interfering RNA; USP21, ubiquitin specific protease 21.

698 genes demonstrated decreased expression in the USP21 siRNA treated sample. Furthermore, it was observed that the major signaling pathways of the differentially expressed genes were associated with the following molecular pathways:

NOD-like receptor signaling, TGF-β signaling pathway, RNA degradation, small cell lung cancer, pathways in cancer and extracellular matrix-receptor interaction. It was observed that USP21 depletion markedly attenuated genes specifically

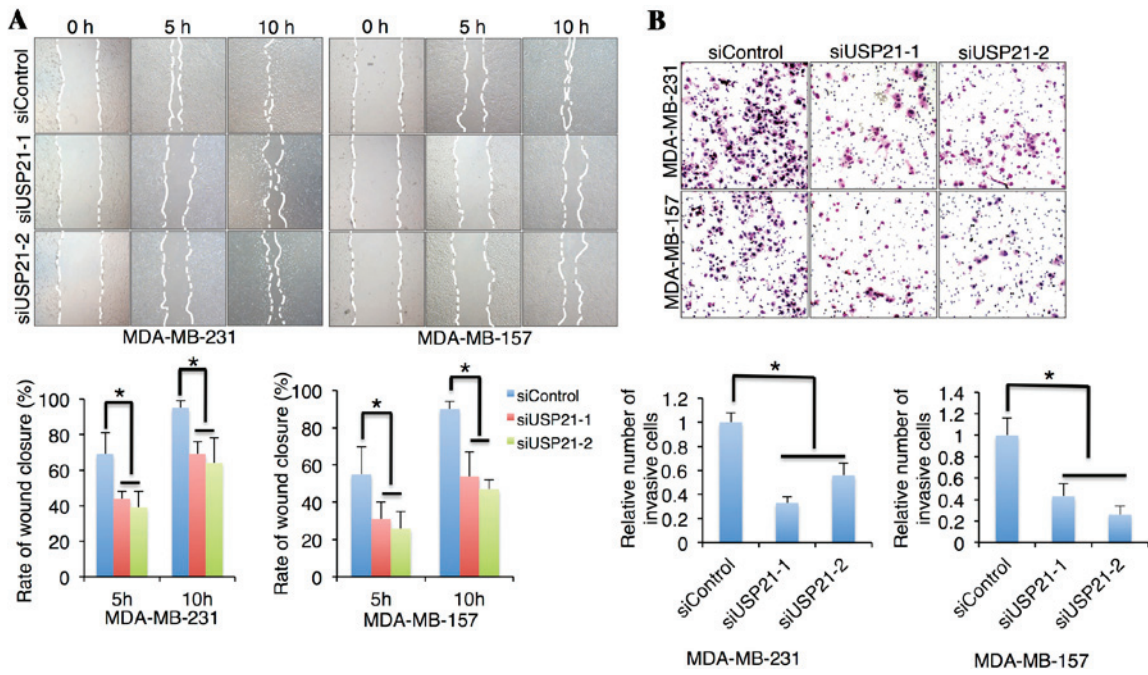


Figure 3. Effect of USP21 on TNBC cell motility. (A) Representative images showing the scratch (wound) at 0, 5 and 10 h for TNBC cells with various treatments. Magnification, x40. The graphs show the percentage of wound closure in MDA-MB-231 and MDA-MB-157 cells with various treatments. Data are presented as the mean \pm SD of three independent experiments. * $P < 0.05$. (B) The invasive ability of MDA-MB-231 and MDA-MB-157 cells 48 h subsequent to transfection with siControl, siUSP21-1 or siUSP21-2, was assayed using a Matrigel-coated transwell chamber. The cells that successfully invaded into the Matrigel were quantified 24 h after plating. Statistically significant differences were detected when control groups were compared with the USP21 knockdown groups. Data are presented as the mean \pm SD of three independent experiments. * $P < 0.05$. USP21, ubiquitin specific protease 21; TNBC, triple negative breast cancer; SD, standard deviation; si, small interfering.

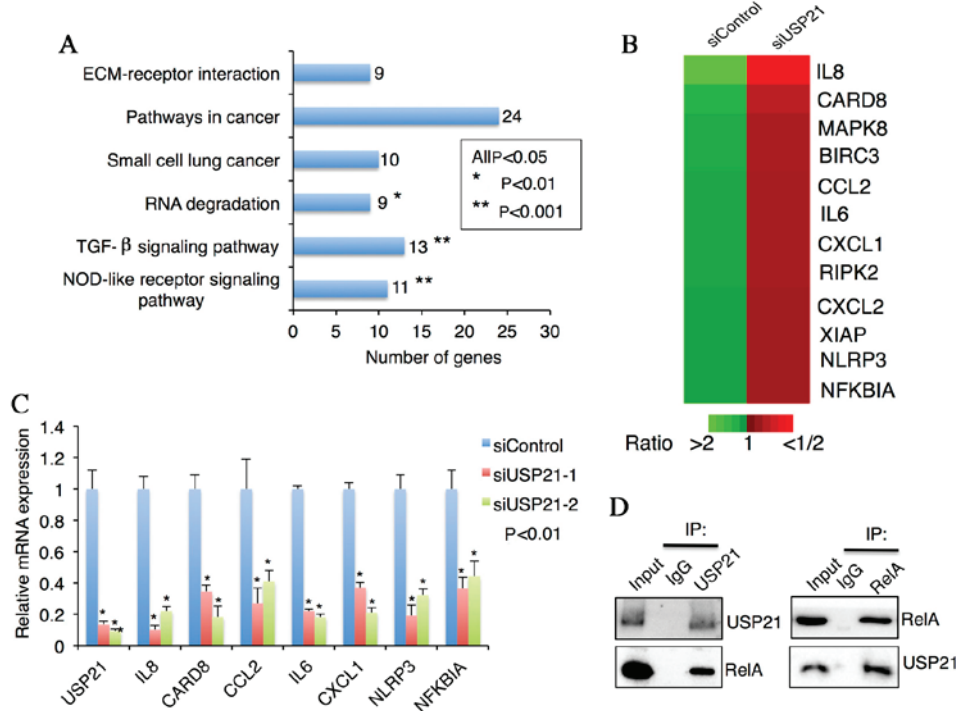


Figure 4. Global gene profiling of MDA-MB-231 following USP21 knockdown. (A) Summary of the kyoto encyclopedia of genes and genomes pathways of genes significantly enriched in response to USP21 knockdown, using database for annotation, visualization and integrated discovery software. (B) Changes in gene expression levels in MDA-MB-231 cells following USP21 knockdown. The heat map depicts relative gene expression changes (siRNA control/siUSP21-1). (C) Confirmation of downregulated genes from the microarray dataset by reverse transcription-quantitative polymerase chain reaction. * $P < 0.05$. (D) Co-immunoprecipitation of USP21 and RelA in MDA-MB-231 cells. USP21, ubiquitin specific protease 21; ECM, extracellular matrix; TGF, transforming growth factor; NOD, nucleotide-binding oligomerization domain; siRNA, small interfering RNA; IL, interleukin; CARD8, caspase recruitment domain family member 8; MAPK8, mitogen-activated protein kinase 8; BIRC3, baculoviral IAP repeat containing 3; CCL2, chemokine (C-C motif) ligand 2; CXCL, chemokine (C-X-C motif) ligand; RIPK2, receptor interacting serine/threonine kinase 2; XIAP, X-linked inhibitor of apoptosis protein; NLRP3, NLR family pyrin domain containing 3; NFKBIA, nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor α ; IP, immunoprecipitation.

associated with the NOD-like receptor signaling pathway [interleukin (IL)6, NLR family, pyrin domain containing (NLRP)3, I κ B α and chemokine (C-X-C motif) ligand (CXCL)8] and stimulated genes associated with the TGF- β signaling pathway (bone morphogenetic protein (BMP)4, BMP type IB receptor, ID1, ID2, ID3, ID4 and TGF- β receptor 1) (Fig. 4A and B). To confirm the microarray results, 8 genes were selected (USP21, IL8, caspase recruitment domain family member 8 (CARD8), chemokine (C-C motif) ligand 2 (CCL2), IL6, CXCL1, NLRP3 and I κ B α) and their expression was measured with RT-qPCR on the same sample used for microarray studies. All of these genes exhibited moderate to high expression and demonstrated high concordance between microarray and PCR data (Fig. 4C).

As USP21 has been reported to be a histone H2A deubiquitinase that initiates transcriptional activity (12), the present study screened the association of USP21 with NOD-like receptor associated transcription factors, including NF- κ B, activator protein 1 or interferon regulatory factors. Notably, it was observed that relA, an essential subunit of the transcriptionally active NF- κ B dimer, may be 'pulled down' with USP21 using a co-IP assay (Fig. 4D). Thus, these results indicate that USP21 may associate with NF- κ B transcription factors.

Discussion

TNBC is an aggressive and deadly subtype of breast cancer and lacks targeted therapies (33). In the present study, it was observed that USP21 is the most altered USP member in breast cancer using online TCGA data sets. Furthermore, the present study demonstrated, for the first time to the best of our knowledge, that silencing of USP21 leads to impaired proliferation, migration and invasion ability of TNBC cells, which indicates that USP21 may be involved in tumor metastasis. The present study also investigated global gene profiling upon depletion of USP21 in TNBC cells. The results of the present study revealed that a subset of genes involved in NLR signaling pathways were significantly repressed when USP21 was knocked down in TNBC cells. It was also observed that USP21 was associated with relA, implying a link between USP21 and NF- κ B in regulating NLR signaling and TNBC progression.

The NLR signaling pathway plays a vital role in human diseases, including cancer (15). In the current study, it was demonstrated that silencing of USP21 repressed several NLR signaling pathway factors, including IL6, IL8, CCL2, CXCL1, NLRP3, I κ B α and CARD8. A number of these genes are involved in TNBC regulation. For example, inhibition of IL-6 and IL-8 expression in TNBC led to a decrease in colony formation and cell survival *in vitro* and inhibited tumor engraftment and growth *in vivo* (34). In addition, RIPK2 can stimulate triple-negative breast cancer cell migration and invasion through NF- κ B and c-Jun N-terminal kinase signaling pathways (35). Thus, it appears likely that the impaired tumorigenic ability in USP21 depleted TNBC cells may be directly associated with this downregulation of NLR signaling pathway members.

Several studies have demonstrated a role of USP21 in inflammation and the NF- κ B signaling pathway (21,36,37). It has been previously shown that USP21 is able to regulate the expression of IL8 and cancer stem cell properties in human

renal cell carcinoma (28). IL8 has been demonstrated to be an important cytokine that is required for growth of TNBC (34), supporting the results of the present study. Through deubiquitinating RIP1, USP21 is able to repress TNF α -induced NF- κ B activation in HeLa cells, suggesting that USP21 may serve as a negative regulator of the NF- κ B signaling pathway (21). An additional study has revealed that depletion of USP21 decreases IL33 protein levels and IL33-mediated NF- κ B p65 promoter activity, indicating USP21 is able to positively regulate the NF- κ B signaling pathway (36). Therefore, although USP21 is involved in NF- κ B signaling transduction activity, the function of USP21 appears to be context dependent. Based on the fact that NLR signaling pathway components were repressed upon USP21 depletion, and USP21 was associated with relA, it is likely USP21 is a positive regulator of the NF- κ B signaling pathway in TNBC cells. If that is the case, the global regulation of histone deubiquitination by USP21 requires further investigation. IL6 and IL8 have critical roles in anchorage-independent growth of TNBC, and function through the NF- κ B signaling pathway (34), meaning that NF- κ B may be a potential therapeutic target in TNBC. A previous report demonstrated that NF- κ B regulates cancer stem cell populations in the basal-like breast cancer subtype of TNBC (38). In addition, TGF- β ligands are often enriched in the TNBC tumor microenvironment and have a role in breast cancer stem cells (39,40). The results of the present study suggest that USP21 may serve as a modulator for the TGF- β signaling pathway in TNBC.

In conclusion, USP21 was observed to be elevated in breast cancer patient samples. The expression of USP21 may promote proliferation, migration and invasion of breast cancer cells. Therefore, USP21 may have a potential role in the prognosis of and be a relevant target in breast cancer.

Acknowledgements

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