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WW domain-containing E3 ubiquitin protein ligase 1 depletion evokes antitumor effect in cutaneous squamous cell carcinoma by inhibiting signal transducer and activator of transcription 3 signaling pathway

Yonghua Xia, Xiao Chang, Shi Lian and Wei Zhu

Abstract

Objectives: WW domain-containing E3 ubiquitin protein ligase 1 (WWP1) has been implicated in tumor progression. We aimed to investigate the role of WWP1 in cutaneous squamous cell carcinoma (CSCC).

Methods: WWP1 gene and protein levels were detected using semi-quantitative reverse transcription-polymerase chain reaction, immunohistochemistry and western blotting. The effects of WWP1 on cell cycle, apoptosis, cell migration and invasion were examined by flow cytometry, wound healing and Transwell assays, respectively. The antitumor efficacy of WWP1 small interfering RNA was determined in CSCC tumor xenografts in mice.

Results: WWP1 expression was significantly higher in CSCC tissues and cells than in normal skin and cells, respectively. WWP1 expression was significantly associated with histological grade, invasion depth and lymph node metastasis in patients with CSCC. High expression predicted metastatic potential and an unfavorable prognosis. WWP1 downregulation suppressed tumor growth *in vitro* and *in vivo*, reduced cell migration and invasion, arrested the cell cycle in G0/G1 and induced apoptosis in A431 cells. WWP1 depletion also decreased phosphorylated signal

Department of Dermatology and Venerology, Xuanwu Hospital, Capital Medical University, Xicheng District, Beijing, P.R. China

Corresponding author:
Wei Zhu, Department of Dermatology and Venerology, Xuanwu Hospital, Capital Medical University, No.45 Changchun Street, Xicheng District, Beijing 100053, P.R. China.
Email: zhuwei_2020@126.com

transducer and activator of transcription 3 (STAT3), matrix metalloproteinase-2, cyclin D1 and Bcl-2, but did not affect total STAT3.
Conclusions: WWP1 is a potential target for the diagnosis, prognosis and therapy of patients with CSCC.

Keywords
WW domain-containing E3 ubiquitin protein ligase 1, cutaneous squamous cell carcinoma, cell growth, cell migration, apoptosis, STAT3 signaling pathway

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Introduction

Non-melanoma skin carcinomas are common skin tumors that can largely be classified into two main histological types: cutaneous basal cell carcinoma (CBCC) and cutaneous squamous cell carcinoma (CSCC).[1, 2] CBCC accounts for more than 70% of all skin tumors, while CSCC, as the second most frequent skin cancer worldwide, accounts for about 20%.[3–5] Although CSCC is less common than CBCC, it tends to grow and spread much faster, and thus demonstrates a higher degree of malignancy than CBCC.[6] Although most patients with primary CSCC have a favorable prognosis and can usually be cured, the invasive and metastatic potentials of CSCC are associated with significant mortality.[7, 8] Chemotherapy is a standard treatment protocol for CSCC and has gained wide clinical attention.[8] However, the obvious side effects of chemotherapy make it necessary to develop novel diagnostic and therapeutic markers to improve the prognosis and therapeutic outcome of patients with CSCC.

WW domain-containing E3 ubiquitin protein ligase 1 (WWP1) was first identified due to its characteristic WW domain.[9–11] Numerous investigations have since implicated WWP1 in the regulation of many cellular and biochemical signaling pathways, including proliferation, apoptosis, cell differentiation, transcriptional regulation and protein degradation.[11, 12] Recent overwhelming evidence has indicated that WWP1 is directly related to tumor progression in many cancers, and it has been recognized as an oncogene in several tumor types, including gastric carcinoma,[13] breast cancer,[14–16] oral cancer[17] and prostate cancer.[18] WWP1, as a key ubiquitin ligase, can manipulate several important substrates, including tumor suppressors such as Smad2,[18] RNF11[19] and p63,[20] which further support tumor progression and expansion. Inhibition of WWP1 will thus indirectly increase the expression of tumor suppressors, and may therefore represent a promising therapeutic strategy in a variety of cancers.[21] However, the role of WWP1 in the development and progression of CSCC remains unclear.

In the present study, we aimed to clarify the role of WWP1 in the development and progression of CSCC by examining WWP1 mRNA and protein expression levels in CSCC compared with normal tissues and cells. We also investigated the effects of WWP1 expression on cell growth, migration and invasion, and on the cell cycle and apoptosis in CSCC cells. The results of this study may have important

implications for the diagnosis and treatment of patients with CSCC.

Materials and methods

Tissue samples

Matched CSCC and normal tissues were obtained from the First Affiliated Hospital of Xinxiang Medical University, Weihui, Henan Province, China. Written informed consent was obtained from all participants and this study was approved by the Committee for Ethical Review of Research in our hospital. No patients had received any preoperative treatment, including radiotherapy or chemotherapy.

Immunohistochemistry (IHC) assay

IHC assay was conducted as described previously.[22] In brief, formalin-fixed, paraffin-embedded tissue sections were cut into 4-μm slices, deparaffinized in xylene and rehydrated through graded alcohols. Antigen retrieval was carried out according to the manufacturer's instructions, and the tissue sections were then incubated with anti-WWP1 primary antibody (1:100 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. Finally, 3′-diaminobenzidine reagent was used to develop the staining signal, with normal horse serum replacing anti-WWP1 primary antibody as a negative control. Finally, the IHC staining signal was evaluated as described previously.[23]

Semi-quantitative reverse transcription polymerase chain reaction (sqRT-PCR)

WWP1 mRNA expression levels in CSCC and matched normal skin tissues and in cells were examined by SqRT-PCR assay, as described previously,[24] using the following primers: WWP1 (NM_007013) F: 5′-GGCTGTTGAAGGCACGAATG-3′ and R: 5′-AGCAACCTGAGACGGAG ATG-3′ (product size: 121bp); β-actin (NM_001101) F: 5′-TCCAGGCTGTGCT ATCCCT-3′ and R: 5′-CGGATGTCCA CGTCACACTT-3′(product size: 463 bp).

Western blotting assay

WWP1 protein expression in CSCC and normal tissues and cells was detected by western blotting assay, as described previously,[24] using primary antibodies against WWP1, phospho-signal transducer and activator of transcription 3 (p-STAT3), total STAT3 (t-STAT3), matrix metalloproteinase (MMP)-2, cyclin D1, Bcl-2 and β-actin (Santa Cruz Biotechnology).

Cell culture

The CSCC cell lines A431 and SCL-1 and the spontaneously immortalized human keratinocyte cell line HaCaT were stored frozen in liquid nitrogen in our laboratory and were thawed at 37°C. The cells were then grown in DMEM supplemented with 10% fetal calf serum, streptomycin (100 U/mL) and penicillin (100 U/mL) in a humidified incubator with 5% CO_2 at 37°C.

Transfection with WWP1 siRNA and control siRNA

A431 cells (4×10^5/well) were seeded into a 6-well plate and transfected with WWP1 siRNA and control siRNA (consiRNA), respectively (both Santa Cruz Biotechnology). The cells were divided into the following three groups for transfection: A431 (untreated group), consiRNA (transfection with consiRNA) and WWP1 siRNA (transfection with WWP1 siRNA). Transfection was carried out with WWP1 siRNA and consiRNA at final concentrations of 100 μM using Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's instructions. The cells were then harvested and

subjected to sqRT-PCR, western blotting, migration experiments, Transwell assays and flow cytometry at 48 hours after transfection.

Cell Counting Kit-8 (CCK-8) assay

Cell proliferation ability was detected using a CCK-8 kit (Beyotime® Biotech, Jiangsu, China) according to the manufacturer's instructions. Briefly, A431 cells (2×10^3) were seeded into 96-well culture plates (three wells/group) and maintained in fresh DMEM medium. Fresh medium harboring 10% CCK-8 in a volume of 100 μL was added to the cells at 0, 24, 48, 72 and 96 hours, for a further 3 hours, and cell proliferation ability was then determined by measuring the absorbance value at 450 nm using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

In vivo *xenograft experiments*

Female athymic BALB/c nude mice (Charles River Company, Beijing, China) were kept in specific pathogen-free conditions. The mice were divided into three groups (n=7 per group) and A431 cells (1×10^7 cells/mouse) were injected subcutaneously into the backs of the mice. When the tumor volume reached approximately 100 mm^3, the tumors were injected with phosphate-buffered saline (PBS), consiRNA (100 μM) or WWP1 siRNA (100 μM), respectively, in a volume of 100 μL. Tumor volume was measured twice weekly using digital Vernier calipers. Tumors were measured for 30 days, or until they reached 2000 mm^3, when the mice were euthanized. All animal care and experimental protocols were conducted according to the guidelines for the Care and Use of Laboratory Animals of Henan Province, China.

Cell migration

Cell migration was assessed by wound healing migration assay, according to previous reports.[25] Briefly, transfected A431 cells were added to 6-well culture plates at a density of 4×10^5. Scratch wounds were made in the cell layer after 24 hours *in vitro* using a 200-μL sterile pipette tip. After scrubbing off the suspended cells, the cultures were photographed immediately under an inverted microscope (0 hours), and then allowed to grow for 24 hours at 37°C, and photographed from the same position at 12 and 24 hours, respectively. Migration distances were measured from the wound edges in at least three independently repeated experiments.

Cell invasion experiment

Cell invasion was assessed in 24-well plate Transwell chambers (Corning, New York, NY, USA) (6.5-mm diameter, 8.0-μm pores), harboring 100 μL of Matrigel basement membrane matrix (BD Biosciences, San Diego, CA, USA) per well, solidified at 37°C for 30 minutes. Briefly, A431 cells (3×10^4 per well) transfected as above were inoculated into the upper chamber inserts pre-coated with Matrigel overnight at 37°C in a CO_2 incubator. Invading cells were fixed with methanol and stained using 0.5% crystal violet for 20 minutes after washing with PBS, and the cell numbers were counted in 10 random visual fields by microscopy. Invasive abilities were assessed in three independently repeated experiments.

Cell cycle

The effects of WWP1 on the cell cycle were assessed by flow cytometry, as described previously.[26] Briefly, 1×10^6 A431 cells transfected as above were collected and rinsed three times in PBS, followed by 70% cold ethanol for 30 minutes. The cells were then

resuspended in 1 mL PBS buffer containing 40 μg propidium iodide (BD Biosciences) and 100 μg of RNase A at 37°C for 30 minutes, after three rinses with cold PBS buffer. Finally, the DNA content was determined to assess cell cycle status using a flow cytometer (BD Biosciences).

Apoptosis

A431 cells transfected as above were digested with trypsin, collected, rinsed using cold PBS, and then stained with Annexin V-FITC (1 μg/mL, BD Biosciences) and propidium iodide (250 ng) in binding buffer for 15 minutes at 37°C in the dark. Finally, apoptosis was investigated by flow cytometry.

Statistical analysis

All data were analyzed by χ^2 tests and one-way ANOVA using SPSS Statistics, version 17.0 (SPSS Inc., Chicago, IL, USA). The association between WWP1 expression level and prognosis in patients with CSCC was determined using Kaplan–Meier curve analysis. All data are presented as means ± standard deviation (SD). $P<0.05$ was considered statistically significant.

Results

Elevated WWP1 expression in CSCC tissues

A total of 95 patients with CSCC were enrolled in the current study. WWP1 protein expression levels were detected in paired CSCC and normal skin tissues using IHC. The incidence of WWP1-positivity was significantly higher in CSCC tissues (76.8%, 73/95) than in normal skin tissues (13.7%, 13/95) ($\chi^2=76.476$, $P<0.001$) (Figure 1a). sqRT-PCR and western blotting also revealed that WWP1 mRNA and protein levels were significantly higher in CSCC tissues than in normal skin ($P<0.05$) (Figure 1b, c, d and e). These findings suggest that WWP1 may play an essential role in the occurrence and development of CSCC.

Association of WWP1 protein expression with clinicopathological features in patients with CSCC

We investigated the association between WWP1 protein expression and clinicopathological characteristics in patients with CSCC to clarify its possible role in the progression of CSCC. WWP1 protein expression was significantly associated with invasion depth, histological grade and lymph node metastasis ($P<0.01$), but not with tumor size (Table 1). These results suggest that WWP1 expression may be correlated with the occurrence and development of CSCC.

High WWP1 level predicted higher metastatic potential and poor prognosis

We further explored the association of WWP1 expression with metastasis and prognosis in patients with CSCC by sqRT-PCR and Kaplan–Meier curve analyses. Relative levels of WWP1 mRNA were significantly higher in patients with lymph node metastasis (1.164 ± 0.089) compared with those without lymph node metastasis (0.712 ± 0.050) ($P<0.01$) (Figure 2a). In addition, WWP1-positive patients with CSCC had significantly shorter survival than WWP1-negative patients (36.83 months vs. 57.32 months, $P=0.04$) (Figure 2b). These results suggest that WWP1 may be a novel predictor for lymph node metastasis and prognosis in patients with CSCC.

Patterns of WWP1 expression in CSCC cell lines

We analyzed WWP1 mRNA and protein expression levels in CSCC cells and

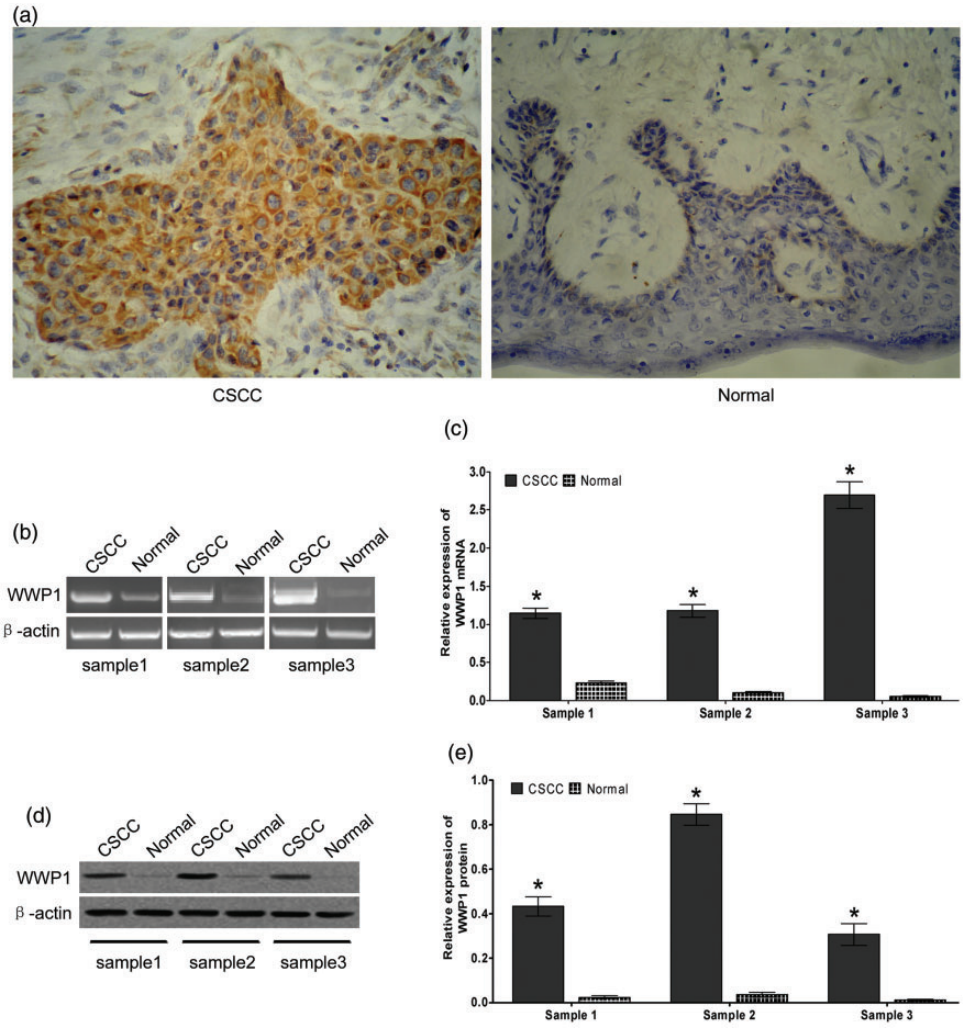

Figure 1. Increased WWP1 expression in human CSCC tissues. (a): WWP1 protein expression in human CSCC and normal skin tissues detected by IHC using anti-WWP1 antibody. Representative figures at 200× magnification. Stained with 3′-diaminobenzidene and counterstained with hematoxylin (b): WWP1 mRNA expression in three randomly selected cases of CSCC and normal skin tissues detected by sqRT-PCR, with β-actin as a loading control. (c): Relative expression levels of WWP1 mRNA in three randomly selected cases of CSCC and normal skin tissues, based on data from three independent experiments. (d): WWP1 protein expression in three randomly selected cases of CSCC and normal skin tissues detected by western blotting, with β-actin as a loading control. (e): Relative expression levels of WWP1 protein in three randomly selected cases of CSCC and normal skin tissues, based on data from three independent experiments. *$P<0.05$, compared with normal skin tissues

normal keratinocytes by sqRT-PCR and western blotting. WWP1 mRNA and protein levels were significantly higher in SCL-1 and A431 cells compared with HaCaT cells ($P<0.05$ and $P<0.01$, respectively), with the highest WWP1 mRNA and protein expression levels in A431 cells (Figure 3a, b, c and d).

Table 1. Relationship between WWP1 protein expression and clinicopathological features in patients with CSCC

Classification	n	WWP1 protein expression		χ^2	*P* value
		+	−		
Histological grade					
Well-differentiated	41	25	16	10.983	0.004
Moderately differentiated	33	28	5		
Poorly differentiated	21	20	1		
Invasion depth					
Interdermis	59	40	19	7.159	0.007
Subcutis	36	33	3		
Tumor size					
<5 cm	63	48	15	0.045	0.833
≥ 5 cm	32	25	7		
Lymph node metastasis					
Yes	31	29	2	7.217	0.007
No	64	44	20		

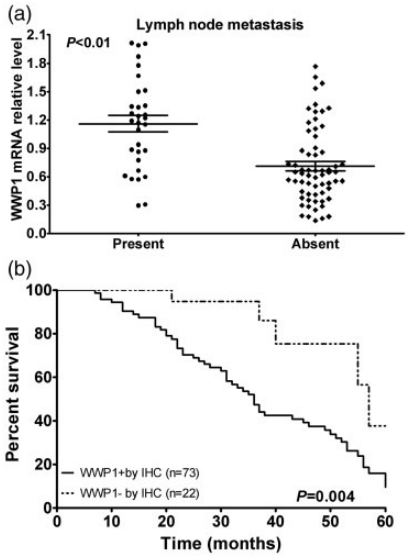

Figure 2. Correlations of WWP1 expression with metastasis and prognosis in patients with CSCC. (a): WWP1 mRNA levels in patients with CSCC (31 with metastasis and 64 without metastasis) detected by sqRT-PCR. WWP1 mRNA levels were calculated relative to β-actin mRNA levels, and based on three independently repeated experiments. Results were expressed as mean $\pm$ SD. (b): High WWP1 protein level predicted poor prognosis in patients with CSCC. Patient survival was evaluated by Kaplan–Meier curves

WWP1 siRNA depleted WWP1 protein expression in A431 cells

To further clarify the role of WWP1 in CSCC cells, we transfected A431 CSCC cells with WWP1 siRNA and analyzed WWP1 protein expression at different time points (24, 48, 72 and 96 hours) by western blotting. WWP1 protein levels were significantly reduced following transfection with WWP1 siRNA at 24, 48, 72 and 96 hours, compared with the untreated and consiRNA groups ($P<0.05$, $P<0.01$, $P<0.01$ and $P<0.05$, respectively) (Figure 4a and b). WWP1 protein levels were lowest at 48 hours compared with the other time points (24, 72 and 96 hours) (Figure 4a and b). These findings indicate that WWP1 siRNA efficiently downregulated WWP1 protein expression in A431 cells.

Downregulation of WWP1 repressed cell growth, migration and invasion in CSCC

We examined the roles of WWP1 in regulating cell growth, migration and invasion in CSCC *in vitro* and *in vivo* by CCK-8 assay, xenografts in nude mice, and

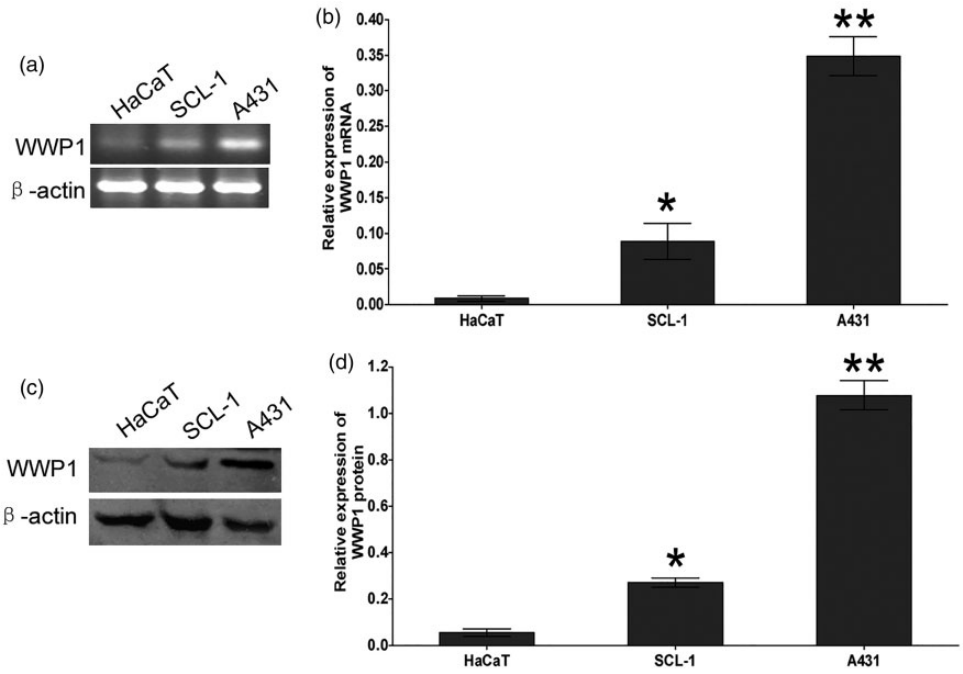

Figure 3. WWP1 mRNA and protein expression levels in CSCC cell lines and normal keratinocytes. (a): WWP1 mRNA levels in SCL-1 and A431 CSCC cells and HaCaT keratinocytes, detected by sqRT-PCR, with β-actin as an internal control. (b): WWP1 mRNA levels were calculated relative to β-actin levels, and expressed as mean $\pm$ SD, based on three independently repeated experiments. (c): WWP1 protein expression in SCL-1 and A431 CSCC cell lines and HaCaT keratinocytes, detected by western blotting, with β-actin as a loading control. (d): WWP1 protein levels were calculated relative to β-actin levels, and expressed as mean $\pm$ SD, based on three independently repeated experiments. *$P<0.05$ compared with HaCaT cells, **$P<0.01$ compared with the other cells

wound healing migration and Transwell chamber assays. WWP1 depletion by siRNA significantly suppressed the growth of A431 cells *in vitro* and tumor xenografts *in vivo* ($P<0.05$) (Figure 5a and b). WWP1 downregulation also significantly reduced cell migration ability after 24 hours ($P<0.05$) (Figure 5c and d). The number of invading cells was also significantly lower in the WWP1 siRNA group compared with untreated cells and cells transfected with consiRNA ($P<0.05$) (Figure 5e and f). These data suggest that WWP1 plays pivotal roles in the regulation of CSCC cell growth, migration and invasion.

Downregulation of WWP1 promoted cell cycle arrest and apoptosis in A431 cells

We investigated the possible roles of WWP1 in regulation of the cell cycle and apoptosis in CSCC by examining changes in cell cycle distribution and apoptosis in transfected A431 cells by flow cytometry. The percentage of cells in G0/G1 phase was significantly higher in the WWP1 siRNA group compared with the untreated and consiRNA groups ($P<0.05$) (Figure 6a and b), while the opposite results were obtained for S phase ($P<0.05$) (Figure 6a and b). Apoptotic cell numbers were also significantly increased in the WWP1

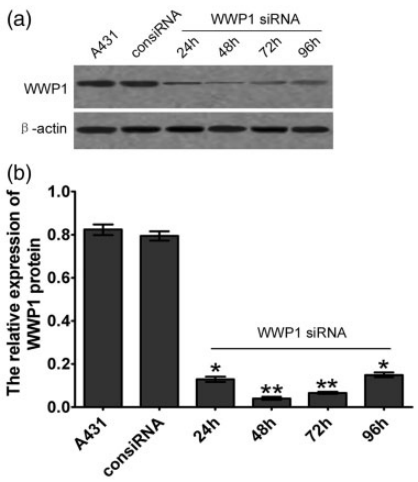

Figure 4. WWP1 siRNA effectively downregulated WWP1 protein levels in A431 cells at 24, 48, 72 and 96 hours. (a): WWP1 protein expression levels in untreated A431 cells and cells transfected with consiRNA or WWP1 siRNA at 24, 48, 72 and 96 hours, detected by western blotting, with β-actin as a loading control. (b): WWP1 protein levels in A431 cells were calculated relative to β-actin levels, and expressed as mean $\pm$ SD, based on three independently repeated experiments. *$P<0.05$ compared with untreated A431 cells and consiRNA group, **$P<0.01$ compared with the other groups

siRNA group compared with untreated and consiRNA cells ($P<0.05$) (Figure 6c). These findings suggest that WWP1 siRNA may trigger changes in the cell cycle and apoptosis in CSCC.

Downregulation of WWP1 evoked inactivation of STAT3 signaling pathway in A431 cells

We further examined the possible mechanisms regulated by WWP1 in CSCC by investigating the status of the STAT3 signaling pathway and related proteins by western blotting. WWP1 downregulation was associated with significant decreases in protein expression levels of p-STAT3 ($P<0.0001$), MMP-2 ($P<0.01$), Bcl-2 ($P<0.001$) and cyclin D1 ($P<0.05$), but not t-STAT3, in CSCC A431 cells, compared with the untreated and consiRNA groups (Figure 7).

Discussion

The results of the current study support the idea that WWP1 functions as an oncogene and might be a promising molecular diagnostic and therapeutic target in patients with CSCC. WWP1 expression levels were increased in CSCC compared with normal tissues, and high WWP1 expression was associated with higher metastatic potential. WWP1 also demonstrated essential roles in the regulation of cell growth, migration, invasion, cell cycle and apoptosis in CSCC. These findings highlighted the critical roles of WWP1 in the initiation, development and progression of CSCC.

Increasing evidence has indicated that WWP1 is an underlying oncogene overexpressed in breast and prostate cancers.[15, 18] Wang et al.[27] recently revealed that WWP1-positive cells were increased in patients with prostate cancer compared with normal tissues. Furthermore, the integrated optical density value of WWP1 was markedly increased in patients with bone metastasis, compared with non-bone metastasis patients, as supported by real-time quantitative PCR, suggesting that WWP1 plays a key role in the progression and metastasis of prostate cancer. Elevated WWP1 mRNA and protein levels were also found in oral cancer patients and in six oral cancer cell lines,[17] suggesting that WWP1 also functions as an oncogene in oral cancer, and may thus may be a potential therapeutic molecular target for oral cancer. WWP1 was also shown to be overexpressed in a wide array of breast cancer cells.[16] Overall, these findings suggest that WWP1 may be a promising therapeutic molecular target in many types of tumors. We confirmed a potential role for WWP1 in

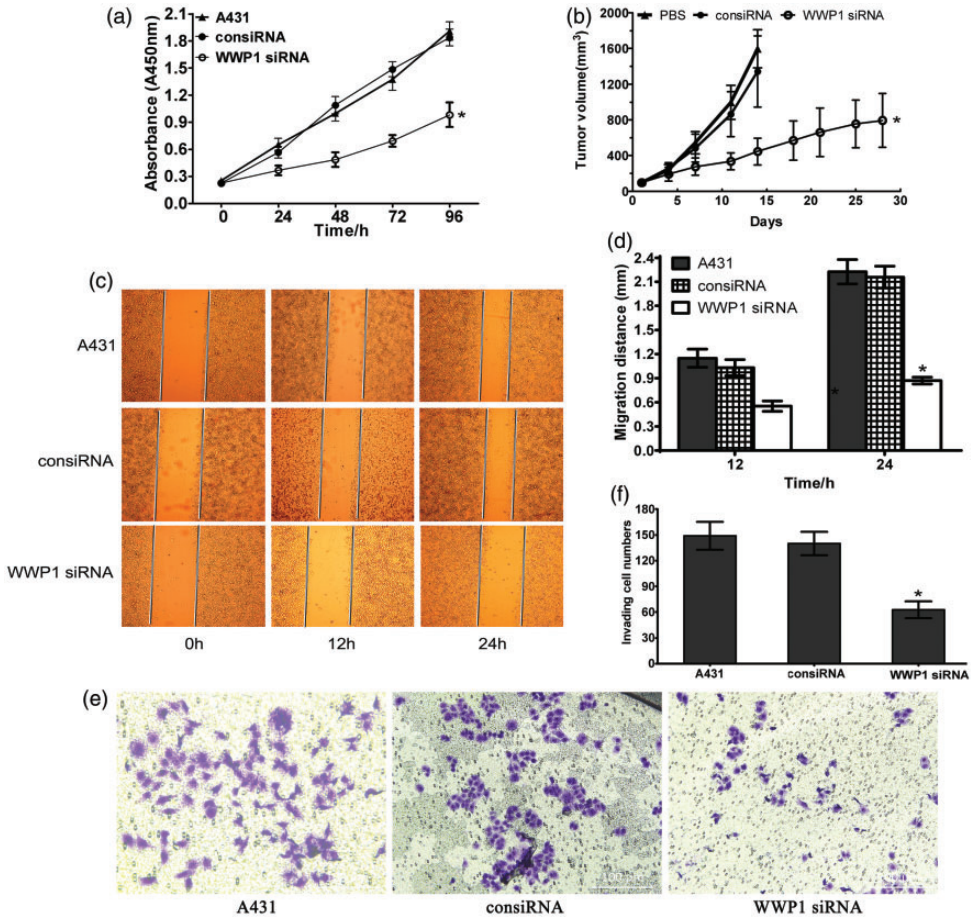

Figure 5. Effects of WWP1 depletion on cell growth *in vitro* and *in vivo*, and cell migration and invasion abilities in A431 cells *in vitro*. (a): WWP1 siRNA significantly suppressed the proliferation of A431 cells. (b): WWP1 siRNA significantly suppressed tumor growth in A431-xenografted nude mice. A431 cells (1×10^7 per nude mouse) were inoculated into the back of right flank of each nude mouse. When the tumor volume reached approximately 100 mm^3 (about on day 5), the tumors were injected with PBS, consiRNA or WWP1 siRNA, respectively. Tumor volumes were measured twice a week and tumor growth curves were plotted. (c): WWP1 knockdown markedly inhibited migration of A431 cells at 12 and 24 hours. (d): Cell migration was assessed by measuring migration distances from the wound edges. *$P<0.05$ compared with untreated A431 cells and consiRNA group. (e): WWP1 downregulation decreased the invasion ability of A431 cells. Stained with 0.5% crystal violet. (f): Invading cells were counted to evaluate the effect of WWP1 siRNA on the invasion ability of A431 cells. *$P<0.05$ compared with untreated A431 cells and consiRNA group

CSCC by demonstrating that WWP1 expression levels were significantly increased in CSCC tissues and cells compared with normal tissues and immortalized human adult keratinocytes, as confirmed by sqRT-PCR and western blotting. In addition, high WWP1 levels were closely correlated with histological grade, invasion depth and lymph node metastasis. Notably, high WWP1 expression predicted a higher metastatic potential and poor prognosis in patients with CSCC, which differed from

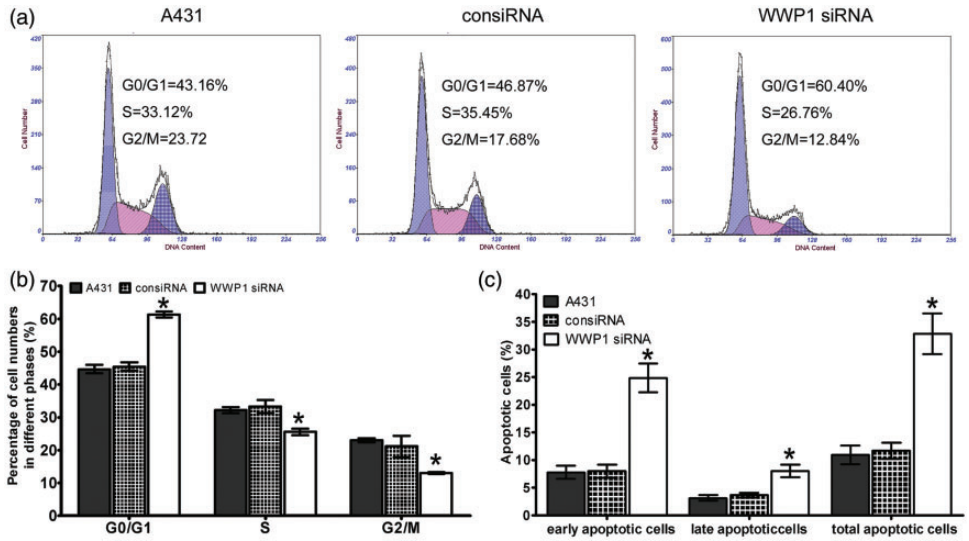

Figure 6. Effects of WWP1 downregulation on cell cycle distribution and apoptosis in A431 cells detected by flow cytometry. (a): Representative cell cycle distributions in untreated A431 cells and cells transfected with WWP1 siRNA or consiRNA. (b): Cell cycle distributions in untreated A431 cells and cells transfected with WWP1 siRNA or consiRNA, based on data from three independent experiments. (c): Apoptosis distributions in untreated A431 cells and cells transfected with WWP1 siRNA or consiRNA. *$P<0.05$ compared with untreated A431 cells and cells transfected with consiRNA

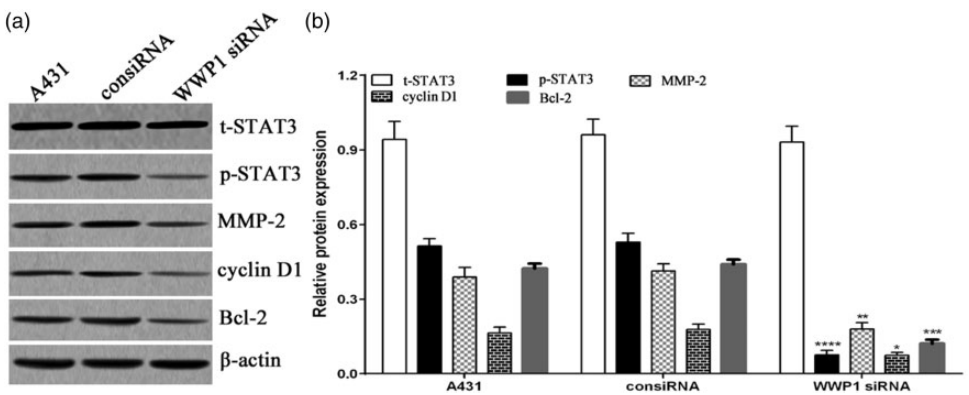

Figure 7. WWP1 downregulation triggered inactivation of the STAT3 signaling pathway in A431 CSCC cells. Total proteins were extracted from untreated A431 cells and cells transfected with WWP1 siRNA or consiRNA, and protein levels were detected by western blotting. (a): Protein levels of t-STAT3, p-STAT3, MMP-2, cyclin D1 and Bcl-2 were detected by western blotting, with β-actin as a loading control. (b): Relative expression levels of t-STAT3, p-STAT3, MMP-2, cyclin D1 and Bcl-2 proteins in A431 cells, based on three independent experiments. *$P<0.05$, **$P<0.01$, ***$P<0.001$ and ****$P<0.0001$ compared with untreated A431 cells and cells transfected with consiRNA

the results of a previous study in breast cancer patients,[16] but was consistent with a study in patients with gastric carcinoma.[13] These findings imply that WWP1 may represent a new biomarker for metastasis and prognosis in patients with CSCC.

Targeting WWP1 is known to suppress tumor growth, progression and metastasis and is thus a promising strategy for tumor therapy. Yeung et al.[28] verified that WWP1 overexpression enhanced the proliferation ability of MCF10A mammary epithelial cells positive for large tumor suppressor kinase 1 (LATS1), while WWP1 knockdown significantly suppressed the proliferation of MCF7 cells harboring wild-type LATS1.[29] In addition, WWP1 knockdown significantly inhibited the growth of human gastric carcinoma cells by suppressing the PTEN-Akt signaling pathway.[13] Furthermore, WWP1 overexpression postponed senescence of human fibroblasts, whereas WWP1 downregulation resulted in premature senescence,[30] further suggesting an important role for WWP1 in cell survival. Recent investigations revealed that siRNA-mediated WWP1 downregulation repressed colony formation, proliferation, migration and invasion of hepatocellular carcinoma cells *in vitro*.[31] However, conversely, WWP1 depletion had no effect on cell growth or survival, but dramatically increased cell migration under a CXC motif chemokine ligand 12 gradient in breast carcinoma cells *in vitro*.[32] We further examined the effects of WWP1 depletion on the proliferation, migration and invasion of CSCC cells and found that WWP1 downregulation dramatically inhibited cell growth *in vitro* and *in vivo*, and reduced CSCC cell migration and invasion abilities *in vitro*. These data suggest that WWP1 plays crucial roles in the processes of growth, migration and invasion of CSCC cells.

Several studies have revealed that WWP1 depletion affected the cell cycle distribution and induced apoptosis in various tumors. Zhang et al.[13] found that downregulation of WWP1 expression significantly promoted cell cycle arrest in G0/G1 phase and apoptosis in gastric carcinoma cells, with similar results for hepatocellular carcinoma cells. Decreased levels of WWP1 also contributed to the inhibition of cell growth and apoptosis in MCF7 and HCC1500 breast cancer cell lines harboring estrogen receptor α,[19] while conversely, MCF10A cells with WWP1 depletion were more resistant to doxorubicin-mediated apoptosis.[20] These data suggest that manipulating WWP1 levels may represent a means of evoking cell cycle alterations and apoptosis in a wide range of tumors. The current results also showed that WWP1 downregulation contributed to cell cycle arrest in G0/G1 phase and apoptosis in CSCC cells, suggesting that WWP1 may be a critical regulatory factor of these processes in CSCC, and may represent a promising strategy for therapy of CSCC.

Numerous studies have indicated that the STAT3 signaling pathway participates in the processes of cell proliferation, cell cycle distribution, apoptosis, invasion, metastasis and tumorigenesis.[33–39] Notably, previous studies have highlighted the critical roles of STAT3 signaling in tumor development and progression, indicating that suppression of STAT3 signaling may be a promising therapeutic target in many types of tumors.[40–44] We therefore clarified the role of STAT3 signaling in the possible molecular mechanisms of WWP1 in the progression of CSCC. We detected the expression levels of t-STAT3 and p-STAT3, as well as those of related proteins including MMP-2, Bcl-2 and cyclin D1 in CSCC A431 cells. Downregulation of WWP1 markedly reduced the levels of p-STAT3, MMP-2, Bcl-2 and cyclin D1 proteins, but had no effect on the level of t-STAT3 in A431 cells. These data suggest that the antitumor

effect of WWP1 downregulation may be achieved via inactivation of the STAT3 signaling pathway in CSCC.

Collectively, the current results demonstrated that WWP1 expression levels were increased in CSCC, and were associated with higher metastatic potential and poor prognosis in patients with CSCC. siRNA-mediated WWP1 downregulation contributed to tumor growth suppression and reduced cell migration and invasion abilities, as well as altering the cell cycle distribution and promoting apoptosis in CSCC cells, possibly via inactivation of the STAT3 signaling pathway. These results suggest that WWP1 may be a promising molecular target for the diagnosis, prognosis and therapy of patients with CSCC.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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ORCID iD

Wei Zhu http://orcid.org/0000-0001-5816-0355

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