



WW and C2 domain-containing protein-3 promoted EBSS-induced apoptosis through inhibiting autophagy in non-small cell lung cancer cells

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Background: WW and C2 domain-containing protein-3 (WWC3) was identified in our previous studies as a tumor suppressor gene, which inhibits the proliferation and invasiveness of lung cancer cells. However, the relationship between WWC3 and autophagy and apoptosis in lung cancer cells is unclear. In this study, we aimed to investigate the potential role of WWC3 in starvation-induced autophagy and apoptosis in non-small cell lung carcinoma (NSCLC) cells.

Methods: The immunoblotting assay and quantitative real-time polymerase chain reaction (RT-qPCR) were used for observing the change of WWC3 protein and mRNA level under starvation condition. The immunoblotting assay and immunofluorescence assay were performed to detect the impact of WWC3 expression on autophagy process induced by Earle's balanced salt solution (EBSS) in lung cancer cells; APC/propidium iodide (PI) apoptosis assay, caspase-3/7 activity assay and MTT assay were used for the apoptosis and proliferation detection of lung cancer cells.

Results: After starvation had been induced with EBSS, WWC3 expression was significantly decreased in the NSCLC cells. Ectopic WWC3 expression weakened the autophagy process in a Beclin1-independent manner and promoted non-small cell lung cancer cell apoptosis via EBSS starvation. Moreover, the inhibition of WWC3 gene knockout was weakened by 3-methyladenine (3-MA), an autophagy inhibitor.

Conclusions: These results indicate that WWC3 promotes apoptosis and death of starved lung cancer cells, at least partly through autophagy.

Keywords: WW and C2 domain-containing protein-3 (WWC3); apoptosis; autophagy; lung cancer

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Introduction

Autophagy is considered to be an evolutionarily conservative process in all eukaryotic cells. It helps cells to maintain homeostasis, adapt to changing nutritional conditions, circulate cytoplasm, generate macromolecular building blocks and energy under pressure, and remove redundant and damaged proteins and organelles (1,2). Autophagy is believed to play a complex role in the

occurrence, development, and treatment of lung cancer (3-5). For example, low Beclin-1 expression has been found to be significantly correlated with tumorigenesis, tumor progression, and poor prognosis in non-small cell lung carcinoma (NSCLC) (6). Rao *et al.* discovered that the development of NSCLC could be accelerated by inactivating autophagy-related 5 (ATG5), an important protein involved in autophagy (7). The inhibition of

autophagy can weaken the proliferative ability of lung cancer cells and promote their sensitivity to chemotherapeutic drug-induced apoptosis (8). Although great progress has been made with our understanding of autophagy regulation to date, the detailed information about the regulation of autophagy remains limited.

WW and C2 domain-containing protein (WWC3) is a member of the WWC protein family (KIBRA/WWC1, WWC2, and WWC3), which maps to the human chromosomal locus Xp22.2 (9). Our previous studies demonstrated that low WWC3 expression is present in both lung cancer cell lines and lung cancer specimens and is associated with low differentiation, advanced pathological tumor-node-metastasis (pTNM) stage, positive lymph node metastasis, and poor prognosis in lung cancer patients. Meanwhile, the ectopic expression of WWC3 has an inhibitory role in the proliferation and invasiveness of lung cancer cells *in vitro* and *in vivo* (10,11). A recent study indicated that KIBRA/WWC1 is involved in autophagy processing in S2 cells and in *Drosophila* larvae (12). These results prompted us to explore the involvement of WWC3 in autophagy and apoptosis in lung cancer cells under starvation or hypoxic conditions.

In this study, we found that forced expression of WWC3 inhibited starvation-induced autophagy and promoted apoptosis of lung cancer cells. Our results provide valuable new insight into the mechanism by which the biological behavior of lung cancer is influenced by WWC3, which may serve as a potential target for the treatment of lung cancer patients.

Methods

Cell culture

The human bronchial epithelial (HBE) cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The NSCLC cell lines A549, H1299 and H460 were purchased from Shanghai Cell Bank (Shanghai, China). The LK2 cell line was a gift from Dr. Hiroshi Kijima (Department of Pathology and Bioscience, Hiroshima University Graduate School of Medicine, Japan). Upon receipt, the cells were frozen and individual aliquots were typically cultured for analysis within 10 passages. All cells were cultured in RPMI 1640 (Hyclone, Logan, UT, USA) containing 10% fetal calf serum (Thermo Fisher Scientific, Waltham, MA, USA), 100 IU/mL penicillin, and 100 µg/mL streptomycin at 37 °C with 5% CO₂ in high

humidity. All cell lines were authenticated by short tandem repeat (STR) DNA profiling.

Plasmids, small interfering RNA (siRNA), and reagents

pEGFP-C2-WWC3 and the corresponding pEGFP-C2 empty vectors were provided by Dr. Joachim Kremerskothen (University of Münster, Germany). siRNA-WWC3 (sc-91139) and control siRNA (sc-37007) were purchased from Santa Cruz Technology Inc. (CA, USA). Lipofectamine 3000 (Thermo Fisher Scientific) transfection reagent was used for plasmid transfection. Earle's balanced salt solution (EBSS, NaHCO₃: 2.2 g/L, glucose: 1.0 g/L, phenol red: 0.011 g/L, #E2888), chloroquine (CQ, #C6628), and 3-methyladenine (3-MA, M9281) were all purchased from Sigma-Aldrich (St. Louis, MO, USA).

Western blot analysis

Total protein from the cell lines was extracted with lysis buffer (Thermo Fisher Scientific) and quantified using the Bradford method. SDS-PAGE (8% and 15%) was used to separate 40 µg of protein. The protein was transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA) before incubation overnight at 4 °C with the following antibodies: WWC3 (#HPA039814, 1:1,000; Sigma-Aldrich); anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; sc-293335, 1:1,000; Santa Cruz Biotechnology); LC3B (#3868, 1:1,000); Beclin-1 (#3738, 1:1,000); P62 (#39749, 1:500); caspase-3 (#9662, 1:500); cleaved caspase-3 (#9664, 1:500); caspase-7 (#9494, 1:500); and cleaved caspase-7 (#9491, 1:500) (all from Cell Signaling Technology, Danvers, MA, USA). After incubation with peroxidase-coupled anti-mouse IgG and anti-rabbit IgG (Santa Cruz Biotechnology) at 37 °C for 2 h, bound proteins were viewed using enhanced chemiluminescence (ECL) (Thermo Fisher Scientific) and detected using BioImaging Systems (UVP Inc., Upland, CA, USA). The relative protein levels were calculated with GAPDH used as the loading control.

Quantitative real-time polymerase chain reaction (RT-qPCR) (SYBR Green method)

The assay was described in our previous study (10). The primer sequences used were: WWC3: forward, 5'-CAAGAGCGCATGTTGAAGGAA-3', reverse: 5'-CGCTGCTGCTTAATCTGGTAGA-3'; GAPDH:

forward: 5'-GGAGCGAGATCCCTCCAAAAT-3', reverse: 5'-GGCTGTTGTCATACTTCTCATGG-3'.

Immunofluorescence

Cells were fixed with 4% paraformaldehyde, blocked with 3% BSA, and incubated with LC3B (#3868, 1:100, Cell Signaling Technology) overnight at 4 °C. Tetramethylrhodamine-5-(and-6)-isothiocyanate (TRITC)-conjugated secondary antibodies were then added for 1 h at room temperature. The cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific). Epifluorescence microscopy was performed using an inverted Nikon TE300 microscope (Melville, NY, USA), and confocal microscopy was carried out with a Radiance 2000 laser scanning confocal microscope (Carl Zeiss, Thornwood, NY, USA).

Labeling and capture of nascent RNA

Click-iT Nascent RNA Capture Kit (Thermo Fisher Scientific) was used to synthesize and isolate new RNA according to the manufacturer's instructions. Briefly, cells were incubated in 0.2 mM of 5-ethynyl uridine (5-EU; an alkyne-modified uridine analog, which was efficiently and naturally incorporated into the nascent RNA) for 4 h, and the total RNA labeled with EU was isolated using TRIzol reagent (Invitrogen). Then, the EU-labeled RNA was biotinylated in a Click-iT reaction buffer with 0.5 mM of biotin azide and subsequently captured on streptavidin magnetic beads.

Detection of apoptosis

Apoptosis induced by EBSS and 3-MA was detected with the Annexin V-APC Apoptosis Detection Kit according to the manufacturer's instructions (BD Biosciences Pharmingen, CA, USA). Briefly, 2×10^5 cells were washed once with ice-cold phosphate-buffered saline (PBS, pH 7.4) and resuspended in 200 mL of binding buffer. The cells were incubated with Annexin V-APC for 30 min and then with propidium iodide (PI) for 5 min at room temperature. Before testing, 400 mL of binding buffer was added. Apoptotic cells were detected by flow cytometry (FACS Calibur, Becton Dickinson, NJ, USA). All experiments were performed in triplicate.

Cell viability

Cells were plated in 96-well plates with medium containing

10% fetal bovine serum (FBS) (2,000 cells/well) 48 h after transfection. Cell viability was determined using the (3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTT) assay. Briefly, 20 μ L of the 5 mg/mL MTT solution (Sigma, cell viability) was added to each well, and the mixture were incubated for 4 h at 37 °C. Then, the medium was removed from each well and the resultant MTT formazan was solubilized in 150 μ L of dimethyl sulfoxide (DMSO). The results were repeated 5 times and quantified spectrophotometrically using a test wavelength of 490 nm.

Caspase-3/7 activity assay

Cell lysates were detected using 3-[[3-cholamidopropyl] dimethylammonio]-1-propanesulfonate (CHAPS) lysis buffer (50 mM HEPES, pH 7.4; 0.1% CHAPS, 0.1% Triton X-100, 0.1 mM EDTA, and 1 mM dithiothreitol) and centrifuged at 12,000 g at 4 °C. DEVDase activity was measured using the specific fluorogenic substrate, Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (DEVD-AMC, Sigma-Aldrich) in the assay buffer (50 mM HEPES, pH 7.4; 100 mM NaCl, 0.1% CHAPS, 10% glycerol, 1 mM EDTA, and 10 mM dithiothreitol), and a fluorescence plate reader was used to obtain real-time recordings of enzyme-catalyzed AMC release. Fluorescence values were converted into picomoles of released AMC using the standard curve generated from free AMC, and the maximum rate of AMC release (pmol/min/mg protein) was calculated. All experiments were performed in triplicate.

Statistical analysis

SPSS 22.0 (SPSS, Chicago, IL, USA) was used to perform all the statistical analyses. The statistical significance of the differences was analyzed by Student's *t*-test. All experiments were performed in triplicate, and the results were presented as the mean \pm standard deviation (SD). A *P* value <0.05 indicated statistical significance.

Results

Starvation could inhibit WWC3 expression in NSCLC cells

To explore the role of WWC3 in autophagy in NSCLC cells, the expression level of WWC3 in A549 and H460 cell lines cultured with complete EBSS, the most direct inducer of typical autophagy, was detected at different

time points (0, 6, 12 and 24 h). In A549 and H460 cells, we observed that the WWC3 protein level had obviously decreased after 6 h of culture with complete EBSS medium. The lowest expression level in the A549 and H460 cells was reached at 12 and 24 h, respectively (Figure 1A,B). There was also a marked decrease in WWC3 mRNA expression after culture with complete EBSS (Figure 1C,D). Because the decrease of WWC3 mRNA level after complete EBSS culture may be attributed to the decrease of mRNA synthesis, a new WWC3 transcription gene was isolated from the A549 and H460 cells using Click-iT Nascent RNA Capture, and analyzed. The results of RT-qPCR showed that starvation-induced by complete EBSS could significantly reduce WWC3 mRNA expression (Figure 1E). Also, when combined with 20 μ M CQ, the lysosomal conversion inhibitor did not alter the reduction of WWC3 in cells cultured with complete EBSS (Figure 1F), which suggests that the activation of autophagy degradation may not promote the reduction of WWC3 mediated by EBSS starvation in lung cancer cells.

WWC3 attenuated EBSS starvation-induced autophagy in NSCLC

The downregulation of WWC3 caused by EBSS starvation led us to explore the potential role of WWC3 involved in starvation-induced typical autophagy. H1299 cells, which have been reported to have low WWC3 expression (10), were transfected with GFP-WWC3 plasmid and then cultured with normal medium or in complete EBSS media for 14 h. The immunoblotting assay demonstrated that WWC3 overexpression eliminated the increased basal LC3-II expression induced by starvation. Under the same conditions, P62/SQSTM1 expression was enhanced in the H1299 cell line and, notably, Beclin1 expression level was not noticeably altered (Figure 2A). In contrast, knockdown of WWC3 by siRNA-WWC3 in A549 cells, a cell line known to have a relatively high expression of WWC3, exerted the opposite effect (Figure 2B). The obvious punctate distribution of endogenous LC3, a marker for autophagosome formation, was also investigated. The results of the immunofluorescence assay showed that forced WWC3 expression was markedly different from the basal punctate distribution in H1299 cells. Moreover, when WWC3 transfected cells were cultured in complete EBSS, even in the presence of 20 μ M CQ for 12 h, the number of LC3 puncta per cell was significantly reduced (Figure 2C,D). These results indicate that WWC3 has a Beclin1-

independent inhibitory effect on starvation-induced autophagy in lung cancer cells.

WWC3 promoted EBSS-induced cell apoptosis in NSCLC

To explore the potential effect of WWC3 on lung cancer cell growth under starvation conditions, we performed an MTT proliferation assay. Compared with the empty vector group, ectopic WWC3 expression substantially inhibited the proliferation of H1299 and LK2 cells when cultured in complete EBSS media, whereas the proliferative ability of A549 and H460 cells was significantly enhanced by WWC3 silencing under starvation conditions (Figure 3A,B). The impact of WWC3 on the activity of caspase-3/7 was also investigated. WWC3 transfection significantly enhanced caspase-3/7 activity under starvation conditions in H1299 and LK2 cells, while WWC3 knockdown in A549 and H460 cells had the opposite effect (Figure 3C,D). The immunoblotting assay demonstrated that WWC3 increased the expression of apoptosis-related molecules, cleaved caspase-3 and cleaved caspase-7 (Figure 3E,F); these results were consistent with those of the caspase-3/7 activity assay. Additionally, we adopted the Annexin-V APC/PI staining method to detect the impact of ectopic WWC3 overexpression on EBSS-induced apoptosis of lung cancer cells. The results showed that WWC3 could promote EBSS-induced apoptosis of H1299 cells (Figure S1). Therefore, WWC3 could potentially be involved in the apoptosis regulation process under EBSS starvation conditions.

The impact of WWC3 knockdown on cell apoptosis under starvation was attenuated by autophagy inhibition

Previous studies have shown that autophagy activation caused by starvation or hypoxia is a mechanism for promoting survival, thus inhibiting cell death (13). Therefore, we hypothesized that WWC3 could promote starvation-induced apoptosis by attenuating autophagy. The effect 3-MA, which is considered to be a negative regulator of PtdIns3K and is widely used as an inhibitor of hunger-mediated autophagy at a preliminary stage, was determined (14,15). We transfected siRNA-WWC3 into A549 cells to silence the WWC3 expression. Western blotting showed that under the media with 3-MA (5 μ M) for 24 h, starvation-mediated P62/SQSTM1 and LC3-II transition expression was inhibited in both the control and WWC3-

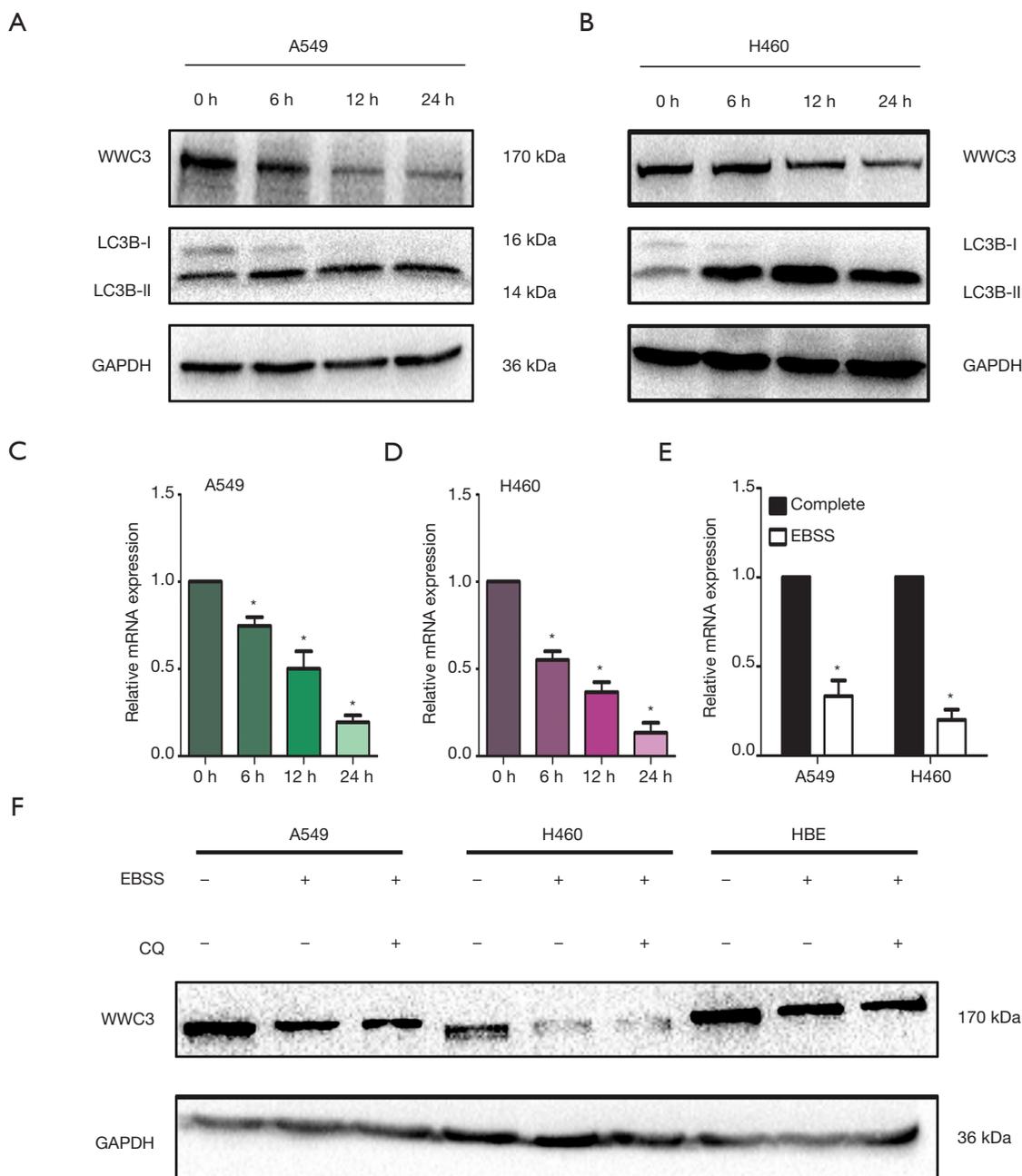


Figure 1 EBSS starvation decreased WWC3 expression in NSCLC cells. (A,B,C,D) A549 and H460 cells were cultured in complete EBSS media for different amounts of time (0, 6, 12, and 24 h). WWC3 protein and mRNA expressions were detected by Western blotting (A,B) and RT-qPCR (C,D); GAPDH was used as the loading control. Data are expressed as the mean \pm SD; *, $P < 0.05$; (E) A549 and H460 cells were cultured in complete EBSS media for 24 h, then Click-iT Nascent RNA Capture was used to capture the newly synthesized RNA. RT-qPCR was performed to detect nascent WWC3 mRNA. The absorbance of triplicate samples was measured at 560 nm. Data are expressed as the mean \pm SD. *, $P < 0.05$; (F) A549, H460, and HBE cells were cultured in normal media or in complete EBSS media with or without 20 μ M of CQ for 12 h. Western blotting was performed to detect the expression of WWC3 protein level. GAPDH served as the loading control. EBSS, Earle's balanced salt solution; WWC3, WW and C2 domain-containing protein-3; NSCLC, non-small cell lung carcinoma; RT-qPCR, quantitative real-time polymerase chain reaction; GAPDH, anti-glyceraldehyde 3-phosphate dehydrogenase; SD, standard deviation.

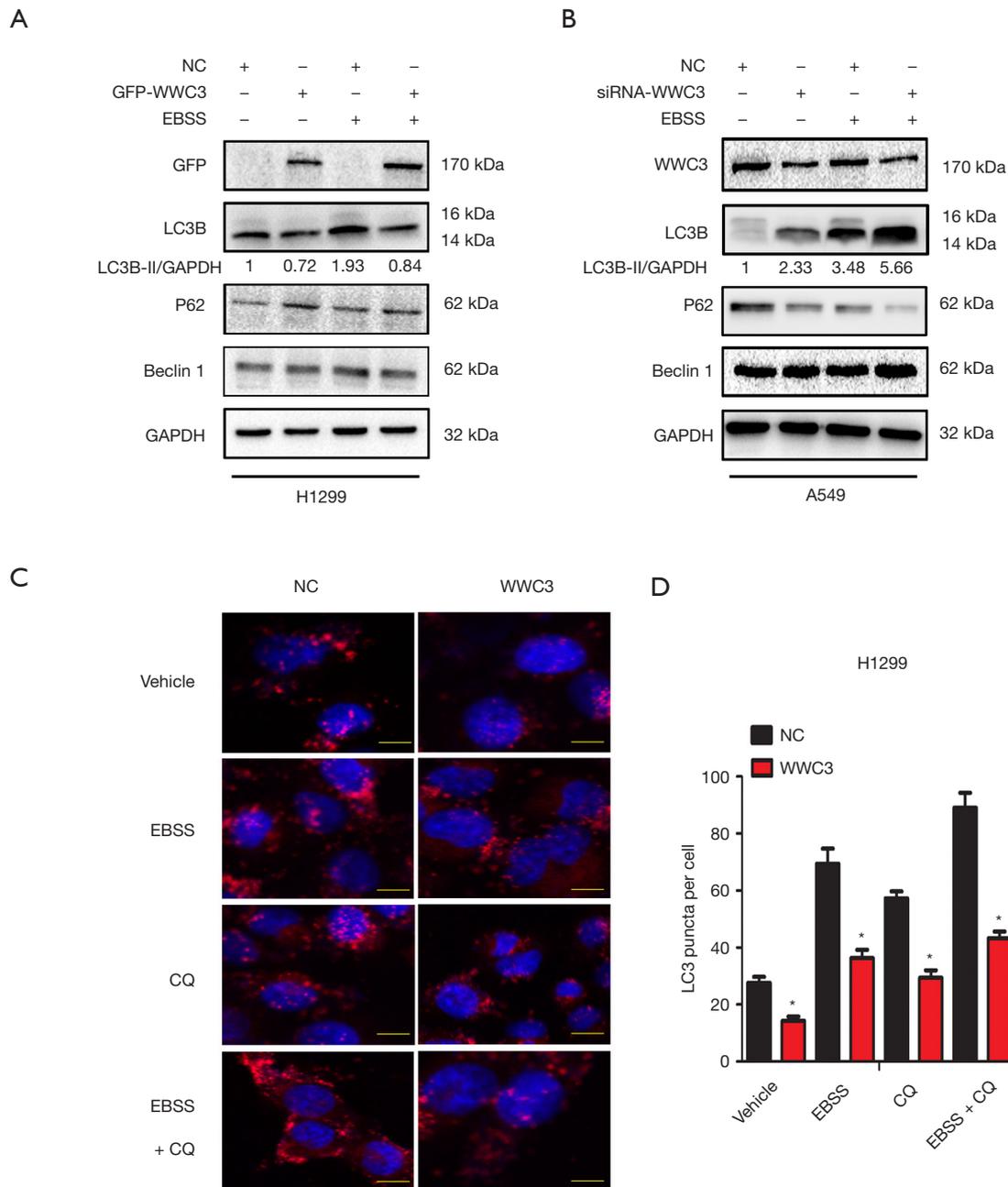


Figure 2 WWC3 attenuated autophagy induced by EBSS starvation in NSCLC. (A,B) H1299 cells were transfected with pEGFP-C2 empty plasmid or pEGFP-C2-WWC3 plasmid (A). A549 cells were transfected with control siRNA or siRNA-WWC3 (B); cells were cultured in normal media or in complete EBSS media for 14 h. Western blotting was performed to detect the changes in LC3-II transition, Beclin-1, and p62/SQSTM1 expression. GAPDH was used as an internal reference; (C,D) NC H1299 cells or with WWC3 plasmid were cultured in normal media or in complete EBSS media with or without 20 μ M of CQ for 12 h. Endogenous LC3 was detected using immunofluorescence (magnification, 400 \times ; scale bar, 50 μ m). The numbers of LC3 puncta per cell were quantified. Data are expressed as the mean \pm SD. *, $P < 0.05$. WWC3, WW and C2 domain-containing protein-3; EBSS, Earle's balanced salt solution; NSCLC, non-small cell lung carcinoma; siRNA, small interfering RNA; GAPDH, anti-glyceraldehyde 3-phosphate dehydrogenase; NC, negative control; CQ, chloroquine; SD, standard deviation.

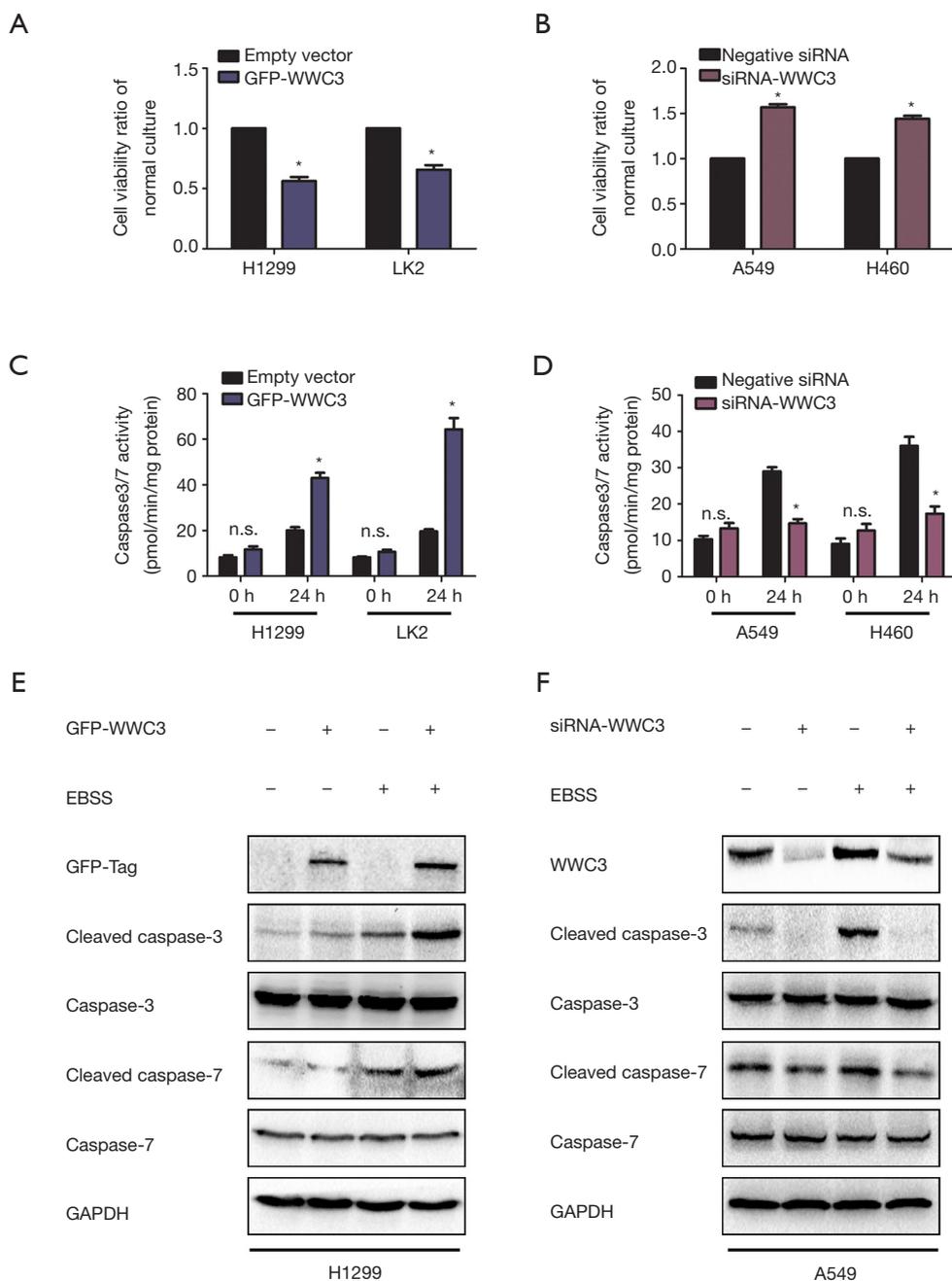


Figure 3 WWC3 promoted EBSS-induced cell apoptosis in NSCLC. (A,B) H1299 and LK2 cells were transfected with empty vector or with WWC3 plasmid and were cultured in complete EBSS for 24 h (A); A549 and H460 cells were transfected with control siRNA or with siRNA-WWC3 and cultured under complete EBSS media for 48 h (B); MTT assay was performed to detect cell viability. Data are expressed as the mean \pm SD; *, $P < 0.05$. Absorbance of 5 repeated samples was measured at 490 nm; (C,D,E,F) H1299 and LK2 cells transfected with empty vector and WWC3 plasmid were cultured under complete EBSS media for 24 h. A549 and H460 cells were transfected with control siRNA and siRNA-WWC3 and cultured under complete EBSS media for 48 h. Then, the activity (C,D) and expression (E,F) of caspase-3 and caspase-7 were detected by immunoblotting. GAPDH was used as a loading control. Data are expressed as the mean \pm SD. *, $P < 0.05$; n.s., not significant. All experiments were performed in triplicate. WWC3, WW and C2 domain-containing protein-3; EBSS, Earle's balanced salt solution; NSCLC, non-small cell lung carcinoma; siRNA, small interfering RNA; SD, standard deviation.

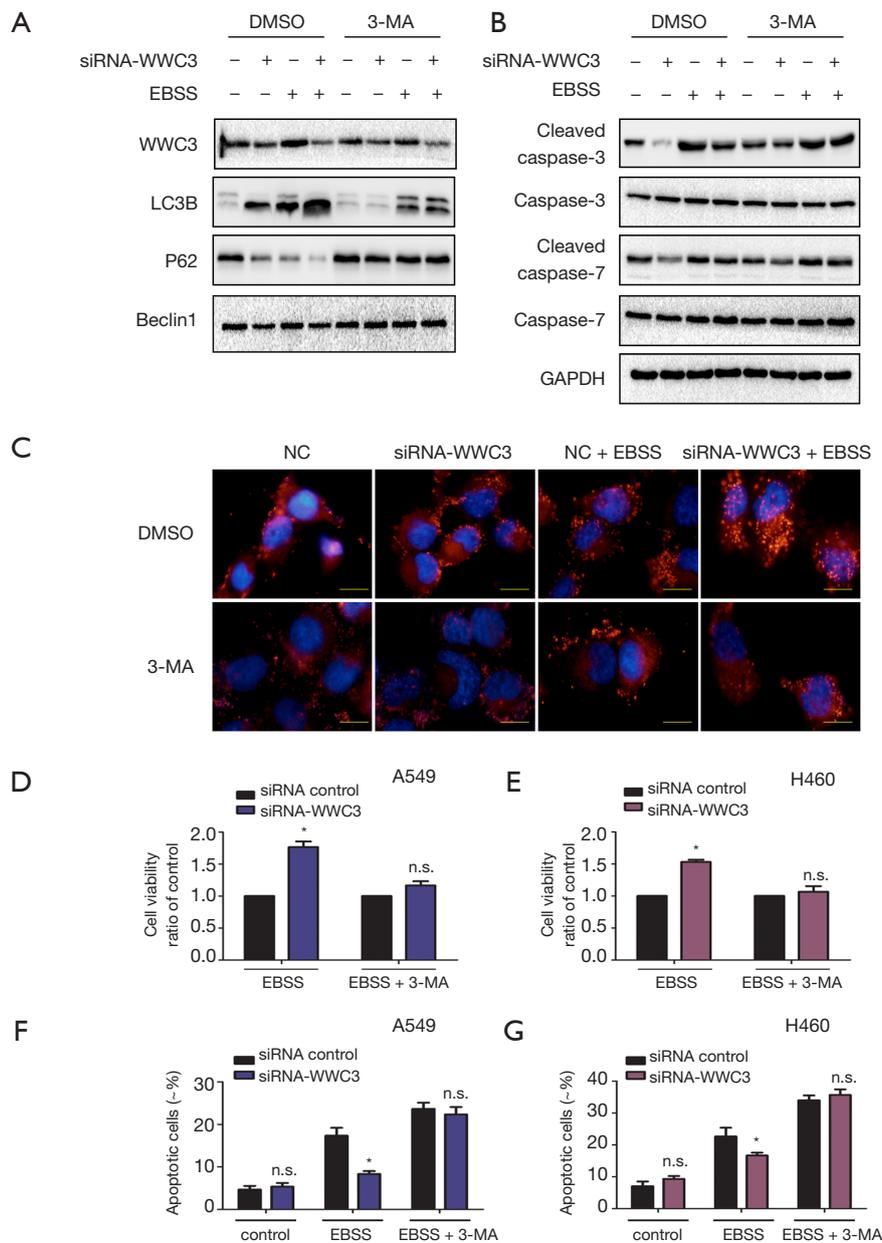


Figure 4 The impact of WWC3 knockdown on cell apoptosis under starvation was attenuated by autophagy inhibition. (A,B) A549 cells were transfected with control siRNA or siRNA-WWC3. After 48 h, the cells were cultured under complete EBSS media with 5 mM 3-MA added for 24 h. Immunoblotting was performed to detect the change in LC3-II transition, Beclin-1, p62/SQSTM1 expression, (A) and the apoptosis-related molecules (B); GAPDH served as a loading control; (C) A549 cells with NC or siRNA-WWC3 were treated under normal media or complete EBSS media with DMSO or 5 mM 3-MA added for 24 h. Endogenous LC3 was detected using immunofluorescence (magnification, 400 \times ; scale bar, 50 μ m). The numbers of LC3 puncta per cell were quantified. Data are expressed as the mean \pm SD. *, $P < 0.05$; (D,E,F,G) A549 or H460 cells transfected with NC or siRNA-WWC3 were treated under normal media or EBSS media with DMSO or 5 mM 3-MA added for 24 h. Cell viability was detected using an MTT assay (D,E); the absorbance of the five repeated samples was measured at 490 nm. Apoptotic cells were measured using flowcytometry (F,G). Data are expressed as the mean \pm SD. *, $P < 0.05$; n.s., not significant. All experiments were performed in triplicate. WWC3, WW and C2 domain-containing protein-3; siRNA, small interfering RNA; EBSS, Earle's balanced salt solution; NC, negative control; 3-MA, 3-methyladenine; GAPDH, anti-glyceraldehyde 3-phosphate dehydrogenase; DMSO, dimethyl sulfoxide; SD, standard deviation.

silenced A549 cells (Figure 4A). We also investigated the impact of 3-MA on apoptosis-related molecules (cysteine-specific proteinases, caspase-3 and caspase-7) after WWC3 knockdown under complete EBSS culture conditions. We found that cleaved caspase-3/7 were significantly downregulated after transfection with siRNA-WWC3 under normal or starvation conditions and that the presence of 3-MA reversed this tendency (Figure 4B). The immunofluorescence assay revealed that treatment with 5 μ M 3-MA for 24 h also reversed the increased LC3 punctate distribution induced by starvation in both the control and knockdown groups (Figure 4C). Moreover, compared with control group, the inhibition of autophagy caused by 3-MA significantly reversed the increase in cell viability under starvation conditions in A549 and H460 WWC3-silenced cells (Figure 4D,E). fluorescence-activated cell sorting (FACS) results showed that treatment with 5 μ M 3-MA for 24 h restored the decreased apoptotic rate of A549 and H460 lung cancer cells induced by WWC3 knockdown (Figures 4F,G,S2). Taking the above results into account, we believe that WWC3 promotes the apoptosis of lung cancer cells induced by starvation, at least partially through the attenuation of autophagy.

Discussion

Autophagy and apoptosis are important physiological processes in cells, and are vital for cell survival and growth (16,17). Autophagy is the key process by which tumorigenesis, tumor stromal interaction, and the effect of tumor therapy are regulated, but it plays a dual and environment-dependent role in tumor inhibition and survival (18). An increasing amount of research has shown that, in some cases, autophagy can protect malignant cells (19-22), while in other cases, autophagy is related to type II programmed cell death (23,24). Understanding and elucidating the molecular mechanism of autophagy, as well as how autophagy can be regulated to kill tumor cells, are still important focuses of tumor therapy. Hence, clarifying the role of WWC3 in autophagy, the role autophagy plays in cell viability, and the mechanisms of autophagy regulation is vital for the development of WWC3-targeted drugs for NSCLC treatment.

The three members of the WWC protein family, KIBRA/WWC1, WWC2, and WWC3, are the upstream molecules of the Hippo signaling pathway (9). Our research and other scholars have shown that WWC3 has an anti-proliferation effect on solid tumor cell lines such as lung

cancer, glioma, and gastric cancer (10,11,25-27). To date, only Jin *et al.* have reported the crucial roles that KIBRA/WWC1 and aPKC perform in regulating starvation-induced autophagy (12), and studies addressing the relationship between WWCs and autophagy are limited. In the present study, we demonstrated that WWC3 served as a negative regulator of autophagy under starvation-induced stress in lung cancer cells, and promoted their apoptosis rate.

First, we found that the expression of WWC3, including at the mRNA and protein levels, markedly decreased in lung cancer cells when cultured in EBSS media. Since the activation of autophagy can promote protein degradation, we then added autophagy inhibitor 3-MA to starvation-conditioned cells. We found that the activation of autophagic degradation of lung cancer cells may not be related to the EBSS starvation-mediated reduction of WWC3. This may be attributed to the inadequate energy intake of lung cancer cells caused by starvation and the obstacle of raw material synthesis.

Subsequently, we examined the effect of WWC3 on the expression of autophagy-related biomarkers, LC3-II and P62, under starvation. The results showed that overexpression of WWC3 could significantly downregulate the expression of LC3-II and inhibit the degradation of the P62 protein. Immunofluorescence also demonstrated that WWC3 overexpression could decrease the number of endogenous LC3 fluorescent spots, which was consistent with the results of the Western blotting. Remarkably, the expression of autophagy-related protein Beclin-1 did not change significantly during this process. This suggests that WWC3 influences autophagy via Beclin-1-independent pathways. However, the specific molecular mechanisms on which WWC3 relies remain unclear.

The WWC protein family members are known to act as classical upstream proteins of the Hippo signaling pathway; they suppress yes-associated protein (YAP) transcriptional activity and trigger Hippo pathway activity. A number of studies have reported the relationship between the Hippo pathway and autophagy (28-30). Whether WWC3 can inhibit autophagy via the Hippo pathway is a question that will be answered in future research.

Finally, we performed FACS assay and Western blotting to demonstrate that WWC3 knockdown could decrease the expression of apoptosis-related molecules such as active caspase-3/7, and hence inhibit the apoptotic rate of starved lung cancer cells. The presence of 3-MA abrogated this effect, indicating that WWC3 enhanced apoptosis of

NSCLC cells caused by EBSS starvation through inhibiting autophagy. It is worth noting that we used siRNA to knock down WWC3 expression to observe the effect of decreased expression of WWC3 on the EBSS-induced apoptosis rate and the apoptosis-related protein expression in A549 cell line. The results showed that EBSS-induced apoptosis could be attenuated by silencing WWC3. Furthermore, we also observed that EBSS could increase the cleaved caspase-3/7 expression in DMSO-treated WWC3 knockdown cells, which may be related to the action time of EBSS. The best time to detect protein changes in the signal pathway with Western blotting is quite different from that for apoptosis assay. In general, the change of protein precedes the appearance of cell phenotype. Therefore, we believe that while the apoptosis-related protein caspase-3/7 can be dramatically upregulated in lung cancer cells after EBSS treatment for 24 h, the degree of apoptosis may not be significantly changed. We also calculated the statistical difference in DMSO-treated WWC3 knockdown cells after EBSS treatment in *Figure 4F*, and in fact, the results showed statistical significance. Combined with the results of *Figure 3E,F*, caspase-3/7 may be closely related to EBSS-induced apoptosis. Undoubtedly, adding caspase-3/7 inhibitor is the most direct way to prove this view. We will attempt to explain and verify this in our future research. To conclude, under tumor cell starvation, by upregulating some mechanisms of WWC3 and combining with 3-MA drugs, it is expected to become a new targeted treatment strategy for lung cancer.

Conclusions

In summary, we demonstrated that WWC3 promoted EBSS starvation-induced apoptosis of NSCLC cells via inhibiting autophagy. 3-MA can potentially be applied in lung cancer treatment strategies to sensitize cells and resist WWC3-targeted therapy by blocking the downregulation of WWC3-mediated protective autophagy.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/jtd-20-966>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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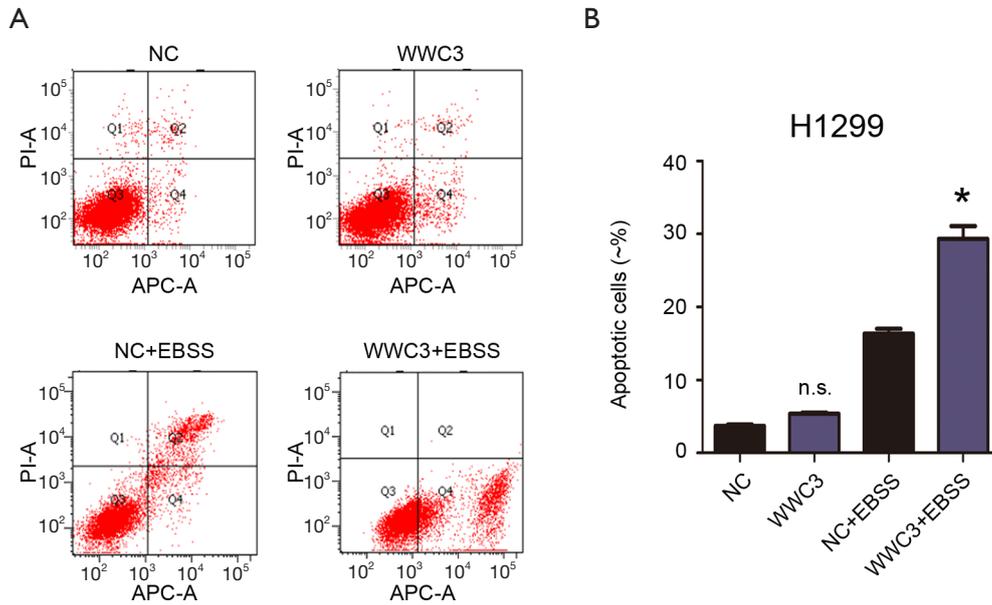
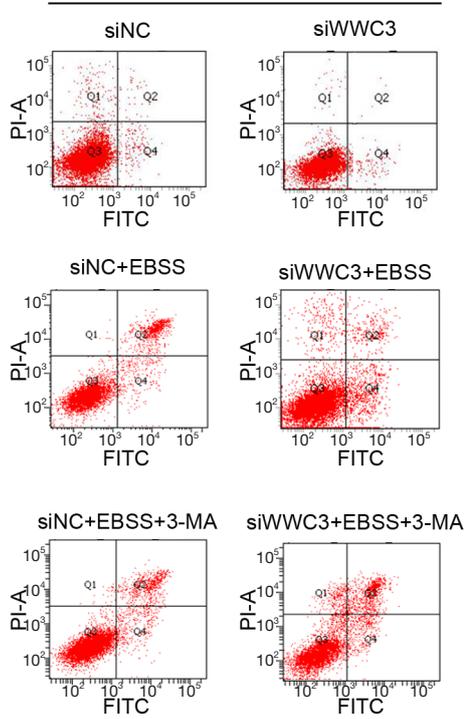


Figure S1 The impact of ectopic WWC3 overexpression on the apoptosis rate of lung cancer cells. (A,B) H1299 cells were transfected with empty vector (or NC) or GFP-WWC3. After 24 h, the cells were treated under normal media or EBSS media for a further 24 h. Apoptotic cells were measured using flowcytometry. Data are expressed as the mean \pm SD; *, $P < 0.05$; n.s., not significant. WWC3, WW and C2 domain-containing protein-3; NC, negative control; EBSS, Earle's balanced salt solution; SD, standard deviation.

A

A549



B

H460

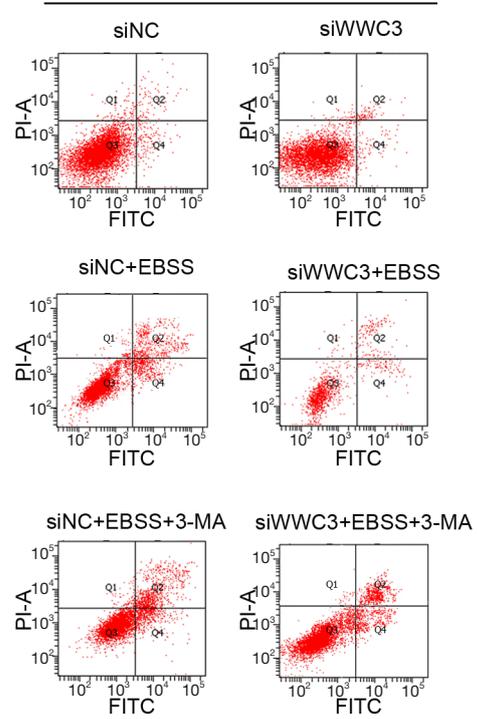


Figure S2 The impact of WWC3 knockdown on cell apoptosis under EBSS treatment was attenuated by 3-MA. (A,B) The representative apoptotic cytograms are shown in *Figure S2*. A549 or H460 cells were transfected with NC or siRNA-WWC3 for 48 h, and then treated under normal media or EBSS media with DMSO or 5 mM 3-MA added for 24 h. Apoptotic cells were measured using flow cytometry. WWC3, WW and C2 domain-containing protein-3; EBSS, Earle's balanced salt solution; 3-MA, 3-methyladenine; NC, negative control; siRNA, small interfering RNA; DMSO, dimethyl sulfoxide.