Ubiquitin-conjugating enzyme UbcH10 promotes gastric cancer growth and is a potential biomarker for gastric cancer

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Abstract. Gastric cancer is a fatal disease and the availability of early diagnostic methods is limited. There is an urgent need to identify effective targets for early diagnosis and therapeutics. UbcH10 is a ubiquitin-conjugating enzyme with high expression in various types of cancers. In the present study, several gastric tumor cell lines with high or low expression of UbcH10 were exploited to study the role of UbcH10 in gastric cancer. Knockdown of UbcH10 expression using siRNA in gastric cancer cell lines with high expression of UbcH10 resulted in reduced proliferation, increased cisplatin-induced apoptosis and reduced serum-induced ERK, Akt and p38 phosphorylation signaling. In agreement, overexpression of UbcH10 in gastric cancer cell lines with low expression of UbcH10 led to enhanced cell proliferation and resistance to cisplatin-induced apoptosis. Most importantly, IHC analyses showed that the UbcH10 protein was expressed at a high level in most patient gastric cancer tissues, but was absent in adjacent mesenchyme tissues. These data suggest that UbcH10 may promote gastric cancer growth and can serve as a biomarker for diagnosis or as a target for novel therapeutics in gastric cancer.

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Introduction

Gastric cancer is a multifactorial and fatal disease, and its overall mortality ranked third among cancer-related deaths worldwide in 2012 (1). The incidence and mortality rate of gastric cancer vary geographically, with the highest in East Asia, including China and Japan (2,3). Currently, surgical resection along with chemoradiation are the standard therapeutic procedures (4,5). More research efforts to identify molecular biomarkers can facilitate early diagnosis or new drug development for gastric cancers. Several critical genes have been discovered to correlate with different types of gastric cancers (intestinal and diffuse types) and have been suggested as potential prognostic biomarkers in previous reviews (6-9).

Ubiquitin-dependent proteolysis is a significance physiological process involved in differentiation, signal transduction and the cell cycle (10,11). Ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3) are the three different enzymes involved in this system. UbcH10 belongs to the E2 gene family coding for a protein of 19.6 kDa and plays an important role in the destruction of mitotic cyclins and cell cycle progression (12-14).

Pulmonary tumors are among the neoplasms with reportedly high levels of UbcH10 and UbcH10 has been shown to play an important role in tumor progression (15). UbcH10 was found to be a useful target for diagnosis and therapy in a subsequent study (16). UbcH10 also presents a high expression level in anaplastic thyroid carcinomas (17), endometrial malignant neoplasms (18), nasopharyngeal carcinoma (19), liver cancer (20), breast (21) and colon carcinoma (22,23), and brain metastasis (24). High expresssion of UbcH10 is correlated with cell invasion and tumor node metastasis (TNM) stage in some of the above-mentioned cancer types. Furthermore, knockdown of UbcH10 expression by RNA interference significantly reduced the proliferation of cancer cells (17,25) and enhanced cell apoptosis *in vitro* (26).

To date, there is no study concerning UbcH10 and gastric carcinoma. To investigate this, two gastric cancer cell lines with a high UbcH10 expression level, KATO III and SGC-7901,

were selected for gene knockdown experiments. UbcH10 was also transfected into NCI-N87 and HS-746T, two cell lines with low UbcH10 expression and low multiplication capacity. Cell proliferation, apoptosis and cell signal transductions were examined in these cell lines.

To examine the clinical relevance of the findings, paraffin-embedded gastric carcinoma tissue samples from 59 patients were analyzed by immunohistochemistry.

Materials and methods

Clinical samples. Tumor samples from 59 cases of gastric carcinoma were obtained from the Minhang Central Hospital, (Shanghai, China) from 2012 to October 2014. The procedure was performed with patient informed consent and in accordance with the Helsinki Declaration and upon the approval of The Institutional Review Board of Minhang Hospital.

Cell lines. The human gastric carcinoma cell lines used in the present study were SGC-7901, AGS, NCI-N87, HS 746T, MKN-45, KATO III, NCI-SNU-1, SNU-5 and SNU-16 and were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). These cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, glutamine and ampicillin/streptomycin (all from Gibco Laboratories, Carlsbad, CA, USA) in a 5% CO_2 atmosphere. GAX023, GAXC031 and GAX066 are primary tumor cell lines established from PDX models (PDX-PDC; patient-derived xenograft, patient-derived cell line) at Shanghai ChemPartner as previously described (27-29).

Reagents. The anti-UbcH10 (A-650) antibody was purchased from Boston Biochem (Boston, MA, USA). Anti-ERK1/2 (#4695), anti-phospho-ERK1/2 (T202/Y204, #4370), anti-p38 MAPK (D13E1, #8690), anti-phospho-p38 MAPK (Thr180/Tyr182, #9211), anti-Akt (#9272), anti-phospho-Akt (Ser473, #9271), anti-actin (13E5, #4970) and anti-GAPDH (14C10) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Annexin V-FITC Apoptosis Detection Kit I (cat. 556547) was obtained from BD Pharmingen (San Diego, CA, USA). Blocking buffer (PBS), goat anti-mouse (IRDye800CW 926-32210) and goat anti-rabbit (IRDye800CW 926-32211) were purchased from Odyssey. Hoechst 33258 (#861405) and MTT reagents (M2128) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Plasmid construction. The CDS sequences (121-660) of UBE2C transcript variant 1 mRNA (GeneBank NM_007019) were chemically synthesized and cloned into mammalian cell expression vector pLVX-UBE2C-IRES-eGFP.

Transfection of small interfering RNA. Gastric carcinoma cells were transfected using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

siRNA directed against the *UbcH10* transcript with the sequence: 5'-CCUGCAAGAAACCUACUCAdTdT3' antisense, and a scrambled siRNA were obtained from GenePharma (Shanghai, China). *In vitro* transfections were performed using 0.8 μg (54 pM) siRNA in 24-well plates using Lipofectamine 2000 transfection reagent ($1x10^5$ cells/well) following the manufacturer's protocol. Transfection of siRNA was performed at least 5 times for each cell line. Each experiment was repeated at least twice. Non-specific siRNAs (scrambled siRNAs) were used as negative controls.

Western blot assay. Cells were lysed in T-PER tissue lysis buffer containing protease inhibitor (both from Thermo Fisher Scientific, Waltham, MA, USA), and phosphatase inhibitor cocktail (Sigma, St. Louis, MO, USA). Twenty micrograms of protein/sample was loaded on 4-12% SDS-PAGE gels (Life Technologies, Carlsbad, CA, USA). Proteins were separated and transferred onto a nitrocellulose membrane. Membranes were incubated with blocking buffer (LI-COR, Inc., Lincoln, NE, USA) and incubated with primary antibodies overnight, followed by the Alexa Fluor 680-conjugated secondary antibody (Invitrogen). Blots were scanned on the Odyssey system (LI-COR, Inc.). Anti-p-ERK1/2 (Thr202/Tyr204), anti-ERK1/2, anti-Akt, anti-p-Akt (Ser473), anti-p38, anti-p-p38 and anti-cyclin D1 antibodies were purchased from Cell Signaling Technology. Anti-actin (Cell Signaling Technology) or anti-GAPDH antibody (Epitomics, Burlingame, CA, USA) was used as a loading control.

MTT assay. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to study the proliferation of the tumor cells. Cells were plated in 96-well plates at a density of 10^4 cells/well to attach for 24 h and transfected with 50 nM different siRNAs. At 0, 24, 48 and 72 h after treatment, 10 μl of MTT reagent (5 mg/ml; Sigma) was added to each well. After a 4-h incubation at 37˚C, the supernatant was gently aspirated and formazan crystals were dissolved in 100 μl of dimethyl sulfoxide (DMSO). The absorbance of each well was measured by an ELISA plate-reader (Bio-Rad, Hercules, CA, USA) at a wavelength of 570 nm.

Cell cycle and apoptosis analysis. The cell cycle or apoptosis distribution was analyzed by flow cytometry. Cells were seeded into a 6-well plate, followed by transfection of siRNA or overexpression plasmids, and cultivation was maintained for 36 h. For cell cycle analyses, the harvested cells were washed with PBS and fixed with ice-cold 70% ethanol at 4˚C overnight. The fixed cells were resuspended in PBS (containing 0.2 mg/ml RNAse and 10 mM PI) and incubated in the dark for 30 min at 4˚C.

For cell apoptosis analyses, the cells were harvested and washed with PBS and stained with Annexin V and PI staining (Annexin V-FITC Apoptosis Detection Kit; BD Biosciences, San Jose, CA, USA). A FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA, USA) was used for data acquisition and FlowJo (TreeStar, Palo Alto, CA, USA) was used for data analysis.

Immunohistochemistry. Protein cellular distribution of UbcH10 was assessed by immunohistochemical analysis. Formalin-fixed, paraffin-embedded, 6-mm tissue sections were deparaffined. Antigen retrieval was performed by water heating method at 100˚C for 10 min with 0.01 M citrate buffer (pH 6.0). Endogenous peroxidase activity was quenched with methanol-hydrogen peroxide (3%) for

15 min. After blocking with unrelated antiserum, the slides were incubated with the UbcH10 primary monoclonal antibody (Boston Biochem, Cambridge, MA, USA) at 1:200 dilution and incubated overnight at 4˚C. After washing with Tris-buffered saline and Tween-20 (TBST), the sections were incubated with secondary antibodies followed by peroxidase-labeled streptavidin (Dako, Carpinteria, CA, USA) for 20 min at room temperature, and visualized with 3,3'-diaminobenzidine and counterstained with hematoxylin. Unrelated IgG was used instead of the primary antibody as a negative control.

Results

UbcH10 expression in 12 gastric carcinoma cell lines. To investigate the role of UbcH10 in gastric cancer, 12 gastric cancer cell lines (SGC-7901, AGS, NCI-N87, HS-746T, MKN-45, KATO III, NCI-SNU-1, SNU-5, SNU-16, GAXC023, GAXC031 and GAXC066) were examined to determine the UbcH10 expression levels by western blot analyses. Among these cell lines, GAXC023, GAXC03 and GAXC066 are primary tumor cell lines established recently in our laboratory from gastric cancer PDX models (30).

Diverse UbcH10 expression levels were observed in these 12 cell lines, with relative high expression levels observed in KATO III, SGC-7901, GAXC-023 and GAXC-031 cells, and much lower expression levels noted in NCI-N87, HS-746T and NCI-SNU-1 cells (Fig. 1A and B).

Knockdown of UbcH10 expression in gastric tumor cells inhibits proliferation. SGC-7901 and KATO III, both of which express UbcH10 at a high level, were selected for the RNA interference study. siRNA transfection led to 90 and 98% inhibition of UbcH10 expression in the SGC-7901 and KATO III cells, respectively (Fig. 2A).

Then, MTT cell proliferation assay was used to test the effect of UbcH10 knockdown. Cell growth was significantly slower in the UbcH10-knockdown SGC-7901 and KATO III cells when compared with those transfected with scramble RNAs (Fig. 2B).

Flow cytometric analysis with PI staining was then used to study cell cycle distribution. UbcH10-knockdown SGC-7901 cells displayed markedly reduced percentages of S and G2/M phase populations (Fig. 2C).

Inhibition of UbcH10 expression reduces cell fitness. Cell proliferation can be induced by changes in signaling pathways. Cells with or without UbcH10 knockdown were analyzed using western blot analyses. The cells with siRNA transfection were seeded in DMEM without serum overnight. Serum (1%) was then used to stimulate cell signaling.

Phospho-ERK, phospho-Akt and phospho-p38 were attenuated with UbcH10 knockdown in the SGC-7901 cells (Fig. 3A and B). Similar changes were also observed in the same experiment using KATO III cells (data not shown).

The decrease in cell signaling and proliferation may be a sign of reduced cell fitness. To investigate this, the role of UbcH10 was then studied in cisplatin (10 μM)-induced gastric tumor cell apoptosis with flow cytometry using FITC-Annexin V and PI staining. Knockdown of UbcH10 in these 2 gastric cancer cell lines resulted in increases in apoptosis and eventual death following exposure to cisplatin treatment (Fig. 3C).

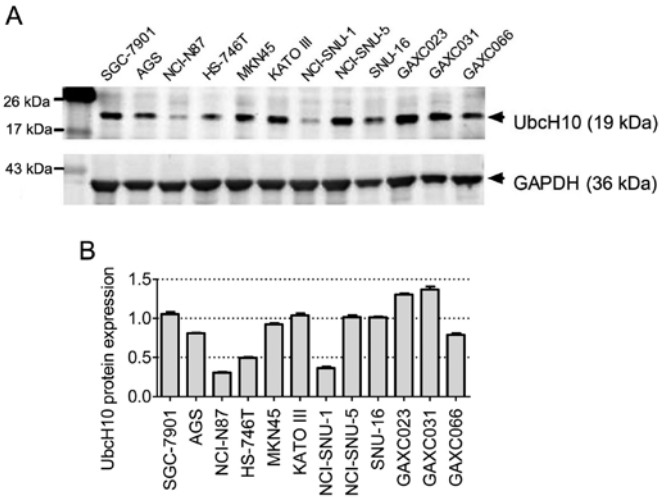

Figure 1. Expression of UbcH10 in 12 gastric carcinoma cell lines. (A) Cell lysates were prepared and analyzed for UbcH10 expression by western blotting. (B) Quantification of UbcH10 protein expression level in 12 cell lines. The ratio of UbcH10 to GAPDH was quantified using software ImageJ and normalized against SGC-7901 which was assigned the expression level of 1.0. Results are representative of 3 independent experiments. Columns, mean (n=3); bars, mean ± SD.

The data suggest that knockdown of UbcH10 in gastric cancer cells reduced tumor cell proliferation, serum-induced signal transduction and increased susceptibility to cisplatin-induced cell death.

Overexpression of UbcH10 in gastric tumor cells promotes cell proliferation and inhibits apoptosis. To confirm these findings, we constructed the UbcH10 expression plasmid for the overexpression studies in the NCI-N87 and HS-746T cells, Two of the gastric cancer cell lines showed low UbcH10 expression (Fig. 1).

The western blot analysis showed the enhanced expression level of UbcH10 in the two cell lines after the transfection (Fig. 4A).

MTT assay was used to test the cell proliferation 24 h after transient transfection of UbcH10. In accordance with the previous gene knockdown data, the overexpression of UbcH10 promoted tumor cell growth (Fig. 4B).

The overexpression of UbcH10 also led to reduced cisplatin-induced cell apoptosis as detected by Hoechst staining and apoptotic nuclei quantification (Fig. 4C).

Immunohistochemical (IHC) analysis of UBcH10 protein expression in gastric carcinoma patient samples. The role of UbcH10 in gastric cancer was supported both by the UbcH10 overexpression and the knockdown studies. Next, clinical gastric cancer patient samples were examined to evaluate the clinical relevance of the findings.

The expression of UbcH10 was examined in 59 cases of confirmed cases of gastric cancers by IHC analysis. Most of the cancer tissues showed different levels of UbcH10 expression at the tumor area; 19 samples displayed clear positive staining

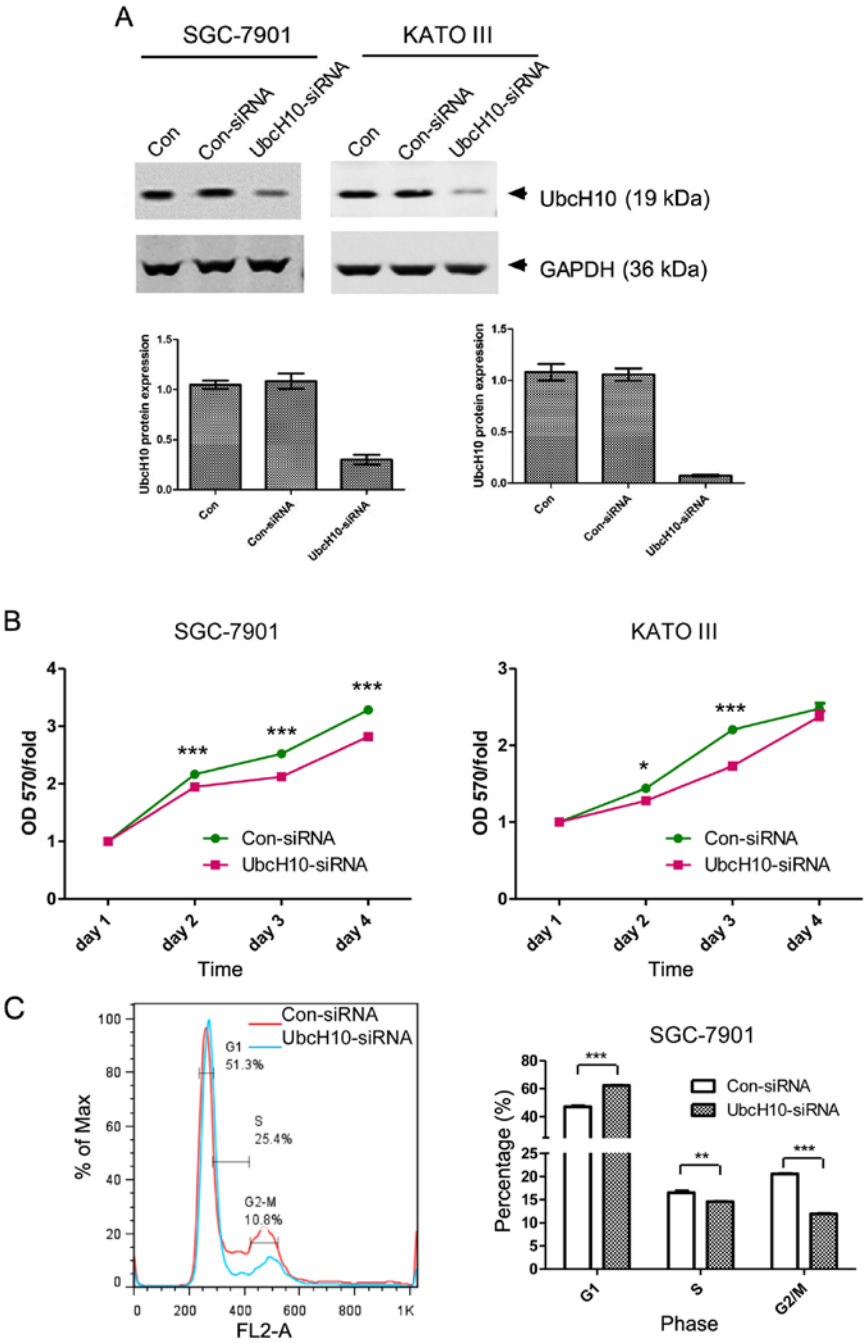

Figure 2. Knockdown of UbcH10 expression by siRNA inhibits cell proliferation and cell cycle progression. (A) Inhibition of UbcH10 expression in SGC-7901 and KATO III cells. Cells were transiently transfected with the scrambled or UbcH10-siRNA using Lipofectamine 2000. Con, untreated cells; Con-siRNA, scrambled siRNA; UbcH10-siRNA, UbcH10 targeted siRNA. Cell lysates were prepared and analyzed for the protein expression of UbcH10 by western blotting 36 h post-transfection. The UbcH10 expression level is presented in the lower panels. The ratio of UbcH10 to actin was quantified using software ImageJ. Columns, mean (n=3); bars, mean ± SD. (B) SGC-7901 and KATO III cells were transfected with scramble or UbcH10 siRNA, respectively, and cell proliferation was analyzed by MTT assay in the subsequent 4 days. Values of optical density (OD) were obtained by the absorbance at the dual wavelengths 570/700 nm (*P<0.05, ***P<0.001; two-way ANOVA with Bonferronni post-tests). (C) SGC-7901 and KATO III cells were harvest in 70% ethanol 36 h after transfection with scramble or UbcH10-siRNA for cell cycle analyses by flow cytometry. Results were analyzed using FlowJo software. Bars, mean ± SD (**P<0.01, ***P<0.001; two-way ANOVA with Bonferronni post-tests).

(score of +), 23 samples displayed strong staining (score of ++) and another 7 samples exhibited very strong staining (score of +++) (Fig. 5). No signals were detected in the neighboring mesenchymal or IgG-negative control. Overall, 49 out of 59 cases (83%) showed significant UbcH10 expression in the cancerous tissues when compared with that noted in the neighboring mesenchyma (Table I).

Discussion

As a critical regulator of cell cycle progression, UbcH10 is considered as a tumor-promoter gene in the conventional view among diverse cancers. As a member of the anaphase promoting complex or cyclosome (APC/C), UbcH10 not only regulates and controls the cell cycle (31), but also takes part in

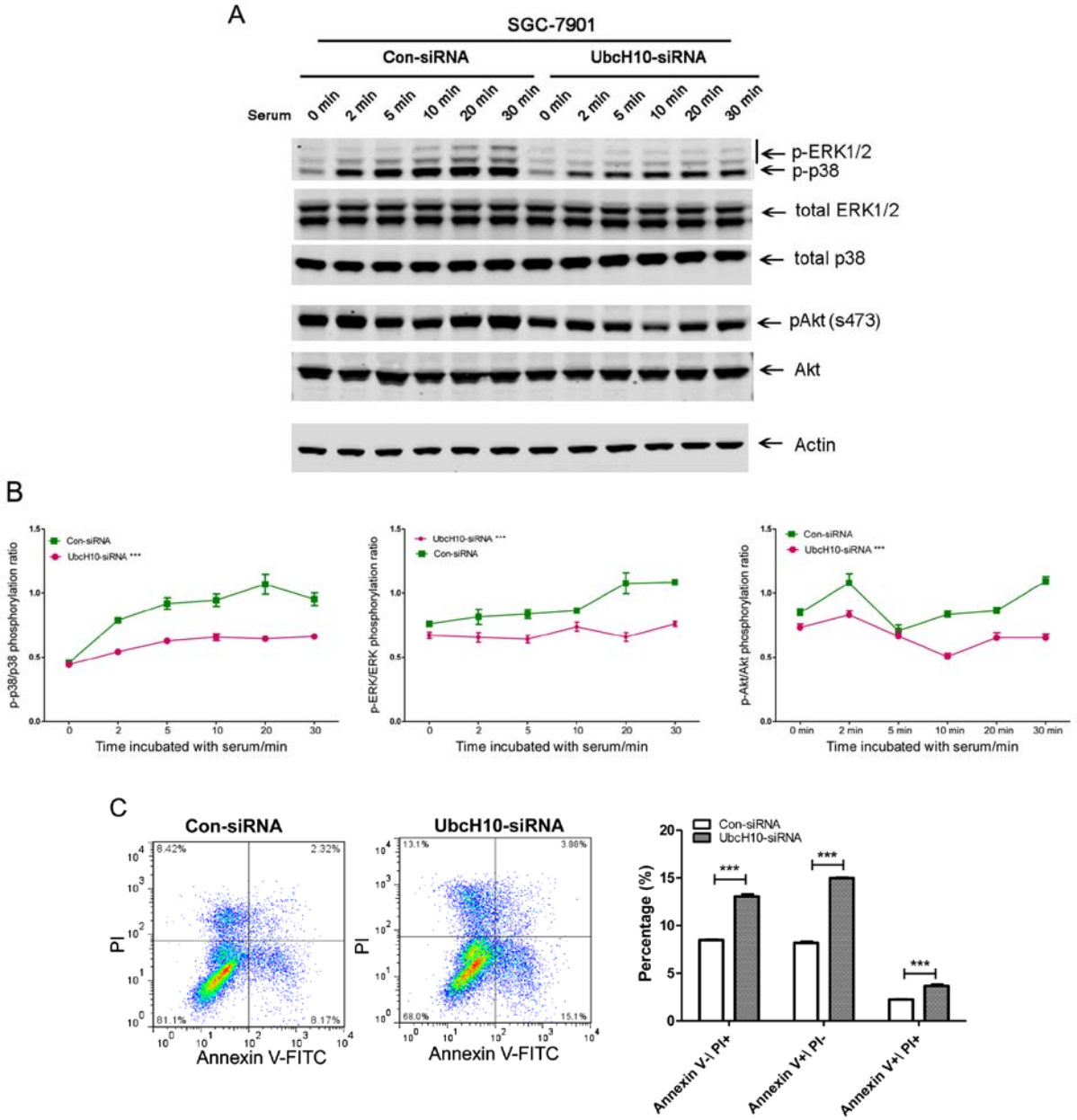

Figure 3. Signaling pathways and promotion of apoptosis. (A) SGC-7901 and KATO III cells were starved in serum-free medium overnight after transfection with siRNAs for 24 h. Cells were harvested for western blot assay after stimulation with 1% serum and harvested at the time points indicated. (B) Quantification of, p38, ERK and Akt phosphorylation in the SGC-7901 cells. Ratios of phosphorylated protein to total protein were analyzed with ImageJ software. ***p<0.0001 compared to con-siRNA; two-tailed ANOVA. (C) KATO III cells were transfected with scramble or UbcH10-siRNA and cultured for 24 h, and then were treated with 10 μM cisplatin for 24 h before analyses of apoptosis/necrosis using flow cytometry. Results were analyzed using FlowJo software. ***p<0.0001 compared to con-siRNA; two-way ANOVA with Bonferronni post-tests.

initiation, progression and transformation (15) and significantly shows increased expression in HCC and other tumor tissues. There are no reports concerning the relevance of UbcH10 in gastric carcinoma. Thus, we utilized several gastric carcinoma cell lines for UbcH10 knockdown and overexpression studies.

In the present study, we found that inhibition of UbcH10 expression suppressed cell proliferation and altered the cell to become more sensitive to apoptosis accompanied by cell cycle change and cell survival signaling attenuation. In agreement with these data, the overexpression of UbcH10 promoted cell proliferation and resistance to cisplatin-induced apoptosis. UbcH10 was also overexpressed in 3 PDX-derived primary cell lines established in our laboratory. These cell lines had only limited passages *in vitro* and may be more closely related to current clinical diseases than traditional cancer cell lines.

More importantly, significant expression of UbcH10 protein was detected in patient tumor tissues, but not in adjacent mesenchyma among 83% of the gastric carcinoma patients (49 out of 59). In addition, with a limited data set, we also noted that the highest UbcH10 expression was also correlated with poorly differentiated cancer, suggesting that UbcH10 expression may be associated with poor prognosis. This issue may require future patient follow-up studies or retrospective analyses of a large volume of clinical data and

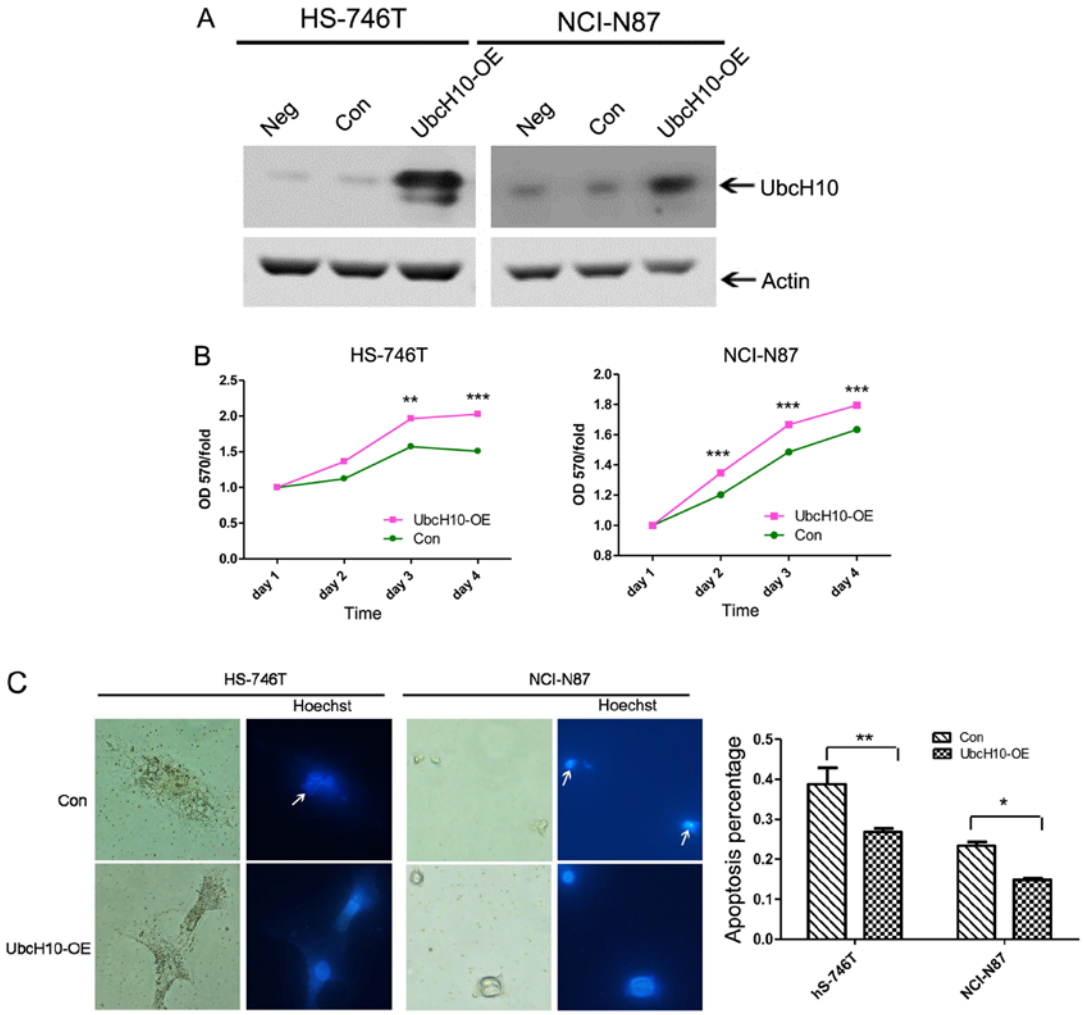

Figure 4. Overexpression of UbcH10 induced by plasmid transient transfection promotes cell proliferation, cell cycle and inhibits apoptosis. (A) FLVX-GFP-IRES-UbcH10 was transiently transfected into HS-746T and NCI-N87 cells, respectively, as UbcH10-OE; cells treated with FLVX-GFP-IRES empty plasmid as well as untreated cells were used as negative controls. (B) HS-746T and NCI-N87 cells were transfected with different plasmids, and cell proliferation was analyzed by MTT assay in the subsequent 4 days. Values of optical density (OD) were obtained by the absorbance at the dual wavelengths 570/700 nm. Bars, mean ± SD (**P<0.01, ***P<0.001; two-way ANOVA with Bonferroni post-tests). (C) HS-746T and NCI-N87 cells transfected with FLVX-GFP-IRES-UbcH10 or control plasmids were treated with 10 μM cisplatin for 24 h. Cells were fixed with 4% paraformaldehyde followed by Hoechst 33258 staining. The images were captured by fluorescence microscope, and quantification is presented in the right panels. Results are representative of 3 independent experiments. Columns, mean (n=3); bars, mean ± SD (*P<0.05, **P<0.01; t-test).

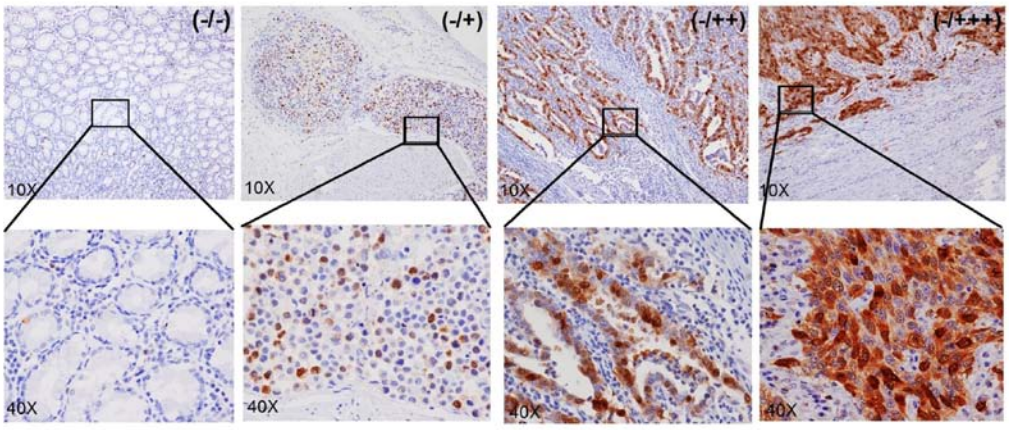

Figure 5. UbcH10 presents a high expression level in gastric carcinoma, but not in adjacent mesenchyma. Upper panels, magnification, x10; lower panels, magnification of x40 of the area shown in the boxes in the upper panels. Left panels, UbcH10 expression level was marked as (-/-) indicating negative for both mesenchyma and tumor area; second panels, UbcH10 was detect in cancer tissue (+) but not in normal mesenchyma tissue (-); third panels, strong UbcH10 expression was detected in cancer tissue (++) and normal mesenchyma tissue was negative (-); right panels, UbcH10 expression level was very strong in cancer tissue (+++) while normal mesenchyma tissues remained negative (-).

Table I. UbcH10 expression in gastric cancer patient tumors and mesenchyme.

Staining of tumors	Staining of neighboring mesenchyme	No. of samples	Percentage of the 59 samples (%)	Accumulative %
+++	-	7	11.86	11.86
++	-	19	32.20	44.06
+	-	23	38.98	83.04
-	-	10	16.95	100.00

samples. If confirmed, Ubch10 expression level can serve as a useful biomarker for diagnosis and prognosis.

Amplifications of certain chromosomal regions have been identified in many types of cancers. The gene *UBE2C* coding UbcH10 was reported to form genomic amplification which led to the increase in UBE2C expression in colon cancer, thyroid carcinoma and prostate cancer (32-34). A *UBE2C* genomic amplification may contribute to the overexpression of UbcH10 observed in at least some of the patient gastric cancer samples and gastric cancer cell lines. A systemic genomic analysis of the patient samples may offer a more definitive answer.

Notably, knockdown of UbcH10 expression in 2 overexpressing cell lines resulted in slightly different responses. In the SGC-7901 cells, such knockdown led to reduced percentages of S and G2M phase cells, while in the KATO III cells, G2/M phases were largely unaffected with changes predominantly in the S phases (data not shown). In addition, the changes in protein phosphorylation were more prominent in the UbcH10-knockdown KATO III cells in the absence of serum stimulation. These data suggest that the exact mechanism of how UbcH10 promotes tumor cell proliferation and resistance to apoptosis also depends on the 'wiring' of the cells, making detailed mechanistic studies more difficult.

Manipulating the ubiquitin pathways in cancer treatment has become one of the current focuses of research, despite that targeting these pathways has been largely premature in the clinic. The tumor promoting role of UbcH10 may make it a valuable therapeutic target.

Overall, our results indicated that overexpression of UbcH10 leads to increased cancer cell proliferation and reduced cancer cell apoptosis in gastric cancer cell lines, in agreement with previous findings in other types of cancers (20,35-38), further validated the tumor-promoting role of UbcH10. These data, and more importantly, its overexpression in gastric cancer patient samples while its absence in adjacent mesenchyma, suggest that UbCH10 may represent a potential biomarker for the diagnosis and prognosis or a potential therapeutic target in gastric carcinoma.

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