Ursolic acid inhibits the metastasis of colon cancer by downregulating ARL4C expression

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Abstract. Ursolic acid (UA), a natural pentacyclic triterpenoid, is known to exhibit various biological activities and anticancer effects. However, the underlying anticancer mechanism is not fully understood to date. The present study aimed to investigate the antimetastatic effect of UA through ADP-ribosylation factor like GTPase 4C (ARL4C) in colon cancer. A lung metastasis model of colon cancer in nude mice was established through tail vein injection. A Cell Counting Kit-8 assay was used to investigate the proliferation of colon cancer cells. Transwell assays were used to detect cell migration and invasion. The expression levels of proteins including ARL4C, matrix metallopeptidase 2 (MMP2), phosphorylated (p)-AKT and p-mTOR were measured using western blot analysis. Immunohistochemistry was used to determine the protein expression level in tissues. ARL4C ubiquitination levels were analysed using immunoprecipitation and western blotting. The results indicated that UA inhibits the metastasis of colon cancer *in vivo* and *in vitro*. The expression of ARL4C in human colon cancer tissue was significantly higher than that in adjacent tissues and its high expression level was associated with lymph node metastases and tumour stage. UA treatment significantly decreased ARL4C and MMP2 protein levels and inhibited the AKT/mTOR signalling pathway. Overexpression of ARL4C reversed the inhibitory effect of UA on the invasion and migration of HCT-116 and SW480 cells, as well as the expression and secretion of MMP2 protein. In addition, UA and an AKT signalling pathway inhibitor (LY294002) induced the ubiquitination of the ARL4C protein, which was reversed by a proteasome inhibitor (MG-132). Collectively, it was revealed in the present study that UA served as a novel solution to relieve colon cancer metastasis by inducing the ubiquitination-mediated degradation of ARL4C by modulating the AKT signalling pathway. Thus, UA may be a promising treatment option to prolong the survival of patients with colon cancer metastasis.

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Introduction

Colon cancer is one of the most common gastrointestinal tumours and it is the second leading overall cause of cancer-related mortality (1). Previous studies have revealed that 20-25% of patients with colon cancer present with metastatic disease at the time of diagnosis (2). Although treatments such as surgery, chemotherapy, radiotherapy and immunotherapy can increase the survival rate, metastasis and relapse remain the leading causes of death in most patients with colon cancer (3,4). Thus, it is necessary to understand the underlying molecular mechanisms of colon cancer metastasis and develop new molecular markers and drugs for improving patient diagnosis and treatment.

ADP-ribosylation factor (ARF) like GTPase 4C (ARL4C), a small GTP binding protein, belongs to the ARF subfamily of proteins (5). Previous studies have demonstrated that ARL4C modulates filopodium formation and cell migration (6,7), participates in cytoskeleton rearrangement (8) and intracellular vesicle transport (9). In addition, as the target gene of the Wnt/β-catenin and EGF/Ras signalling pathways, ARL4C is a regulator coordinating carcinogenesis and epithelial tubule formation (10). There is evidence that ARL4C enhances the stem-like characteristics of glioblastoma cells and is markedly increased in patients with high-grade glioma (11). Patients with glioma and low ARL4C levels have longer overall survival and progression-free survival (11). High expression of ARL4C is reported to be a poor prognostic factor in gastric cancer and is related to the depth of tumour invasion and peritoneal metastasis (12). Moreover, ARL4C silencing resulted in cell cycle arrest and increased apoptosis in gastric cancer cells through inhibition of MDM2 protein expression in the p53 pathway (13). The growth of primary and metastatic liver tumours in mice can be effectively reduced by subcutaneous

injection of oligonucleotide drugs targeting ARL4C (14). These findings indicated that targeting ARL4C may be an effective and precise cancer treatment for tumours.

Currently, Chinese herbal medicine is regarded as an important source for the development of new anticancer drugs (15). With its high efficiency and low toxicity, it has gradually become the focus of attention. Ursolic acid (UA) is a triterpenoid compound in natural plants that is widely employed in Traditional Chinese Medicine, such as *Hedyotis diffusa*, *Gardenia* and *Prunella vulgaris*. UA possesses antioxidant, anti-inflammatory and antitumorigenic biological activities (16). In the case of cancer treatment, UA exhibits preventive and therapeutic effects in several different cancer types, including breast cancer (17), lung cancer (18), oral squamous cell carcinoma (19), prostate cancer (20) and colorectal cancer (21). It was reported that UA promoted apoptosis, autophagy and chemosensitivity to gemcitabine in pancreatic cancer (22). Moreover, UA was shown to inhibit the metastasis of prostate cancer to the lung and liver by downregulating the expression of C-X-C motif chemokine receptor 4 (CXCR4) in the prostate tissue of TRAMP mice (23). Mechanistically, the anticancer effect of UA was associated with the inhibition of cell proliferation (24) and invasion (25), reversal of drug resistance (26), induction of apoptosis (27) and regulation of the tumour microenvironment and cancer immunity (28). Concerning colon cancer, several studies have investigated the effects and mechanisms of UA in cell proliferation, angiogenesis and apoptosis (29-31). However, the effect of UA on colon cancer invasion and migration and its corresponding molecular mechanism remains unclear. The present study aimed to investigate the effect of UA on invasion and migration *in vitro* and *in vivo* and revealed the pharmacological effect and mechanism of UA on ARL4C expression. The present study may provide a potential molecular target and novel insights into UA, in the effective treatment of colon cancer metastasis.

Materials and methods

Cell culture and reagents. Human colon cancer cell lines, HCT-116 and SW480, were purchased from the The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences. The cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS (Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 g/ml streptomycin (HyClone™; Cytiva) at 37˚C in a 5% CO_2 incubator. UA (Selleck Chemicals) was dissolved in dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA) and stored at -20˚C. LY294002, rapamycin and MG-132 were purchased from Selleck Chemicals.

Cell proliferation assays. HCT-116 and SW480 cells were seeded in 96-well plates at a density of $1x10^4$ cells/well and incubated overnight at 37˚C. The cells were treated with various concentrations of UA (0, 5, 10, 15, 20, 25, 30 and 40 μmol/l) for 24 or 48 h. Subsequently, serum-free medium containing 10% Cell Counting Kit-8 (Dojindo Laboratories, Inc.) reagent was added and incubated at 37˚C for 2 h. The absorbance was measured at 450 nm with a microplate reader.

Transwell migration and invasion assays. For the migration analysis, HCT-116 and SW480 cells ($5x10^4$ cells in RPMI-1640 serum-free medium) pretreated with different concentrations of UA were evenly seeded in the Transwell upper chamber. A total of 500 μl of medium containing 10% FBS was added to the lower chamber. After 36 h of incubation at 37˚C, the migrating cells were fixed with 4% paraformaldehyde at room temperature for 30 min and stained with crystal violet solution at room temperature for 10 min. The number of cells in three random fields was recorded using a light microscope (Leica Microsystems GmbH). Regarding the invasion analysis, 100 μl Matrigel (BD Biosciences) were placed on the bottom of the Transwell upper chamber in advance and incubated at 37˚C for 6 h. The remaining steps were the same as those for the migration analysis.

Tissue samples. Human tissue samples were obtained from a tissue microarray (cat. no. HCol-Ade060CS-01) provided by Shanghai Xinchao Biological Technology Co., Ltd., including 40 colon cancer tissue samples and 40 adjacent normal tissues. The present study was approved (approval no. PTEC-A-2021-29-1) by the Ethics Committee of Putuo Hospital affiliated to Shanghai University of Traditional Chinese Medicine (Shanghai, China).

Immunohistochemistry. The mouse lung tissue was fixed in 4% paraformaldehyde (Ruibaohe Biotechnology, Inc.) at room temperature for 24 h, and then was dehydrated in an automatic dehydrator. After being embedded in paraffin, the tissue was sliced into 5-μm-thick slices. Subsequently, immunohistochemical staining was performed according to the manufacturer's instructions. The slides were placed in 100% xylene for dewaxing and were then rehydrated in the graded ethanol series (70, 80, 90, 95, and 100%; Shanghai Lingfeng Chemical Reagent Co., Ltd.). For heat-mediated antigen retrieval, the slides were heated at 100˚C for 15 min and 50˚C for 6 min in citrate buffer (0.01 mmol/l, pH 6.0). Tissue sections were incubated with endogenous peroxygenase inhibitor (cat. no. PV-9001; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.) for 10 min at room temperature and subsequently with 5% BSA (cat. no. ST025; Beyotime Institute of Biotechnology) at 37˚C for 30 min. The sections were incubated with primary antibody against ARL4C (1:200; cat. no. ab122025, Abcam) at 4˚C overnight and then incubated with a secondary, horseradish peroxidase (HRP)-labelled antibody at 37˚C for 30 min. DAB staining solution was added (Fuzhou Maixin Biotechnology, Inc.) and the sections were incubated at room temperature for 3 min. Finally, the slides were counterstained with hematoxylin at room temperature for 10 sec, washed with running water, soaked in deionized water for 30 min and dried overnight at room temperature. The slides were observed and images were captured under a light microscope (BX43; Olympus Corporation). Analysis of the pathological changes was conducted using Image-Pro Plus software (v 6.0; Media Cybernetics, Inc.).

Western blot analysis. Cells were lysed with the use of RIPA buffer (Beyotime Institute of Biotechnology) containing a protease inhibitor on ice for 15 min. Cell lysates were subjected to centrifugation at 8,051 x g for 15 min at 4˚C and

the supernatant was drawn. The total protein was quantified using a BCA kit (Beyotime Institute of Biotechnology). A total of 20 μg protein was separated using 10% SDS-PAGE and transferred onto PVDF membranes (MilliporeSigma). The membranes were blocked with 5% non-fat milk at room temperature for 2 h and then incubated with primary antibodies against β-actin (1:5,000; cat. no. ab6276, Abcam), ARL4C (1:1,000; cat. no. ab122025, Abcam) and matrix metallopeptidase 2 (MMP2) (1:1,000; cat. no. ab92536, Abcam), p-AKT (1:1,000; cat. no. 4060S, Cell Signaling Technology, Inc.) and p-mTOR (1:1,000; cat. no. 5536, Cell Signaling Technology, Inc.) overnight at 4˚C. The membranes were washed three times with TBST buffer containing 0.05% Tween-20 (cat. no. ST1726, Beyotime Institute of Biotechnology) and incubated with anti-rabbit IgG, HRP-conjugated secondary antibody (1:1,000; cat. no. 7074, Cell Signaling Technology, Inc.) for 2 h at room temperature. Finally, an enhanced chemiluminescence reagent (Bio-Rad Laboratories, Inc.) was added to visualize the immunoreactive bands. The band intensity was analysed with ImageJ software (version 1.51J8; National Institutes of Health) using β-actin as the loading control.

Construction of the ARL4C lentivirus. To generate the ARL4C-overexpressing cell lines (ARL4C-OE), the 2nd generational system was used in the lentivirus transfection experiment. Briefly, the ARL4C coding sequence was cloned into the GV358 (11.6 kb) lentiviral vector (Shanghai GeneChem Co., Ltd.) to form recombinant plasmids. Subsequently, the recombinant GV358, Helper 1.0 and Helper 2.0 plasmids were co-transfected into 293T cells at 37˚C. The quantity of plasmids, Helper 1.0 (packaging) and Helper 2.0 (envelope) were 20, 15 and 10 μg, respectively. After 72 h, the culture supernatant was harvested, concentrated and purified (82,700 x g for 2 h at 4˚C). The concentration of lentivirus was $1.5x10^9$ TU/ml. The HCT-116 and SW480 cells ($5x10^4$) were then seeded on a 12-well plate wherein cells were transfected with lentivirus containing human ARL4C-Flag cDNA or lentiviral vectors (Ubi-MCS-SV40-puromycin; cat. no. con254; Shanghai GeneChem Co., Ltd.) for 24 h at 37˚C (multiplicity of infection=10). Subsequently, the transfection solution was replaced with complete medium, and the stable strain selection experiment was carried out after culturing for 96 h with puromycin at 37˚C. The concentration of puromycin for induction was 5 μg/ml determined in the preliminary experiment and that for maintenance was 2 μg/ml. As the lentiviral vector contains Flag elements instead of enhanced green fluorescent protein (EGFP), western blot analysis detecting Flag antibody and PCR detecting ARL4C gene expression were conducted for determining the success of the transfection. The time interval between transduction and subsequent experimentation was ~2 weeks. The stable strain expressing ARL4C, and the negative control were named ARL4C-OE and ARL4C-NC respectively.

ELISA analysis. According to the manufacturer's instructions, the secretion of MMP2 in cell supernatant was detected using an ELISA kit (cat. no. orb1494431, Biorbyt, Ltd.).

In vivo model of lung metastasis. The male BALB/c-nude mice (5 weeks old; weighing 15-18 g; n=18) used in the present study were purchased from the Shanghai SLAC Laboratory Animal Co., Ltd. The mice were randomly divided into three groups with 6 mice in each group as follows: Control, UA (10 mg/kg), UA (20 mg/kg). According to institutional guidelines, the mice were raised in a specific pathogen-free level barrier system [22˚C; 50% humidity; 12:12 light/dark cycle; food and water were freely available (*ad libitum*)]. HCT-116 cells ($5x10^6$) were injected into the lateral tail vein of male nude mice to establish the lung metastatic model of colon cancer. After 1 week, the mice were injected intraperitoneally with different concentrations of UA (10 and 20 mg/kg) every other day for 4 weeks. After 6 weeks, all of the mice were sacrificed by cervical dislocation and the lung tissues were fully excised. In addition, the number of lung metastases was measured. The lung samples were processed into paraffin-embedded sections and stained with haematoxylin and eosin (H&E) to visualize the presence of metastasis. All animal experimental protocols were reviewed and approved (approval no. TJ-HB-LAC-2020-92) by the Animal Experimental Ethics Committee of Shanghai Tongfeng Huiji Biological Medicine Technology Co., Ltd. (Shanghai, China).

H&E staining. H&E staining was performed using an H&E Staining Kit (cat. no. G1120, Beijing Solarbio Science & Technology Co., Ltd.). Briefly, the steps of tissue fixation and slicing were same as those described for immunohistochemistry. After dewaxing and rehydrating, the sections were stained with hematoxylin for 2 min and with differentiation solution for 10 sec at room temperature. Subsequently, eosin staining was carried out for 30 sec. Finally, the sections were treated with dehydration, transparency and sealing. Images were captured using a light microscope at the magnification of x40 and x100.

The Cancer Genome Atlas (TCGA) data analysis. TCGA data analysis was performed using the publicly accessible UALCAN database (https://ualcan.path.uab.edu/index.html). The mRNA values of ARL4C were collected from healthy (normal) individuals and colon cancer patients, and the mRNA levels of ARL4C related to lymph node metastasis and tumor staging in patients with colon cancer were analyzed based on the TCGA dataset.

In vitro protein ubiquitination assay. HCT-116 and SW480 cells with ARL4C-OE and ARL4C-NC were treated with LY294002 (15 μmol/l) or UA (20 μmol/l) for 8 h at 37˚C. The cells were lysed with 200 μl Pierce™ IP Lysis Buffer (cat. no. 87787, Thermo Fisher Scientific, Inc.) on ice for 15 min. Subsequently, the samples were immunoprecipitated with anti-Flag magnetic beads (cat. no. B26101, Selleck Chemicals) overnight at 4˚C to pull down ARL4C protein. The immunoprecipitated beads were then washed three times with PBST containing 0.5% Tween-20 for 5 min at room temperature. The beads with 50 μl SDS-PAGE sample loading buffer (cat. no. P0015A, Beyotime Institute of Biotechnology) were then boiled for 5 min at 100˚C. The resulting complexes were subjected to immunoblotting with anti-ubiquitin antibody (1:100; cat. no. ab134953; Abcam).

Statistical analysis. SPSS 21.0 software (IBM Corp.) was used for statistical analysis. All data were obtained from three independently replicated experiments and are expressed as

the mean ± standard deviation. Comparisons between two groups were performed using an unpaired Student's t-test or the Mann-Whitney U test, while comparisons among ≥3 groups were performed using either one- or two-way ANOVA followed by the Fisher's least significant difference (LSD) test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of UA on the proliferation, migration and invasion of colon cancer cells. First, the present study investigated the effect of UA on the proliferation of HCT-116 and SW480 cells. By increasing the UA concentration, the proliferation of HCT-116 and SW480 cells gradually decreased at 24 or 48 h (Fig. 1A and B) and the pharmacological effect of the drug at 48 h was improved compared with that at 24 h. The IC_{50} value of UA for the HCT-116 cell line at 48 h was 20.47 μM while for the SW480 cell line it was 25.18 μM. Thus, three concentrations lower than this dose were selected in the subsequent experiment. Subsequently, Transwell assays were performed to verify the inhibitory effect of UA on the migration and invasion of colon cancer cells. The results revealed that UA inhibited the migration and invasion of HCT-116 and SW480 cells in a dose-dependent manner (Fig. 1C and D). These findings indicated that UA significantly inhibited the proliferation, migration and invasion of HCT-116 and SW480 cells.

UA inhibits the lung metastasis of colon cancer in vivo. The present study aimed to evaluate the effect of UA on colon cancer metastasis *in vivo*. To achieve this, a lung metastatic model of colon cancer was established by injecting HCT-116 cells into the tail vein of male BALB/c-nude mice. The organs of the lung were excised after 6 weeks and the number of lung metastatic tumours was measured. The findings indicated that UA could relieve the weight loss caused by colon cancer on day 42 (Fig. 2A). A significant decrease in the number of lung metastases was observed after administering low (10 mg/kg) and high (20 mg/kg) doses of UA, particularly at 20 mg/kg (Fig. 2B and C). Similarly, the presence of lung metastases from colon cancer was confirmed using H&E staining and a significant improvement in lung metastases was observed after UA treatment (Fig. 2D). Taken together, these results demonstrated that UA could inhibit the lung metastasis of colon cancer *in vivo*.

UA inhibits the expression of ARL4C in colon cancer in vitro and in vivo. A previous study reported that high expression of ARL4C is associated with poor prognosis, depth of tumour invasion and peritoneal metastasis (10). Therefore, UA may inhibit metastasis by targeting ARL4C. First, the present study analyzed the expression of ARL4C in normal samples and samples from patients with colon cancer by employing the UALCAN database from The Cancer Genome Atlas (TCGA). In Fig. 3A, the expression of ARL4C was revealed to be increased in colon cancer tissues compared with normal tissues. Subsequently, the association between ARL4C expression and nodal metastatic status or individual cancer stages was analysed using data from TCGA database. The data indicated that the expression level of ARL4C was positively associated with the number of lymph node metastases (Fig. 3B) and cancer stage (Fig. 3C). To further determine the role of ARL4C in human colon cancer tissue, the protein expression of ARL4C in 40 paired human colon cancer specimens was detected through immunohistochemistry. The results revealed that ARL4C was highly expressed in colon cancer tissues compared with the adjacent tissues (Fig. 3D and E). The effects of UA on the expression of ARL4C in HCT-116 and SW480 cells were then evaluated. Western blot analyses demonstrated that the expression level of ARL4C was significantly decreased after UA treatment (Fig. 3F-H). In addition, the protein expression of MMP2 was also decreased (Fig. 3F-H). Similarly, the effect of UA on the expression of ARL4C in mouse lung metastases was examined. The immunohistochemical results (Fig. 3I and J) revealed that the expression of ARL4C was higher in lung tissues not treated with UA while it was significantly decreased after treatment with high-dose UA. These findings indicated that ARL4C was highly expressed in human colon cancer tissue and was associated with local lymph node metastasis and tumour stage. UA effectively inhibited colon cancer metastasis *in vivo* and *in vitro* and the mechanism may be associated with the inhibition of ARL4C expression.

Overexpression of ARL4C reverses UA-inhibited migration and invasion. To explore whether the UA-inhibited migration and invasion was dependent on the ARL4C protein, ARL4C-overexpressing HCT-116 and SW480 cell lines were constructed using lentivirus transfection. The transfection efficiency was verified using western blot analysis (Fig. 4A-C). Subsequently, Transwell assays revealed that overexpression of ARL4C reversed the inhibitory effect of UA on cell migration and invasion, compared with cells only treated with UA (Fig. 4D and E). To rule out any toxic effect of UA in determining the decrease in cell migration and invasion, the assays were also performed at the 90% viability (IC_{10}) concentration of UA. The results in Fig. S1 showed that low concentrations of UA inhibited cell migration and invasion, while overexpression of ARL4C reversed the inhibitory effect of UA. Western blot analysis revealed that the protein expression of ARL4C and MMP2 in the UA group was lower than that in the ARL4C overexpression group combined with the UA treatment (Fig. 4F and H). As MPP2 is a type of secreted protein (32), the secreted MPP2 was detected using ELISA with the cell supernatant. As expected, UA treatment decreased the secretion of MPP2, which was reversed by ARL4C overexpression (Fig. 4G and I). These findings indicated that the pharmacological effect of UA on colon cancer invasion and migration was mediated through the downregulation of ARL4C protein expression.

UA promotes ubiquitination of the ARL4C protein through the AKT/mTOR pathway. It was reported that the AKT/mTOR pathway regulated the expression of ARL4C in glioblastoma and lung cancer (33,34). Therefore, the present study further investigated whether the inhibitory effect of ARL4C by UA is associated with the inhibition of AKT signalling activation. First, ARL4C protein expression was decreased in the

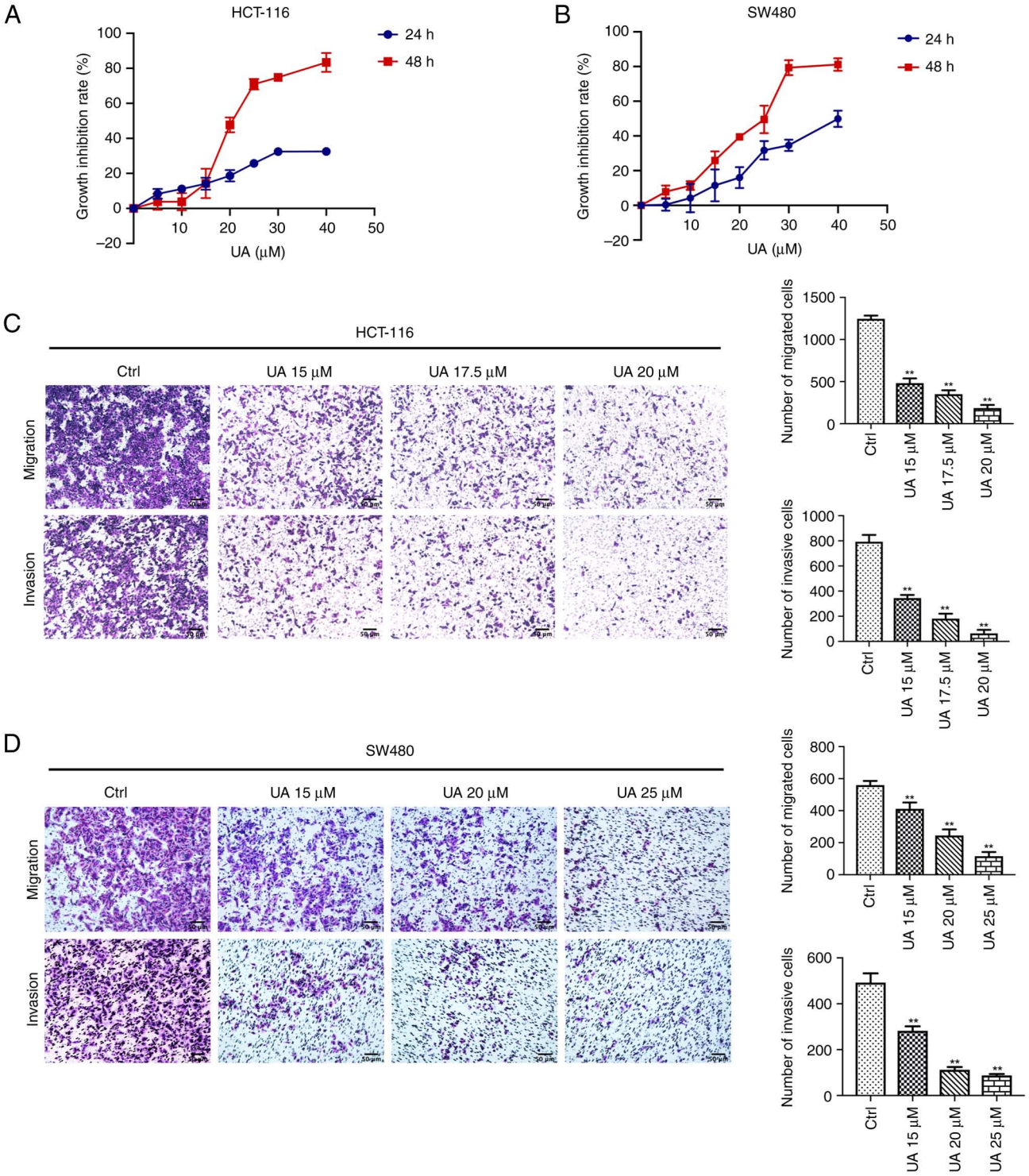

Figure 1. Cell proliferation and metastasis inhibition by UA in colon cancer cells. (A and B) The antiproliferative effect of UA (0, 5, 10, 15, 20, 25, 30, and 40 μmol/l) for 24 and 48 h on HCT-116 and SW480 cells was evaluated using Cell Counting Kit-8 assay. (C and D) Transwell assays revealing the migration and invasion abilities of HCT-116 and SW480 cells treated with UA for 48 h. **P<0.01 vs. the control group. UA, ursolic acid; Ctrl, control.

presence of the AKT inhibitor, LY294002 (Fig. 5A-C), or the mTOR inhibitor, rapamycin (Fig. 5D-F). In addition, UA significantly inhibited the phosphorylation of AKT and mTOR in HCT-116 and SW480 cells (Fig. 5G-J).

However, the mechanism by which AKT regulates ARL4C expression still requires investigation. As the stability of ARL4C is controlled by ubiquitination in hippocampal morphogenesis (35), MG-132, a proteasome inhibitor, was utilized. The results demonstrated that MG-132 reversed the decreased expression of the ARL4C protein caused by UA or LY294002 (Fig. 6A-F). Finally, using an immunoprecipitation experiment, it was observed that the ubiquitination level of ARL4C was increased after the administration of UA or LY294002 in HCT-116 and SW480 cells (Fig. 6G). Overall, these findings indicated that UA increased the ubiquitination of ARL4C by inhibiting the AKT pathway.

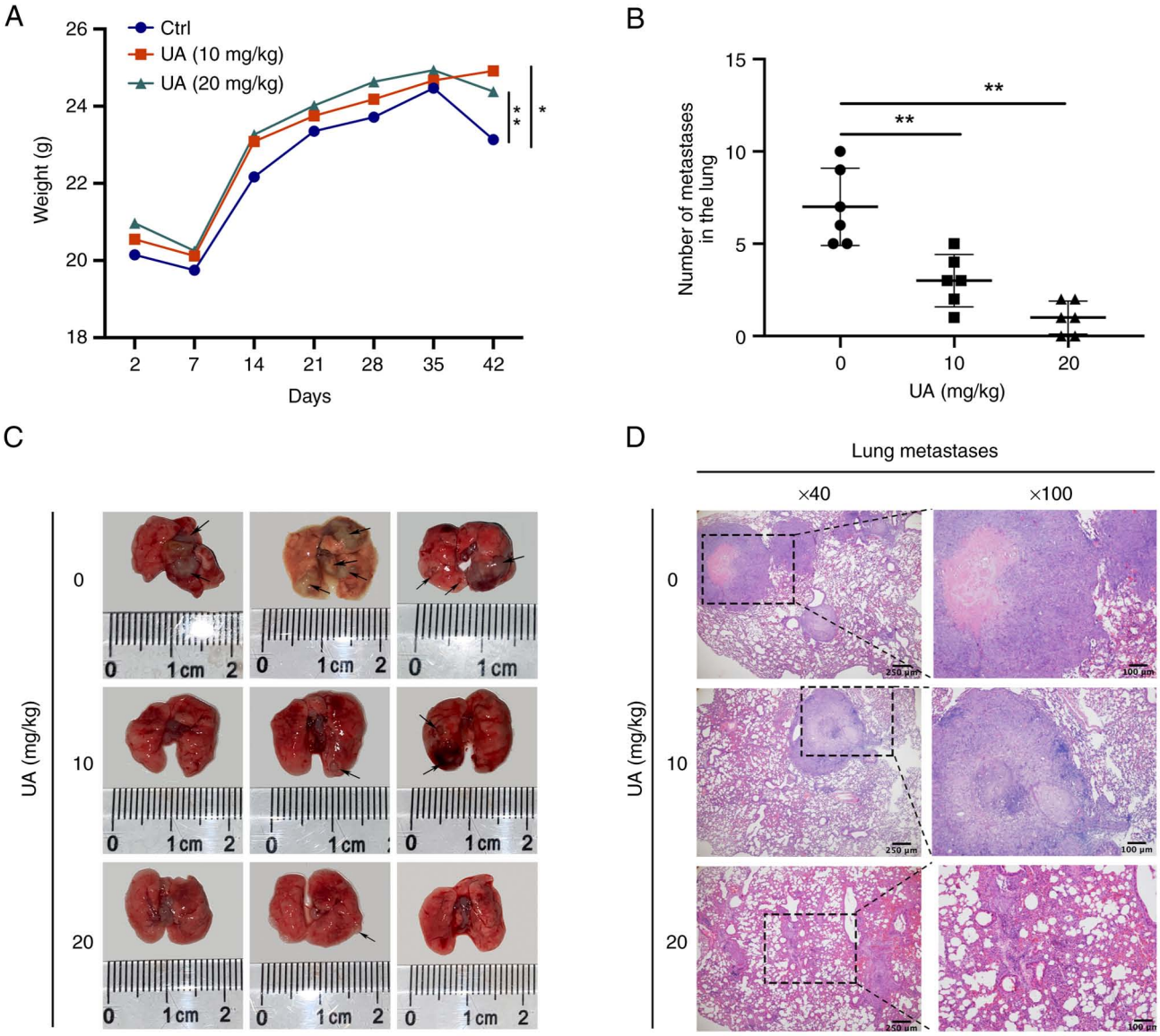

Figure 2. UA inhibits the lung metastasis of colon cancer *in vivo*. (A) Growth curve of the body weights of the nude mice. (B) Statistical analysis of the number of lung metastases 6 weeks after tumour cell inoculation. (C) Representative images of lung metastatic tumours in the lung. (D) Lung metastatic lesions were observed using H&E staining. *P<0.05 and **P<0.01 vs. the control group. UA, ursolic acid; Ctrl, control.

Discussion

The occurrence of colon cancer metastasis causes poor prognosis and increased mortality in patients with colon cancer. Therefore, identifying specific molecules and highly effective drugs is key to improving the survival rate. The present study demonstrated that UA significantly inhibited the migration and invasion of colon cancer cells by inhibiting the AKT signalling pathway and increasing the ubiquitination of the ARL4C protein.

Several previous studies have confirmed that ARL4C is involved in tumour growth and metastasis (36,37). The function and molecular mechanism of ARL4C have been revealed to be significantly different in different tumour types. For instance, ARL4C recruits IQ motif containing GTPase activating protein 1 (IQGAP1) and its downstream effector MMP14 to invasive pseudopodia in pancreatic cancer cells, which induces extracellular matrix degradation. This process is an important condition for the invasion of pancreatic cancer cells (38). Another study demonstrated that the downregulation of ARL4C significantly inhibits the proliferation, migration and invasion of clear cell renal cell carcinoma cells. Moreover, ARL4C promoted the progression of clear cell renal cell carcinoma and the occurrence of epithelial-mesenchymal transformation (EMT) (39). By contrast, other studies have showed that ARL4C is a tumour suppressor in ovarian and breast cancer and low expression of ARL4C was associated with poor prognosis in these patients (40,41). The present study revealed through TCGA database analysis that ARL4C was highly expressed in patients with colon cancer compared with healthy individuals. Moreover, the high expression of ARL4C was closely associated with colon cancer metastasis. Subsequently, the expression level of ARL4C was assessed in human colon cancer tissues. The findings of the present study revealed that the expression of ARL4C was higher in colon cancer tissues than in paracancerous tissues. This is consistent

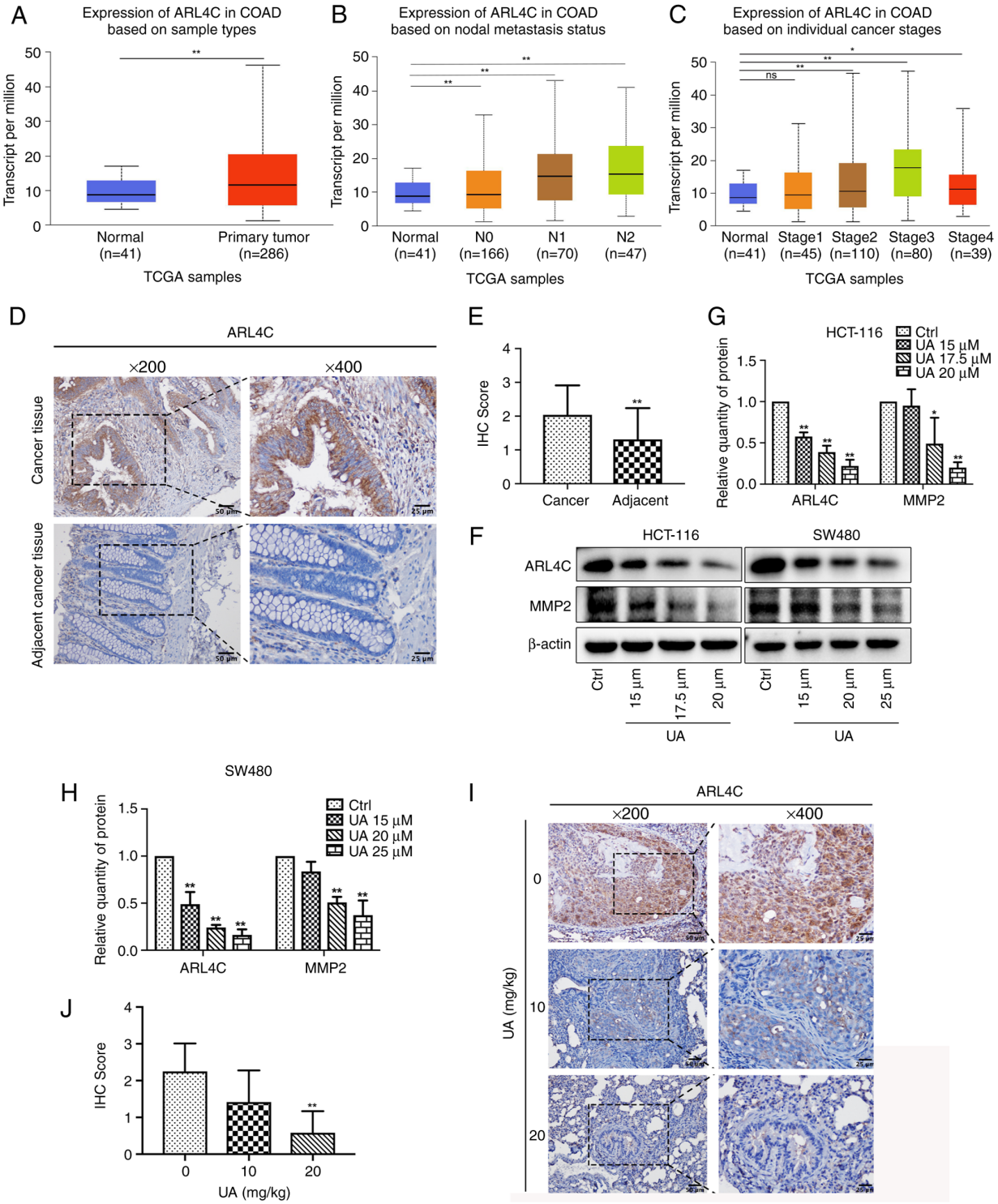

Figure 3. UA inhibits the expression of ARL4C in colon cancer *in vitro* and *in vivo*. (A) Expression of ARL4C in COAD based on sample types from the UALCAN database. (B) Expression of ARL4C in COAD based on nodal metastatic status from the UALCAN database. (C) Expression of ARL4C in COAD based on individual cancer stages from the UALCAN database. (D) Immunohistochemical assessment of ARL4C levels in human colon cancer tissues and adjacent tissues. (E) The average score of ARL4C immunostaining in colon cancer tissues and adjacent tissues. (F) Western blot analyses of ARL4C and MMP2 protein expression after treatment with various concentrations of UA for 48 h in HCT-116 and SW480 cells. (G and H) Relative quantity of ARL4C and MMP2 proteins in HCT-116 and SW480 cells. (I) Immunohistochemical assessment of ARL4C levels in lung metastatic tissues of colon cancer in mice treated with UA. (J) Average score of ARL4C immunostaining in mouse lung metastatic tissues. *P<0.05 and **P<0.01 vs. the control group. UA, ursolic acid; ARL4C, ADP-ribosylation factor like-4C; COAD, colon adenocarcinoma; MMP2, matrix metallopeptidase 2; TCGA, The Cancer Genome Atlas; IHC, immunohistochemical; Ctrl, control; ns, not significant.

with the results revealed in a study by Chen *et al* (42). In addition, the present study demonstrated that overexpression of ARL4C can promote the invasion and metastasis of colon cancer cells and significantly increase the expression and secretion of MMP2 protein. These results indicated that ARL4C may be a potential target for colon cancer therapy.

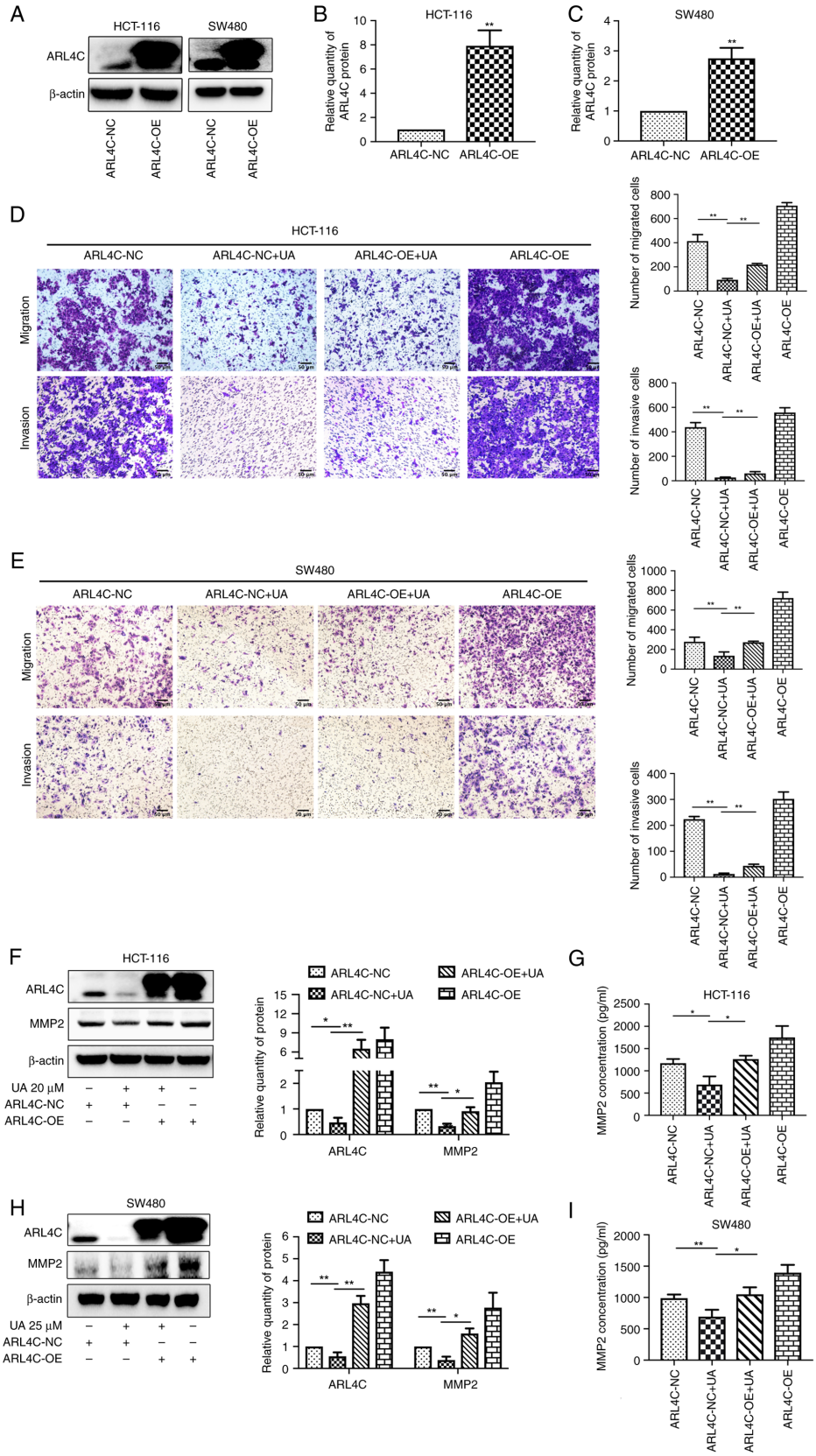

Figure 4. Overexpression of ARL4C reverses the effect of UA. (A) Western blot analyses of ARL4C expression levels after transfection of the ARL4C overexpression lentivirus in HCT-116 and SW480 cells. (B) Relative level of ARL4C protein in HCT-116 cells. (C) Relative level of ARL4C protein in SW480 cells. (D) The migration and invasion of HCT-116 cells treated with or without UA (20 μmol/l) for 48 h after overexpressing ARL4C. (E) The migration and invasion of SW480 cells treated with or without UA (25 μmol/l) for 48 h after overexpressing ARL4C. (F) The protein expression of ARL4C and MMP2 in HCT-116 cells treated with or without UA (20 μmol/l) for 48 h after overexpressing ARL4C. (G) The concentration of MMP2 in HCT-116 cells treated with or without UA (20 μmol/l) for 48 h after overexpressing ARL4C. (H) The protein expression of ARL4C and MMP2 in SW480 cells treated with or without UA (25 μmol/l) for 48 h after overexpressing ARL4C. (I) The concentration of MMP2 in SW480 cells treated with or without UA (25 μmol/l) for 48 h after overexpressing ARL4C. *P<0.05 and **P<0.01 vs. the control group. ARL4C, ADP-ribosylation factor like-4C; UA, ursolic acid; MMP2, matrix metallopeptidase 2; NC, negative control; OE, overexpression.

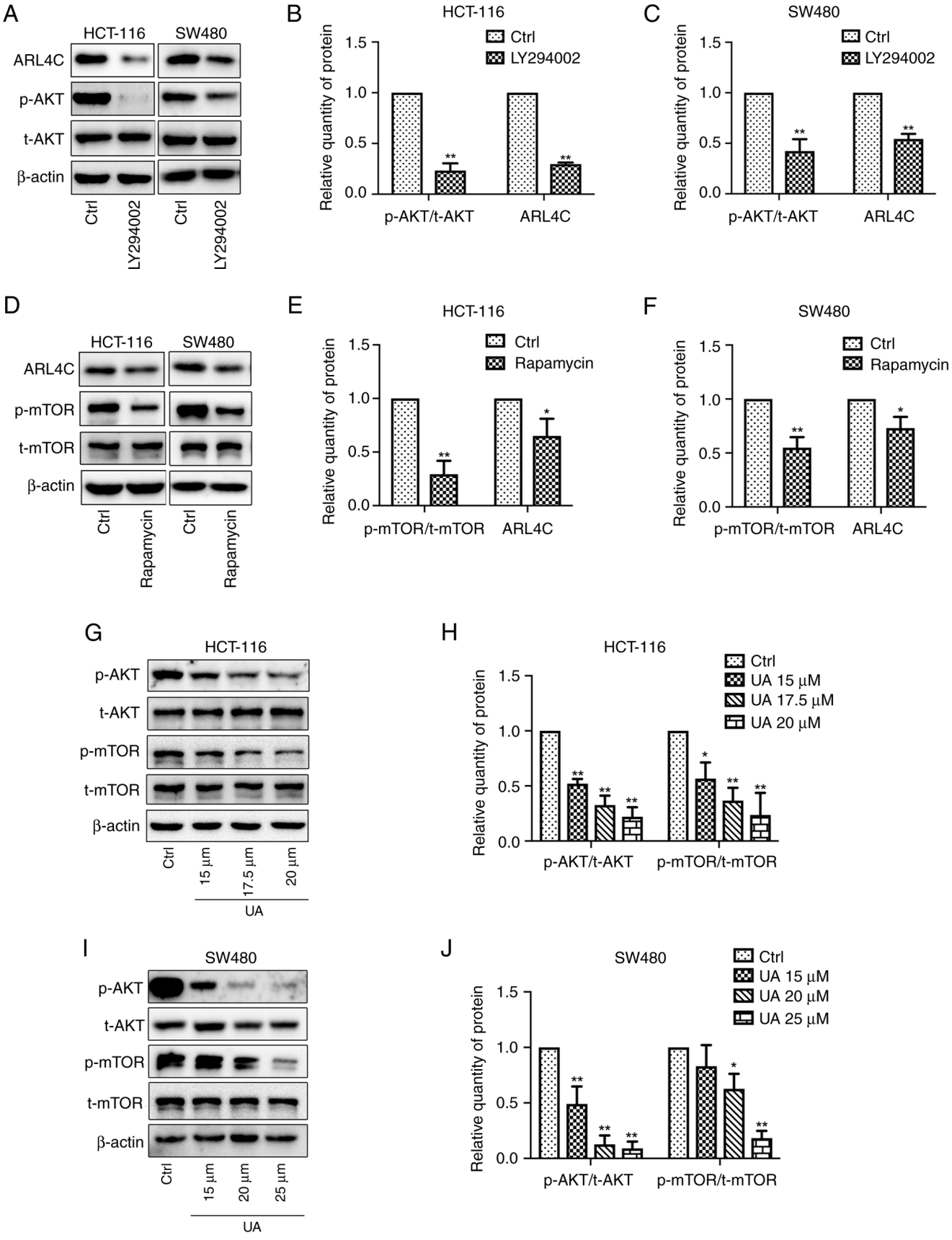

Figure 5. UA inhibits ARL4C protein expression through the AKT pathway. (A) The protein expression of ARL4C and p-AKT in HCT-116 and SW480 cells treated with LY294002 (15 μmol/l) for 24 h. (B and C) Relative levels of ARL4C and p-AKT protein in HCT-116 and SW480 cells. (D) The protein expression of ARL4C and p-mTOR in HCT-116 and SW480 cells treated with rapamycin (50 nmol/l) for 12 h. (E and F) Relative levels of ARL4C and p-mTOR protein in HCT-116 and SW480 cells. (G) The protein expression of p-AKT and p-mTOR in HCT-116 cells treated with UA (20 μmol/l) for 48 h. (H) Relative levels of p-AKT and p-mTOR protein in HCT-116 cells. (I) The protein expression of p-AKT and p-mTOR in SW480 cells treated with UA (25 μmol/l) for 48 h. (J) Relative levels of p-AKT and p-mTOR protein in SW480 cells. *P<0.05 and **P<0.01 vs. the control group. ARL4C, ADP-ribosylation factor like-4C; p-, phosphorylated; UA, ursolic acid; t-, total; Ctrl, control.

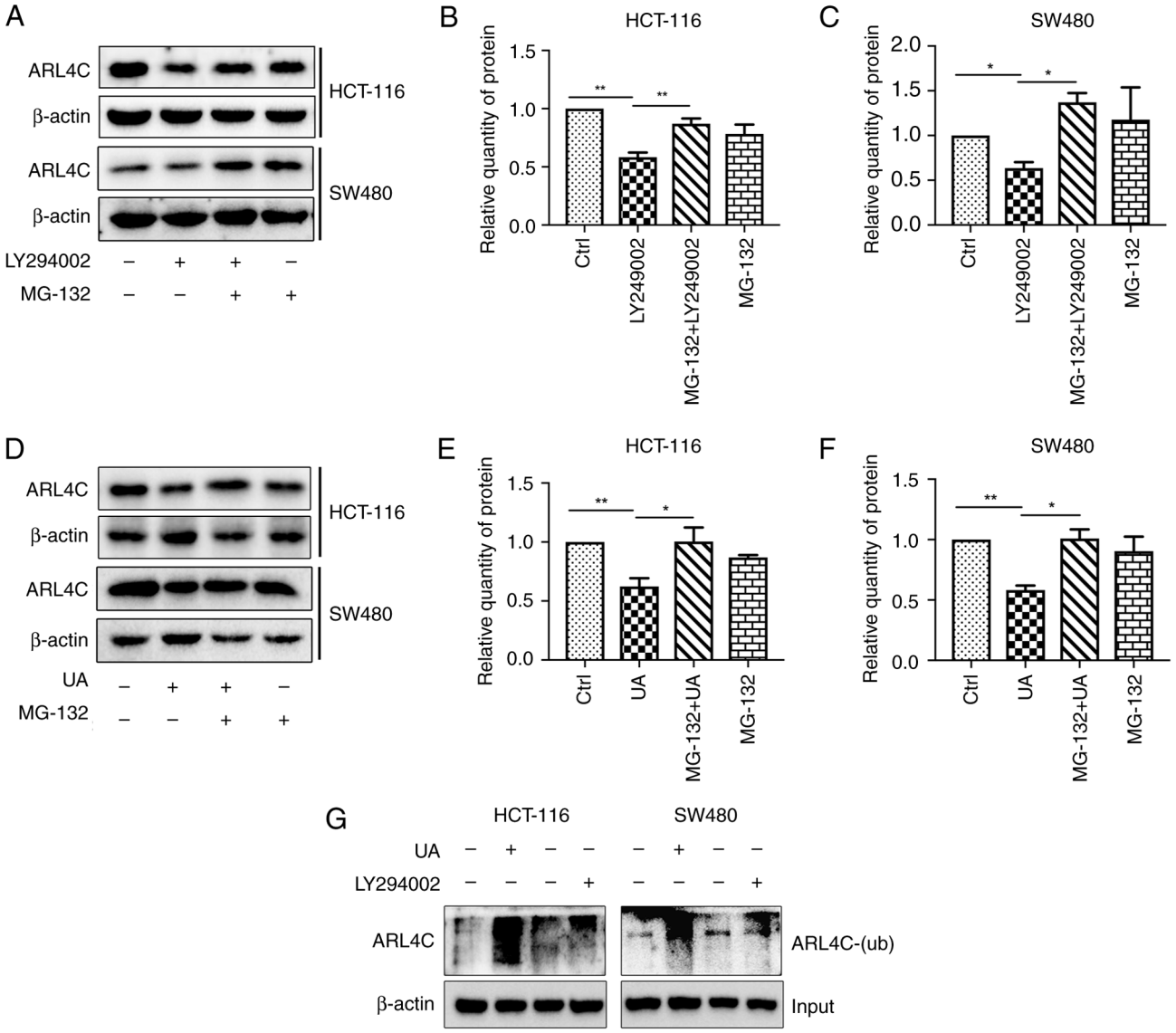

Figure 6. UA promotes ubiquitination of the ARL4C protein through the AKT pathway. (A) Western blot analyses of ARL4C in HCT-116 and SW480 cells treated with or without LY294002 (15 μmol/l) for 24 h after pretreatment with MG-132 (10 μmol/l) for 1 h. (B and C) Relative level of ARL4C protein in HCT-116 and SW480 cells. (D) Western blot analyses of ARL4C in HCT-116 and SW480 cells treated with or without UA (20 μmol/l) for 24 h after pretreatment with MG-132 (10 μmol/l) for 1 h. (E and F) Relative level of ARL4C protein in HCT-116 and SW480 cells. (G) Anti-Flag immunoprecipitation and western blot analysis of ARL4C ubiquitination levels in HCT-116 and SW480 cells treated with UA (20 μmol/l) or LY294002 (15 μmol/l). *P<0.05 and **P<0.01 vs. the control group. ARL4C, ADP-ribosylation factor like-4C; UA, ursolic acid; Ctrl, control; ub, ubiquitinated.

UA is an effective anticancer agent originating from natural sources that is widely distributed in a large variety of traditional medicinal herbs, vegetables and fruits. Previous studies have reported that UA plays a role in cancer cell proliferation, metastasis, apoptosis and angiogenesis, which is due to its wide regulation of intracellular signalling pathways, including Wnt/β-catenin (43), the Hippo signalling pathway (44), the PI3K/AKT pathway (45) and STAT3 (21). For example, UA was revealed to inhibit the proliferation of colon cancer HT-29 cells and induce cancer cell apoptosis by inhibiting the EGFR/MAPK pathway (46). UA can target multiple signalling pathways to inhibit angiogenesis in colorectal cancer (31). The present study investigated the potential anticancer mechanism of UA in colon cancer metastasis both *in vitro* and *in vivo*. To the best of the authors' knowledge, it was determined for the first time that UA significantly suppresses colon cancer metastasis by downregulating the expression of ARL4C *in vivo* and *in vitro*. In addition, the overexpression of ARL4C reversed the inhibitory effect of UA. Thus, decreasing ARL4C expression is one of the mechanisms of action of UA in the treatment of colon cancer metastasis.

The AKT/mTOR pathway is a central node of several signalling pathways and is frequently deregulated in several types of human cancers (47). Activation of the AKT signalling pathway can promote angiogenesis, EMT and metastasis of tumour cells (48). Deng *et al* (49) reported that the activated PI3K/AKT/mTOR signalling pathway can induce EMT and enhance the expression of cancer stem cell markers in ovarian cancer. Zhang *et al* (50) reported that the PI3K/AKT/mTOR signalling pathway is an important regulator of the proliferation, migration and invasion of LoVo colon cancer cells. Previous studies demonstrated that

the regulation of ARL4C expression is involved in multiple carcinogenic signalling pathways, such as Wnt/β-catenin (39), p53 (13), RAF1-MEK/ERK (51) and AKT/mTOR (34). In the present study, AKT or mTOR inhibitors regulated ARL4C protein expression in HCT-116 and SW480 colon cancer cells, which further identifies the critical role of AKT in regulating the ARL4C protein. Moreover, UA significantly inhibited the activation of the AKT/mTOR signalling pathway, which reveals the role of AKT in the antitumour effect of UA in colon cancer. In addition, MG-132 reversed the decreased expression of ARL4C protein caused by UA or LY294002, and UA and AKT signalling pathway inhibitors could induce the ubiquitination level of ARL4C. These data indicated that UA promotes the ubiquitination-mediated degradation of ARL4C through the AKT signalling pathway, which further clarifies the antitumour mechanism of UA.

The present study demonstrated that ARL4C is highly expressed in colon cancer tissues and promotes the occurrence of colon cancer metastasis. The AKT signalling pathway could regulate ARL4C expression and its ubiquitination. Furthermore, UA decreased ARL4C expression and inactivated the AKT signalling pathway, thus inhibiting the migration and invasion of colon cancer cells. Collectively, UA served as a novel solution to relieve colon cancer metastasis by inducing the ubiquitination-mediated degradation of ARL4C by modulating the AKT signalling pathway. UA may be a promising treatment option to prolong the survival of patients with colon cancer metastasis.

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Availability of data and materials

The data used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XK, RW and MZ conceived and designed the project. FX, YS and MZ completed the experiments and acquired the data. YS and RL wrote the article and prepared the images of the figures. QL and QG analyzed the data. MZ and FX confirm the authenticity of all the raw data. All authors read and approved the final version of this manuscript.

Ethics approval and consent to participate

The animal experimental protocols were reviewed and approved (approval no. TJ-HB-LAC-2020-92) by the Animal Experimental Ethics Committee of Shanghai Tongfeng Huiji Biological Medicine Technology Co., Ltd. (Shanghai, China). All procedures performed in studies involving human samples were approved (approval no. PTEC-A-2021-29-1) by the Ethics Committee of Putuo Hospital affiliated to Shanghai University of Traditional Chinese Medicine (Shanghai, China).

Patient consent to publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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