

Overexpression of ATP1B2 promotes cancer cell migration and inhibits apoptosis in patients with esophageal squamous cell carcinoma

FANG-FEI LIU^{1*}, HUI WEN^{2*}, XIAO-BO LIU^{2,3*}, SHENG-BAO LI², SHU JIN², ZI-YE GAO⁴ and QIANG TONG²

¹Department of Intensive Care Unit, Yuebei People's Hospital, Shaoguan, Guangdong 512000, P.R. China;

²Department of Gastroenterology, Taihe Hospital, Hubei University of Medicine, Shiyan, Hubei 442000, P.R. China;

³Hubei Key Laboratory of Embryonic Stem Cell Research, Taihe Hospital, Hubei University of Medicine, Shiyan, Hubei 442000, P.R. China; ⁴Department of Oncology, Taihe Hospital, Hubei University of Medicine, Shiyan, Hubei 442000, P.R. China

Received August 9, 2023; Accepted May 14, 2025

DOI: 10.3892/or.2025.8929

Abstract. The present study aimed to investigate the expression of ATP1B2 in esophageal squamous cell carcinoma (ESCC) and its biological effects. A total of 44 patients with ESCC who underwent surgical resection at Taihe Hospital between December 1, 2017 and December 1, 2018 were enrolled. The expression levels of ATP1B2 in cancerous and adjacent normal tissues were assessed. The present study also examined the associations between ATP1B2 expression and clinicopathological features and patient prognosis. The influence of ATP1B2 on ESCC cell proliferation, migration, cell cycle progression and apoptosis was evaluated using the methylcyclopentadienyl manganese tricarbonyl assay, plate cloning, scratch assay and flow cytometry. Furthermore, the effects of ouabain on these cellular processes were investigated. The results demonstrated that patients with high ATP1B2 expression exhibited significantly shorter overall survival than did those with low ATP1B2 expression (37.3 months vs. 43.1 months; $Z=7.52$; $P<0.05$). ATP1B2 expression, tumor invasion and lymph node metastasis were significantly associated ($P<0.05$). Notably, the overexpression of ATP1B2 correlated with reduced survival rates. ATP1B2 knockdown hindered cell migration and induced apoptosis, whereas ATP1B2 overexpression facilitated migration and impeded apoptosis. Ouabain treatment suppressed proliferation and migration in cells overexpressing ATP1B2 and caused

cell cycle arrest in the G₁/S phase. In conclusion, ATP1B2 overexpression is associated with poor prognosis in patients with ESCC by enhancing cancer cell migration and reducing apoptosis. Ouabain is a potential targeted therapeutic agent for ESCC.

Introduction

Na⁺/K⁺-ATPase plays a crucial role in maintaining the ion balance inside and outside the cell and mediate complex cell signal transduction by binding with the tyrosine kinase Src family joint receptor (1,2). It is composed of the α , β and γ subunit. The α subunit is the catalytic subunit of the enzyme and is involved in all processes of ion transport inside and outside the cell (3). The β subunit has three subtypes: β_1 , β_2 and β_3 . It consists of an extracellular domain and a transmembrane helix. The α subunit interactions jointly maintain the ion balance inside and outside the cell (4). β_1 is expressed in all tissues and cells, β_2 is expressed mainly in the skeletal muscle and nerve cells, and β_3 is expressed in the retina, optic nerve, sciatic nerve, lung and liver (5).

The Na⁺/K⁺-ATPase has been implicated in the occurrence and progression of various cancers. Ouabain, a potent inhibitor of Na⁺/K⁺-ATPase, has shown efficacy in the treatment of heart failure. When combined with low-dose ouabain, Na⁺/K⁺-ATPase can activate intracellular signal transduction without affecting its own function, leading to the apoptosis of cancer cells (3). Ouabain has also been found to promote the phosphorylation of Src, ERK and Akt, inhibit the growth of kidney cancer, breast cancer, and activate the proteasome in breast cancer cells, thereby inhibiting the signal transduction of 17 β -estradiol and inducing apoptosis (6,7). Additionally, ouabain has dose- and time-dependent inhibitory effects on the proliferation and migration of human cervical and pancreatic cancer cells while promotes apoptosis (8). In the present study, the expression of ATP1B2 in patients with esophageal squamous cell carcinoma (ESCC) was evaluated and its association with the clinical pathological indicators was explored. Changes in the proliferation, migration and apoptosis of the ESCC cells were compared before and after

Correspondence to: Professor Qiang Tong or Professor Xiao-Bo Liu, Department of Gastroenterology, Taihe Hospital, Hubei University of Medicine, 32 South Renmin Road, Shiyan, Hubei 442000, P.R. China
E-mail: xinl2012@163.com
E-mail: liuxb1818@163.com

*Contributed equally

Key words: esophageal squamous cell carcinoma, Na⁺/K⁺-ATPase, ouabain, ATP1B2, P53

ouabain treatment and the preliminary role of ATP1B2 in ESCC was investigated.

Materials and methods

Clinical information. Between December 1, 2017 and December 1, 2018, a total of 44 patients who underwent surgical treatment at Taihe Hospital were recruited for the present study. The cohort consisted of 37 male and 7 female patients, with an age range of 43-74 years (median age, 56 years). The inclusion criteria are as follows: confirmation of pathological tissue as ESCC, compliance with the eighth edition of the AJCC International TNM staging criteria for ESCC, no prior radiotherapy, chemotherapy, biological therapy, or immunotherapy, ability to participate in the study, and availability of complete medical records. The exclusion criteria included the presence of other tumors, severe cardiovascular diseases precluding surgery, incomplete clinical and pathological data. The present study was approved (approval nos. 2016KS001 and 2022KS038) by the Ethics Committee of Taihe Hospital (Shiyan, China), and informed consent was obtained from the patients and their families.

Cells and main reagents in the experiment. The esophageal cancer cell line EC109 was purchased from China center for type culture collection (cat. no. GDC0207). The esophageal cancer cell line KYSE150 and Het-1A were purchased from National Collection of Authenticated Cell Cultures (cat. nos. TCHu236 and GNHu51, respectively). The CEC2 cell line was gifted by Professor Li Jinsong from Biomedical Engineering at Beijing Institute of Technology. The main reagents used was the anti-ATP1B2 antibody (Atlas Antibacteries; <https://www.atlasantibodies.com/>). The additional reagents used include DAB staining solution (MaxVision Biology; <http://www.maxim.com.cn/>), Opti MEM and RPMI-1640 medium, the transfection reagent Lipofectamine 3000 (Thermo Fisher Scientific, Inc.), fetal bovine serum (Zhejiang Tianhang Biotechnology Co., Ltd.), TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), puromycin, propidium, ribonuclease A (Guangzhou Saiguo Biotech Co., Ltd.), DMSO (Bioproxx; NeoFroxx), plasmid (Geogene; www.geogene.com), trypsin and PBS (Invitrogen; Thermo Fisher Scientific, Inc.). A reverse transcription kit (Takara Bio, Inc.), methylcyclopentadienyl manganese tricarbonyl (MTT) cell proliferation test kit, Hoechst 333258 (Beyotime Institute of Biotechnology), RT-qPCR kit, DEPC (Tiangen Biotech Co., Ltd.), GoldView nucleic acid dye (Coolpo; <https://www.coolaber.com/>), rabbit anti- β -actin antibody, Goat anti-Mouse IgG (H+L)-HRP, Goat anti-Rabbit IgG (H+L)-HRP (LABLEAD; <https://www.lablead.cn/>), ECL luminescence reagent (Absin) for western blotting, and ouabain (cat. no. HY-B0542; MedChemExpress) were used for the assays.

Immunohistochemical staining. MaxVision was used to detect the expression of the ATP1B2 protein. In the preliminary experiment, ATP1B2 was diluted at 1:250, 1:100, and 1:50 for staining comparison. The best staining effect was achieved at a dilution ratio of 1:100. Accordingly, PBS was diluted to 1:100 according to the manufacturer's instructions. Esophageal tissue was embedded in paraffin, fixed with Carnoy solution

(Ethanol, acetic acid, and trichloromethane mixed in a volume ratio of 6:3:1) at room temperature for 12 h, and sliced with a thickness of 4 μ m. Then, it was dewaxed and hydrated with xylene and gradient ethanol. The slices were placed in ETDA (pH 9.0) antigen repair buffer and repaired with high-pressure steam for 6 min. Then, they were rinsed with distilled water for 1 min. Thereafter, an oil pen was used to mark the area of the tissue to be tested. Finally, the tissue was incubated with 3% H₂O₂ for 10 min to block endogenous peroxidase activity. Endogenous peroxidase blocker of 100 μ l was added to the delineated area of each slice and incubated at room temperature for 10 min. Then, the slices were rinsed three times with PBS solution for 3 min each time. After removing PBS, non-specific antibody blocker of 100 μ l was added to the delineated area of each slice and incubated at room temperature for 10 min, and the slices were rinsed three times with PBS solution for 3 min each time. After removing PBS, the primary antibody (mouse anti-human ATP1B2 polyclonal antibody; 1:100) was added to cover the entire tissue. Incubation was conducted in a 25°C environment for 60 min. Thereafter, the slices were rinsed three times with PBS solution for 3 min each time. After removing PBS, enzyme Goat anti-Mouse IgG (H+L)-HRP of 100 μ l was added to completely cover the tissue. Incubation was conducted at room temperature for 15 min, followed by three rinses with PBS solution for 3 min each time. After removing PBS, freshly prepared DAB color solution of 100 μ l was added and incubated for 3 min at room temperature. The tissue was rinsed with distilled water (tap water) for 1 min and was incubated with 100 μ l hematoxylin staining solution for 10 sec at room temperature until the nucleus was stained dark. Finally, the steps of dehydration, transparency and sealing were completed. All slices were observed, and images were captured under the Leica DM2500 light microscope (Leica Microsystems GmbH).

A total of five areas were randomly selected and imaged under a microscope, and the area and integrated option density (IOD) of the target protein in the immunohistochemical image were calculated using the ImageJ plus 6.0 software (National Institutes of Health). The formula was mean density=IOD/area (9). The cutoff value of ATP1B2 was subsequently determined based on the ROC curve. The highest Jordan index was obtained when the relative expression of ATP1B2 was 2.1. Using the relative expression of ATP1B2 as the critical value, the 44 cases of ESCC were divided into the high-expression group (relative expression ≥ 2.1 ; 28 cases) and low-expression group (relative expression < 2.1 ; 16 cases).

Cell culture, transfection and construction of stable cell line. Immortalized epithelial esophageal cells (Het-1A) and ESCC cells (EC109, KYSE150 and CEC2) were cryopreserved in the laboratory. The cells used in the experiment were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (penicillin 100 U/ml; streptomycin 100 μ g/ml) in a 5%-CO₂ incubator at 37°C.

Based on the test results of qPCR, two cell lines of esophageal cancer EC109 and KYSE150 were selected for subsequent experiments. Before conducting cell transfection experiments, esophageal cancer EC109 and KYSE150 cells were observed under a microscope after being treated with antibiotics and puromycin. The concentrations of the two drugs gradually

decrease in the order of 2, 1.5, 1, 0.5, and 0 $\mu\text{g/ml}$. Based on the standard of culturing for 3 days with the majority of cell death concentrations, the optimal suitable concentration for EC109 cells was selected as 1 $\mu\text{g/ml}$, while the optimal concentration for KYSE150 cells was 0.5 $\mu\text{g/ml}$. Short hairpin (shRNA) was used to silence the ATP1B2 gene. The construction of the plasmid was synthesized and constructed by Shanghai Jikai Gene Chemical Technology Co., Ltd. The interfering sequence of the shRNA of the ATP1B2 gene was shATP1B2: 5'-GAA TGTAAGATGTCGCATCAA21bp-3' (10). Cell transfection experiments were conducted under constant temperature conditions of 25°C. Then, incubation was performed in a 37°C incubator for 72 h. Next, the expression of green fluorescence was examined under a microscope. Finally, a substance containing puromycin of 1 $\mu\text{g/ml}$ was used to screen stable cell line and the maintenance concentration of puromycin is half of the screening concentration.

Cells in the logarithmic growth phase were seeded in a 12-well plate, and the cell density reached 60% within 24 h. The cells were then transfected with 500 ng/well small interfering RNA/plasmids according to the experimental instructions provided by the Lip3000 transfection agent. After transfection, puromycin (0.5 $\mu\text{g/ml}$) was used to screen the cells and establish a stable cell line, with the empty sh-vector served as the negative control group.

Reverse transcription-quantitative PCR (RT-qPCR) and western blot analysis. The ATP1B2 nucleic acid sequence template was queried in the primer design software Primer 6.0 (NCBIGene ID: 482; <https://premiergroup.ca/>), and the primer sequence was synthesized by a manufacturing company. RT-PCR was performed to detect the expression of the ATP1B2 gene following transfection. The primers used are included in Table I. In RT-qPCR, our reagents were purchased from China Tiangen Biochemical Technology Co., Ltd. and relevant research was conducted according to the manufacturer's instructions. Total RNA was extracted from the samples using the TRIzol reagent, after which the RNA concentration and purity were assessed (acceptable 260/280 nm ratio >1.9). The RNA was reverse transcribed into complementary DNA using a reverse transcription kit (HiFi Script CDNA Synthesis Kit) at 42°C for 50 min, followed by incubation at 85°C for 5 min and an infinite cycle at 4°C. Fluorescence-based qPCR was performed using a US BIO-RAD C1000 PCR instrument. For each sample, five replicate wells were used, and the reaction conditions were as follows: 95°C for 10 min, 95°C for 15 sec, and 60°C for 1 min. A total of 39 cycles were carried out for amplification. Gene expression was determined using the $2^{-\Delta\Delta C_q}$ method for data analysis (11,12). This experiment was repeated 6 times.

Western blotting was performed according to the standard protocol. Proteins were extracted using BBproExtra® RIPA Buffer BB-3201 and concentration measured using BCA assay kit. A total of 40 μg of protein was loaded per lane and subjected to SDS-PAGE (10%). Proteins were transferred to a PVDF membrane which was blocked using 1X TBS-T and 10% skimmed milk at room temperature for 1 h. Primary antibodies for mouse anti human ATP1B2 polyclonal antibody (1:1,000; Atlas antibodies), rabbit anti- β -actin (1:3,000; Abcam), rabbit anti-E-cadherin (1:1,000; Abcam),

Table I. Primer sequences used for reverse transcription-quantitative PCR.

Gene name	Primer sequence (5'-3')	Product length, bp
ATP12B	F: GTCCCAAAGCCAGCCGATGT R: GCCGTTCTGTCAACCAAATA	240
GAPDH	F: TCGGAGTCAACGGATTGTTGGT R: TTCCCGTTCTCAGCCTTGAC	181
TP53	F: TGTGACTTGCACGTACTCCC R: ACCATCGCTATCTGAGCAGC	199
RAC1	F: GGTGGGAGACGGAGCTGTA R: AGAACACATCTGTTTGCGGA	212
MMP-7	F: AAGTGGTCACCTACAGGATCGTA R: CTACCATCCGTCCAGCGTTC	286
MMP-10	F: TTTGGCTCATGCCTACCCAC R: CAGGGGGAGGTCCTAGAGA	263

F, forward; R, reverse.

rabbit anti-N-cadherin (1:1,000; Abcam) and rabbit anti-P53 (1:1,000; Abcam) were incubated overnight at 4°C. Proteins were detected with ECL luminescence reagent by Bio-Rad ChemiDoc (Bio-Rad Laboratories, Inc.). This experiment was repeated 3 times. Densitometric analysis was performed using ImageJ 2.0 software (National Institutes of Health).

Measurement of cell migration and repair ability by scratch test. Logarithmic-phase cells were suspended and seeded in a six-well plate. After the cells had adhered to the plate, they were cultured for 12 h to form a uniform monolayer. A 10- μl pipette tip was used to create a vertical scratch perpendicular to the horizontal line. The samples were then rinsed with PBS three times. Serum-free medium was added, and the samples were incubated at 37°C in an incubator with 5% CO₂. Leica's inverted microscope was used in wound healing assays. ImageJ 6.0 software was used to calculate the scratch area and measure cell migration and healing ability. The scratch area of cells was measured at 0, 6, 12 and 24 h. The calculation was cell scar healing rate=(scratch area at 0 h-scratch area at each time point) divided by scratch area at 0 h. This experiment was repeated 3 times.

Detection of cell cycle and apoptosis by flow cytometry. The cell cycle experiment was conducted by centrifuging at 0.8 x g for 5 min at an indoor temperature of 25°C. The logarithmic cells were obtained to create a cell suspension, and the cells were accurately counted. The cell density was adjusted to 20,000 cells/ml, after which a 2 ml/well cell suspension was obtained and inoculated into a six-well plate. The cells in the six-well plate were cultured in an oven at a constant temperature of 37°C under 5% CO₂. When the cell confluence reached 80%, the cells were collected in an Eppendorf tube. Then, 200 μl of precooled PBS and 1 μl of ribonuclease A were added. The cells were incubated in a water bath at 37°C for 30 min. Then,

1 μ l of PI was added, and the cells were placed in a refrigerator at 4°C in the dark for 30 min. Afterward, 300 ml of precooled PBS was added, and the cells were filtered using a machine. The cells were collected following the aforementioned steps. Then, 200 μ l of precooled PBS was added to the suspension cells, and 1 μ l of Hoechst 33258 dye solution was added at a concentration of 1 μ g/ μ l. The solutions were mixed evenly, and the samples were incubated in an incubator at 37°C for 10 min. The dye solution was discarded, and 2 ml of PBS was added to the samples. The cells were rinsed and centrifuged at 0.8 x g for 5 min. These steps were repeated twice. Then, 500 μ l of PBS was added to the cells, and PI was added. The suspension was mixed evenly, incubated at 4°C for 15 min, and filtered. Both the cell cycle and apoptosis were detected on the BD FACSaria III flow cytometer and analyzed by FlowJo software (FlowJo LLC). This experiment was repeated 3 times.

MTT. A blank control group, negative control group, ATP1B2 knockdown group and ATP1B2 overexpression group were established with multiple wells. Cells were transfected in 96-well plates. After 24, 48, 72 and 96 h of treatment, newly prepared 5 g/l MTT culture solution was added to each well under closed light conditions. The cells were routinely incubated in a 37°C incubator for 4 h. The culture plates then removed, and the supernatant was discarded. Then, 100 μ l of DMSO solution was added to each well to dissolve the purple crystals in the cells. The samples were placed on a shaking table and shaken at 100 times per minute for 5 min (5% CO₂ at 37°C). After mixing, a microplate reader was used to detect the absorbance at 570 nm. The experiment was repeated three times. This experiment was repeated 3 times.

Plate cloning experiment. During the logarithmic growth period, single-cell suspension was prepared from EC109 and KYSE150 cells. A total of 100 cells were inoculated in each well of a six-well plate, after which 2 ml of culture medium was added. The mixture was gently shaken and incubated. A total of three wells were used for each group, and the cell status was observed daily. The culture medium was changed as needed. After 11 days of culture, the cells were collected when colonies were visible to the naked eye. The cells were washed with 2 ml PBS twice. Then, in an indoor environment at 25°C, 1 ml of 4% paraformaldehyde was added and cells were fixed for 15 min. Paraformaldehyde was poured out, then rinsing with distilled water three times. Staining was conducted using 1 ml of crystal violet for 15 min at room temperature. Then, wells were rinsed again with distilled water three times. Finally, the cells were placed in a well-ventilated area and dry for 20 min. The number of cloned cells was counted under the microscope. A colony was defined as having >50 cells, and the colony formation rate was calculated using the following formula: Colony formation rate=(number of clones/number of inoculations) x100. This experiment was repeated 3 times.

Statistical analysis. SPSS 25.0 software (IBM Corp.) was used for statistical analysis. Normally distributed data were expressed as the mean $\pm$ standard deviation, while counting data were expressed as cases and percentages. Independent sample t-test were used to compare two groups. Analysis of variance followed by LSD test was used for multiple groups,

and chi-square test was used for comparison between two groups. When the expected frequency in any group was <5, Fisher's exact test was employed. Skewed distribution data are presented as medians (lower quartile, upper quartile). The non-parametric Wilcoxon rank sum test was used for comparisons between two samples, and the Kruskal Wallis H test was used for comparisons among multiple groups. A statistically significant difference was considered at P<0.05.

Results

Expression of ATP1B2 in the ESCC and normal adjacent tissues. The ATP1B2 protein was primarily expressed in the nucleus and stained brown (Fig. 1A). Some proteins are expressed in the cytoplasm and stained light yellow (Fig. 1B). The expression of the ATP1B2 protein in ESCC tissues was significantly greater than that in normal adjacent tissues [3.54 (0.42, 4.89) vs. 1.40 (0.20, 2.39); P<0.01, Table II]. The incidence of lymph node metastasis and invasion of vessels or nerves in patients with ESCC with high ATP1B2 expression was significantly higher than that in patients with low ATP1B2 expression (all P<0.05, Table III). There were no significant differences in sex, age, smoking history, drinking history, tumor stage, or maximum tumor diameter among patients with different ATP1B2 expression levels (P>0.05, Table III). Follow-up was conducted for 44 patients for 4-48 months, for a median follow-up time of 35 months. The survival time of patients with ESCC with high ATP1B2 expression was higher than that of patients with low ATP1B2 expression (P<0.01, Table III). K-M-single-factor survival analysis demonstrated that the survival rate of patients with ESCC with high ATP1B2 expression was significantly lower than that of patients with low ATP1B2 expression (P<0.01, Fig. 1C).

Expression of ATP1B2 in the ESCC before and after shRNA transfection. The expression levels of ATP1B2 in EC109, CEC2 and KYSE150 cells (0.35 $\pm$ 0.12, 0.30 $\pm$ 0.21, and 0.18 $\pm$ 0.10) were significantly greater than those in Het-1A (0.17 $\pm$ 0.12), but the difference was statistically significant only in EC109 (t=-2.59; P=0.028; Fig. 1D). Thus, EC109, a cell line with the highest ATP expression, was targeted for knockdown experiments in esophageal cancer cells, while KYSE150, a cell line with the lowest ATP expression, was selected for overexpression experiments to optimize the intervention's impact on the cells. In EC109 cells, the expression level of ATP1B2 mRNA in the knockdown group was significantly lower than that in the empty-vector group (t=-0.76; P<0.05; Fig. 1E), and the expression level of ATP1B2 protein in the knockdown group was also significantly lower than that in the empty-vector group (t=113.734; P<0.05, Fig. 1G and H). In KYSE150 cells, the expression level of ATP1B2 mRNA in the overexpression group was significantly higher than that in the empty-vector group (t=-2.31; P<0.05; Fig. 1F), and the expression level of ATP1B2 protein in the overexpression group was significantly higher than that in the empty-vector group (t=-9.342; P<0.05; Fig. 1G and I).

Effect of ATP1B2 on the biological behavior of ESCC cells: Migration ability, cell cycle, apoptosis and proliferation. The scratch test revealed that the cell migration ability of

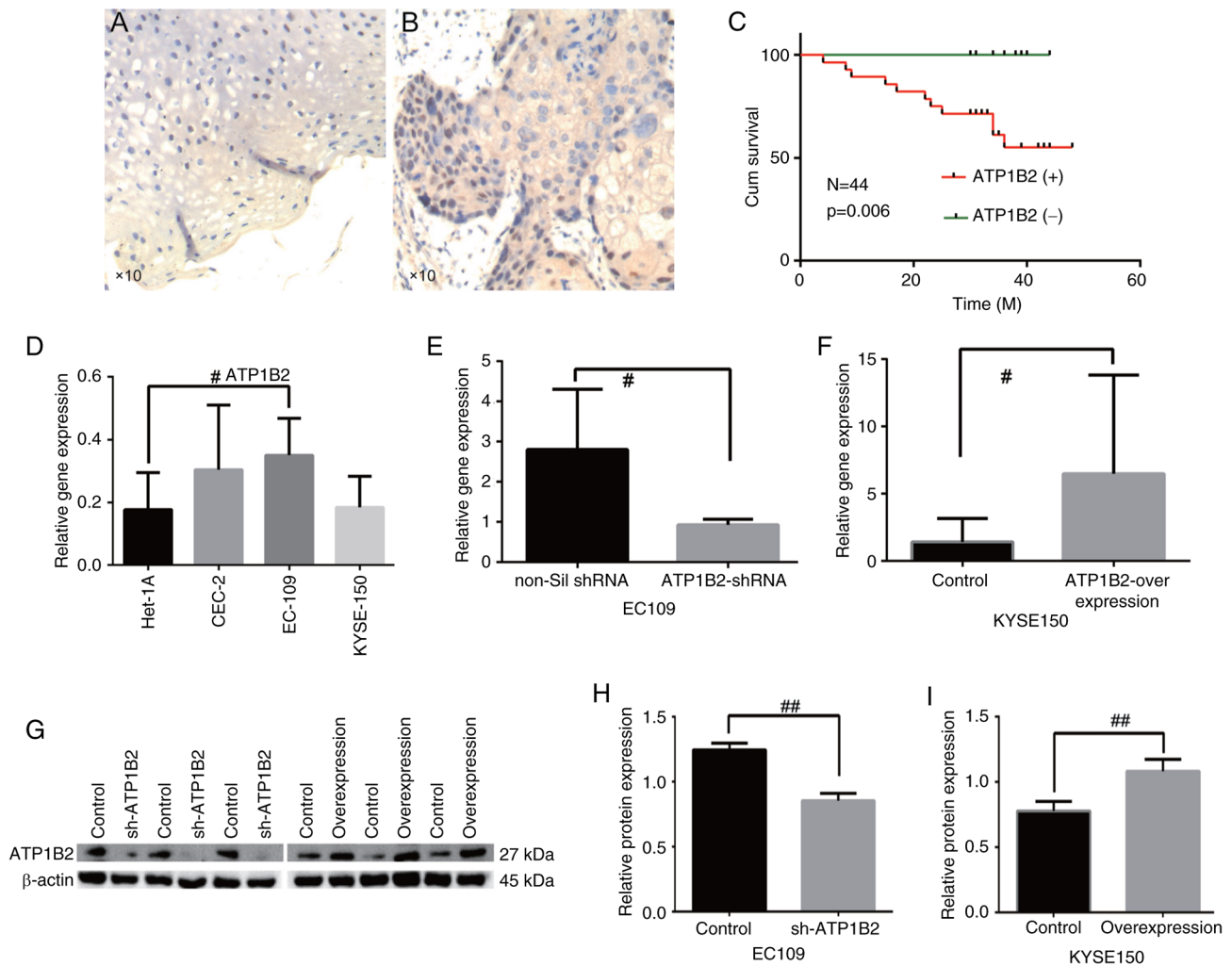


Figure 1. ATP1B2 expression in esophageal cancer and adjacent tissues. (A) In the tissues of ESCC, ATP1B2 protein is mainly located in the nucleus, stained brown, and partially located in the cytoplasm, stained light yellow. (B) Expression of ATP1B2 protein in tissues adjacent to ESCC. (C) Kaplan-Meier method was used to analyze the survival of patients with ESCC with different levels of ATP1B2 expression. The survival rate of the high expression group was significantly lower than that of the low expression group. (D) The expression level of ATP1B2 in immortalized ESCC cell lines was significantly higher in EC109 cells than in Het-1A. (E) In esophageal cancer EC109, the expression of ATP1B2 mRNA in ATP1B2-shRNA group was significantly lower than that of non-Sil shRNA. (F) In esophageal cancer KYSE150 cells, the expression of ATP1B2 mRNA in ATP1B2-over expression cells was significantly higher than that in control group. (G) Western blotting detected the overexpression and silencing of ATP1B2 protein expression. (H and I) Densitometric analysis of western blotting of (H) silencing of ATP1B2 protein and (I) overexpression of ATP1B2 protein. *P<0.05 and **P<0.01. ESCC, esophageal squamous cell carcinoma; shRNA, short hairpin RNA.

the ATP1B2 knockdown group was significantly lower than that of the empty-vector control group at 6, 12 and 24 h (10,963.25±311.53 vs. 15,854.75±2,187.5; 19,371.25±3,804.25 vs. 25,677.25±2,135.76; 33,332.25±5,237.49 vs. 39,618.5±4,191.08; all P<0.05, Fig. 2A and C). The cell migration ability of the ATP1B2 overexpression group was significantly greater than that of the empty-vector control group at 6 and 12 h (81,909,383±50,721.35 vs. 736,194±66, 803.26; 1,552,102.6±80,499.28 vs. 1,257,579.0±144,188.72; all P<0.05, Fig. 2B and D). The cell cycle analysis demonstrated that the overexpression of ATP1B2 and the empty-vector control had no significant effect on the proportions of KYSE150 ESCC cells in the G₀ (47.58±0.94 vs. 47.74±7.74), S (49.1±3.19 vs. 49.37±7.37), or M (3.22±2.26 vs. 2.88±1.48) groups. Similarly, in the cell cycle analysis of EC109 cells in ATP1B2 knock-down group and the empty-vector control group, there was no significant difference in G₀ phase (47.94±0.08 vs. 48.43±1.04),

S phase (49.24±0.28 vs. 48.67±0.66) and M phase (2.82±0.26 vs. 2.89±0.87), but these difference were not statistically significant (all P>0.05; Fig. 2E-J).

The ATP1B2 knockdown group exhibited significantly increased apoptosis compared with the empty-vector control group in EC109 cells (t=3.137; P<0.05; Fig. 3A, B and E). Compared with those in the empty-vector control group, apoptosis was significantly lower in the ATP1B2 overexpression experimental group (t=-2.906; P<0.05; Fig. 3C, D and F). According to the plate cloning results, the cell clone proliferation ability of the ATP1B2 knock-down group was significantly greater than that of the empty-vector control group (170.67±9.50 vs. 103.33±7.64; t=9.57; P<0.05; Fig. 4A, B and E). The cell colony formation ability of the ATP1B2-overexpression cells was significantly lower than that of the empty-vector control cells (83.3±4.16 vs. 160.3±9.45; t=-12.91; P<0.05, Fig. 4C, D and F). After

Table II. Gene expression in esophageal cancer and adjacent tissues.

ATP1B2						
Tissue sample	Tissue sample	Upper quartile	Lower quartile	$\bar{x}$	Z	P
Normal tissue	44	0.2	2.39	1.470	-4.103	0
Esophageal squamous cell carcinoma	44	0.42	4.89	3.541		

Table III. Relationship between the expression level of ATP1B2 and clinicopathological features in patients with esophageal squamous cell carcinoma.Clinical characteristics.

	N	(+)	(-)	χ^2	P-value
Sex					
Male	37	24 (64.86)	13 (35.14)	a	0.690
Female	7	4 (57.14)	3 (42.86)		
Age, years					
>60	16	10 (62.5)	6 (37.5)	0.140	0.910
≤60	28	18 (64.29)	10 (35.71)		
Smoking					
Yes	30	19 (63.33)	11 (36.67)	0.004	0.950
No	14	9 (64.29)	5 (35.71)		
Drinking					
Yes	31	18 (58.06)	13 (41.94)	a	0.314
No	13	10 (76.92)	3 (23.07)		
T stage					
I + II	17	8 (47.06)	9 (52.94)	3.290	0.070
III + IV	27	20 (74.07)	7 (25.93)		
N stage					
N ₀	17	15 (88.24)	2 (11.76)	a	0.010
N ₁ + N ₂	27	13 (48.15)	14 (51.85)		
Lymph node metastasis					
Yes	17	15 (88.24)	2 (11.76)	a	0.010
No	27	13 (48.15)	14 (51.85)		
Invasion of vessels and nerves					
Yes	14	12 (85.71)	2 (14.29)	a	0.049
No	30	16 (53.33)	14 (46.67)		
Tumor diameter					
>3 cm	24	18 (75.0)	6 (25.0)	2.950	0.086
≤3 cm	20	10 (50.0)	10 (50.0)		
Survival status					
Alive	33	17 (51.51)	16 (48.28)	a	0.003
Death	11	11 (100)	0 (0)		

^a, the expected frequency for this group of samples is <5. Fisher's exact test was used, and the chi-square test was not applied.

72 and 96 h after the EC109 ESCC cells were transfected, the absorbance value of the ATP1B2 knockdown group was significantly greater than that of the empty-vector control group (72 h, 5.34±0.17 vs. 4.74±0.16, $t=-6.37$; 96 h, 7.90±0.23 vs. 7.5±0.17, $t=-3.50$; all $P<0.05$; Fig. 4G). After the KYSE150 ESCC cells were transfected for 48 and 72 h,

the absorbance value of the ATP1B2 overexpression group was significantly lower than that of the empty-vector control group (48 h, 3.38±0.16 vs. 3.69±0.27, $t=-2.50$; 72 h, 6.58±0.17 vs. 7.16±0.49, $t=-2.77$; $P<0.05$; Fig. 4H). Thus, it is evident that ATP expression significantly suppresses the proliferative capacity of esophageal cancer cells.

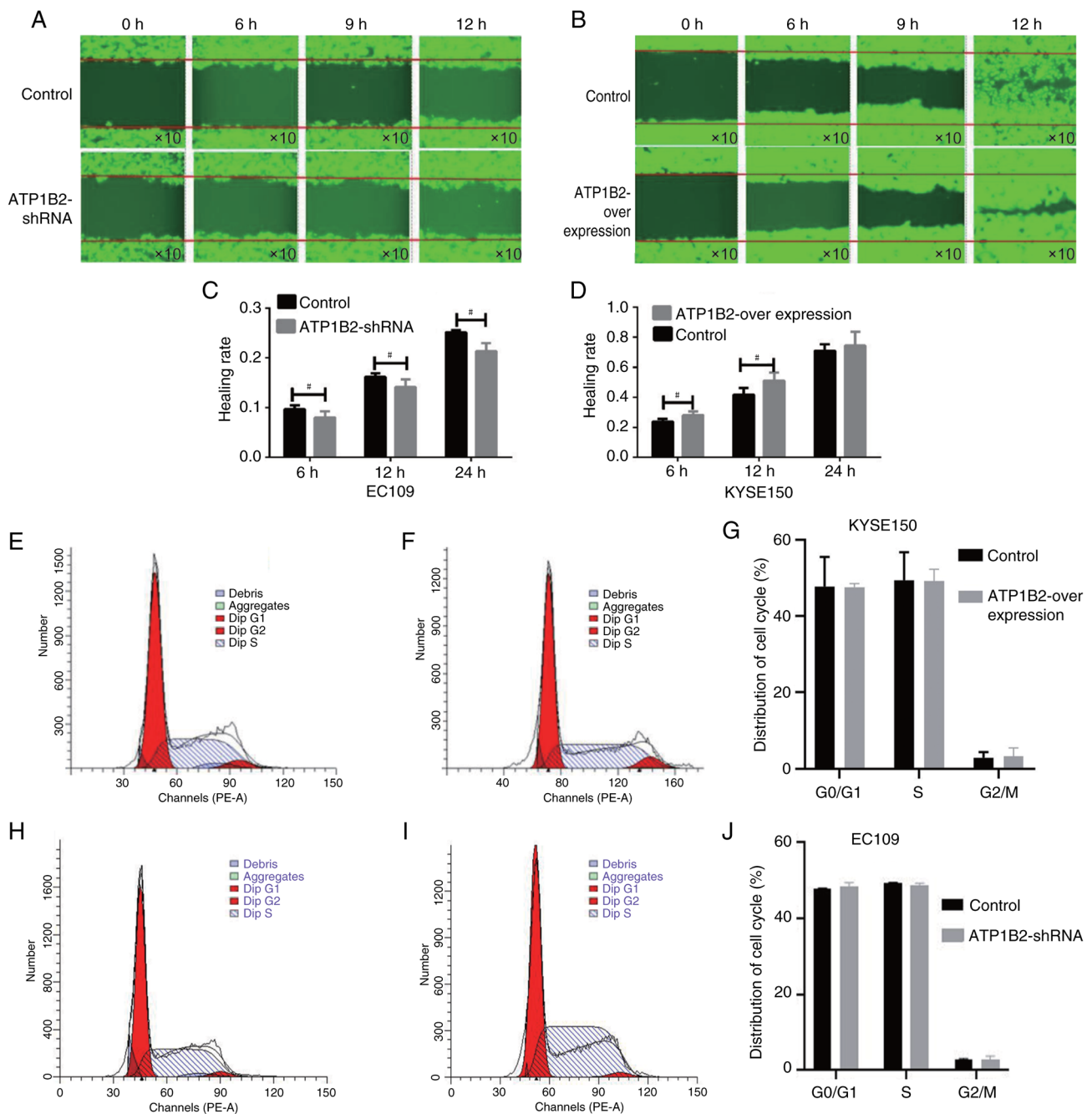


Figure 2. Effect of ATP1B2 on biological behavior of esophageal squamous cell carcinoma cells: invasion, migration ability and cell cycle. (A and B) Observation of cell scratch healing in knockdown and overexpression ATP1B2 and empty-vector control group under fluorescence microscopy. (C and D) Histogram of cell scratch healing in knockdown and overexpression ATP1B2 and empty-vector control group. (E and F) Cell cycle distribution of overexpression ATP1B2 and empty-vector control group. (G) Histogram of cell cycle of ATP1B2 overexpression and empty-vector control group. (H and I) Cell cycle distribution of ATP1B2 knockdown and empty-vector control group. (J) Histogram of cell cycle of ATP1B2 knockdown and empty-vector control group. shRNA, short hairpin RNA. * $P < 0.05$.

Determination of gene expression related to epithelial-mesenchymal transformation (EMT) by RT-qPCR. Following successful transfection of the plasmid, RNA was extracted for reverse transcription. RT-qPCR revealed that the genes RAC1, MMP7 and MMP10 were associated with EMT. The expression of genes related to migration was significantly upregulated in the ATP1B2 overexpression group compared with the control group ($P < 0.05$, Fig. 5A-C). Therefore, overexpression of ATP1B2 was observed to promote cell migration. Additionally, the overexpression

of ATP1B2 significantly inhibited the expression of P53 ($P < 0.05$, Fig. 5D).

Expression of EMT-related proteins is detected by western blotting. After knocking down ATP1B2, the expression levels of E-cadherin increased in EC109 cells, and the difference was statistically significant ($P < 0.05$; Fig. 6A and C). Although the difference was not statistically significant, it cannot be ignored that knocking down ATP1B2 reduced the expression level of N-cadherin (Fig. 6A and B) whereas it increased P53

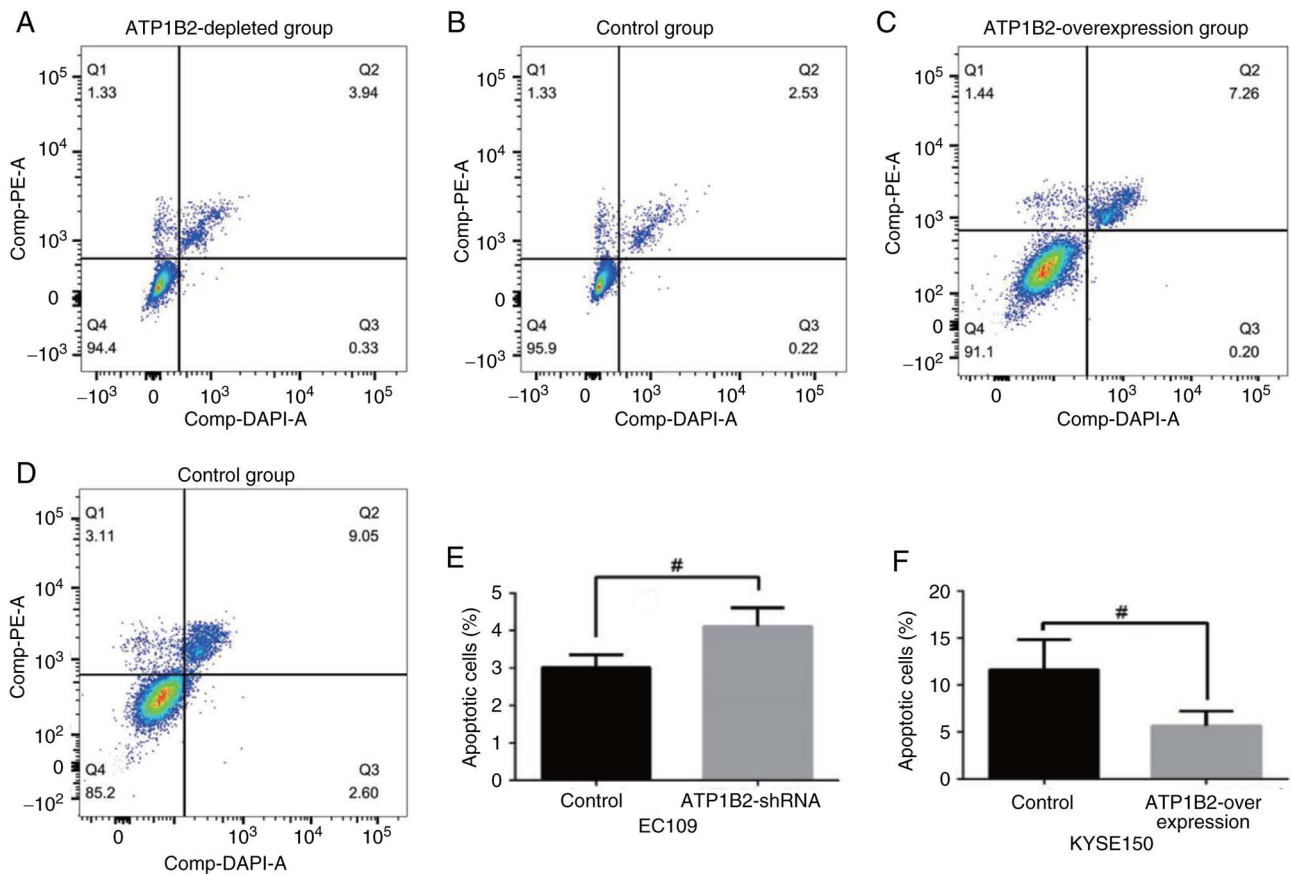


Figure 3. Effect of ATP1B2 on biological behavior of esophageal cancer cells: Proliferation, invasion, migration ability, cell cycle and apoptosis. (A and B) Comparison of apoptosis between the experimental group and the empty-vector control group with ATP1B2 knockdown in ESCC cell line EC109 cells. (C and D) Comparison of apoptosis between the experimental group with overexpression of ATP1B2 and the empty-vector control group in ESCC cell line KYSE150 cells. (E) EC109 cell apoptosis was higher than that of control group after ATP1B2 knockout. (F) After overexpression of ATP1B2, the apoptosis of KYSE150 cells was lower than that of the control group. #P<0.05. ESCC, esophageal squamous cell carcinoma; shRNA, short hairpin RNA.

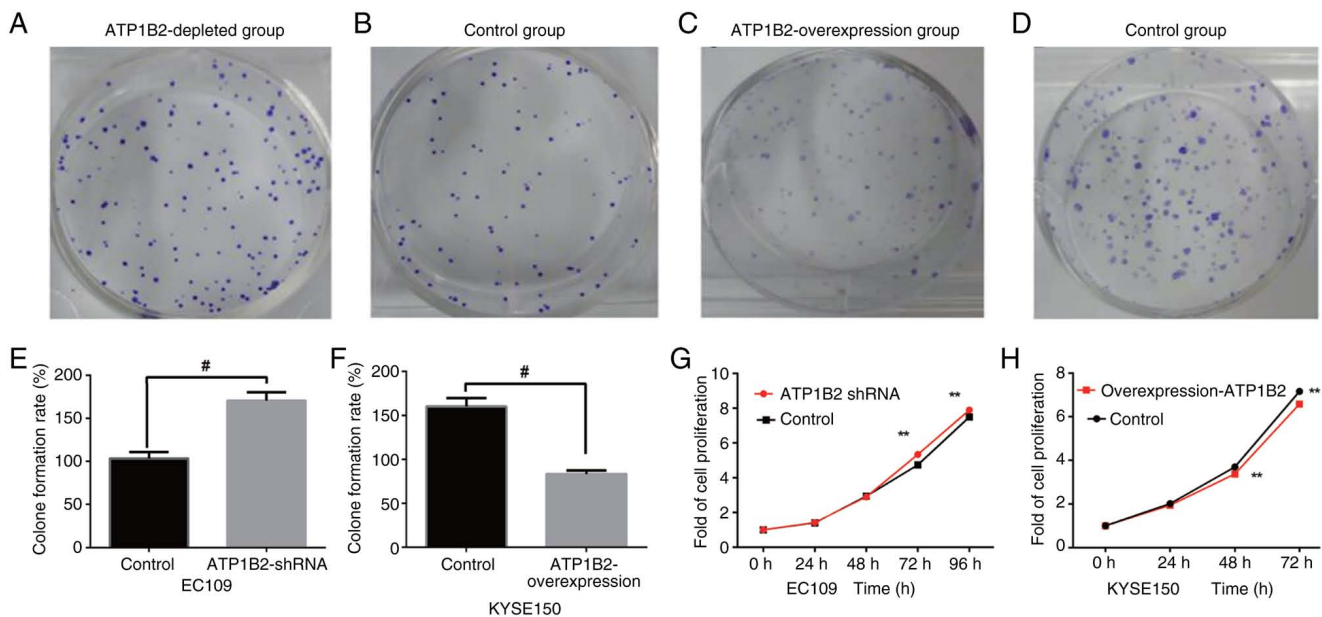


Figure 4. Effect of ATP1B2 on biological behavior of esophageal cancer cells: Proliferation. (A and B) The research results in the human ESCC cell line EC109. (A) Knockdown of the ATP1B2 experimental group; (B) empty-vector control group. The results showed that knocking down ATP1B2 can promote the proliferation of EC109 cells. (C and D) The study in the human ESCC line KYSE150. (C) Experimental group overexpressing ATP1B2; (D) control group. The results revealed that overexpressing ATP1B2 can inhibit the proliferation of KYSE150 cells. (E) Plate cloning experiment demonstrated that knockdown of ATP1B2 gene promoted the proliferation of EC109 cells. (F) Plate cloning experiment showed that overexpression of ATP1B2 inhibited the proliferation of KYSE150 cells. (G) MTT identified that knockdown of ATP1B2 gene promoted the proliferation of EC109 cells. (H) MTT showed that overexpression of ATP1B2 inhibited the proliferation of KYSE150 cells. #P<0.05 and **P<0.01. ESCC, esophageal squamous cell carcinoma; shRNA, short hairpin RNA.

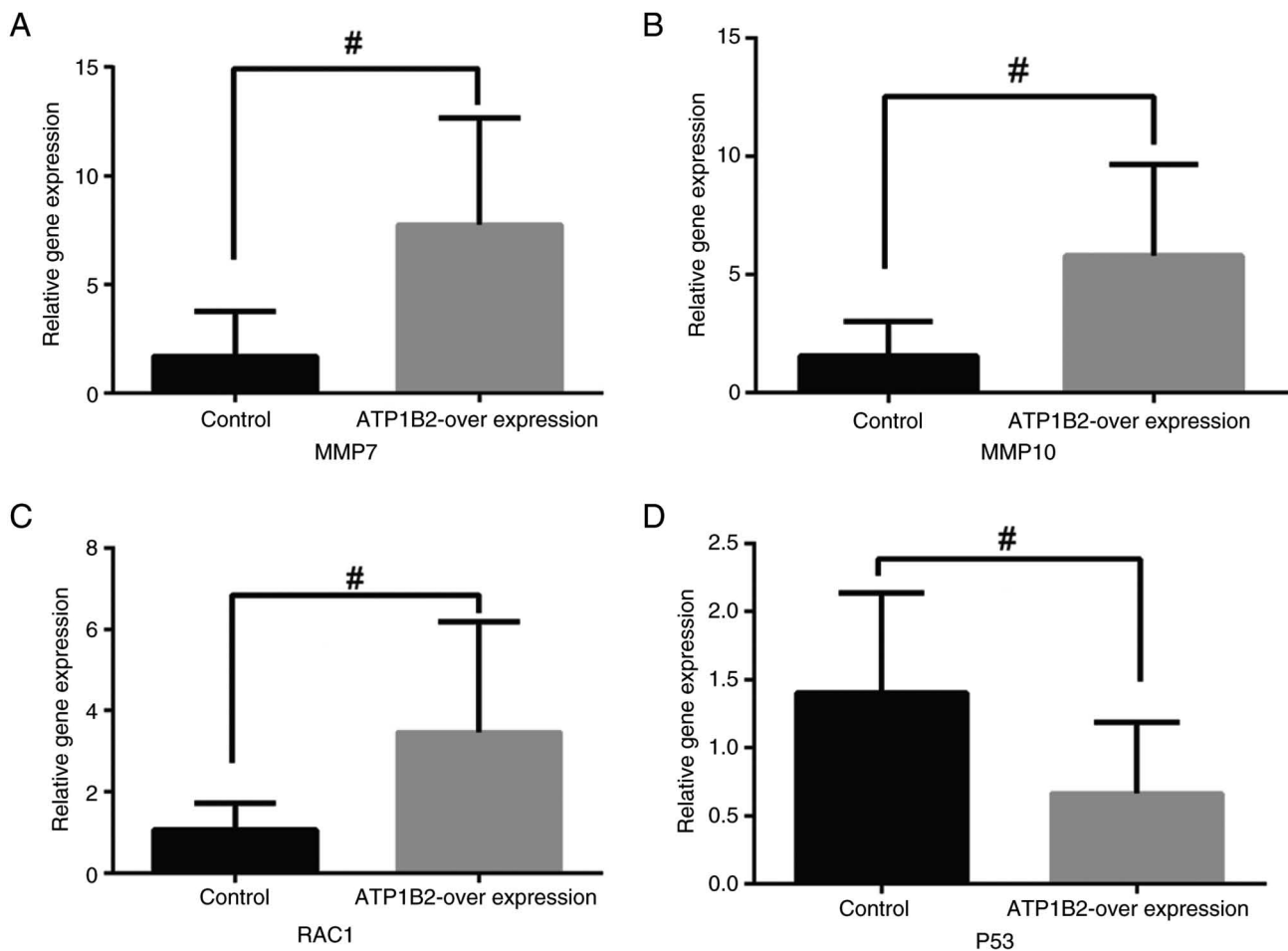


Figure 5. RT-qPCR determination of expression content of epithelial-mesenchymal transformation-related genes. (A-D) RT-qPCR detection in the experimental group overexpressed with ATP1B2 and the empty-vector control group of (A) MMP7; (B) MMP10 (C) RAC1 and (D) P53. *P<0.05. RT-qPCR, reverse transcription-quantitative PCR.

(Fig. 6A and D). In KYSE150 cells, overexpression of ATP1B2 resulted in increased RAC1 protein expression and decreased p53 protein expression, consistent with trends observed in RT-qPCR analysis, although these differences were not statistically significant (P>0.05, Fig. 7A-C).

Significantly enhanced inhibitory effect of ouabain on cell proliferation and migration. A total of three different concentrations of ouabain (20, 40 and 60 nmol/l) were tested. MTT assays demonstrated that ouabain significantly enhanced the inhibitory effect of ATP1B2-overexpressing ESCC cells on proliferation in response to increasing concentrations and durations of exposure compared with those in the control group (P<0.05; Fig. 8). In the scratch test, 60 μ l/l ouabain was added to the ATP1B2 overexpression group and the control group containing the empty vector. The migration rate of the cells was measured at 6, 12 and 24 h, and flow cytometry was used to detect the cell cycle. The inhibitory effect of ouabain on the migration ability of ATP1B2-overexpressing cells was significantly greater than that on the migration of control cells (P<0.01; Fig. 9A and B). Furthermore, the inhibition effect of ouabain on ATP1B2-overexpression ESCC cells resulted in arrest in the G₁/S phase of the cell cycle, which was also significantly enhanced (P<0.05; Fig. 9C and D). Ultimately, the inhibition of ATP1B2 cell migration was significantly enhanced (P<0.01; Fig. 9E).

Discussion

Gastrointestinal malignancies account for ~30% of the global cancer incidence rate and 40% of the mortality rate, with ESCC mortality ranks fourth (13,14). The clinical manifestation of early ESCC is not evident, and the diagnosis rate is low. Some patients already have lymph node metastasis at the initial diagnosis, missing the optimal time for surgery. Even if surgery is performed, complete removal of the focus is not always possible, resulting in short postoperative survival and poor quality of life (15). Therefore, it is crucial to explore the mechanism of invasion and metastasis in ESCC for its treatment and prognosis. The intron 17P13.1 in the ATP1B2 subunit is located in the 3'-region of TP53 and can regulate TP53 function, increasing the probability of ESCC (16). The ATP1B2-PRAKCB gene fusion has been detected in intraductal eosinophilic papillary tumors of pancreas and bile duct (17). ATP1B2 expression is greater in glioma tissues than that in adjacent tissues. Patients with glioma and high expression of ATP1B2 are more prone to cancer cell infiltration and lymph node metastasis, resulting in a shorter overall survival period and poor prognosis (10). Additionally, ATP1B2 expression in ESCC tissues was significantly greater than that in adjacent tissues (P<0.05).

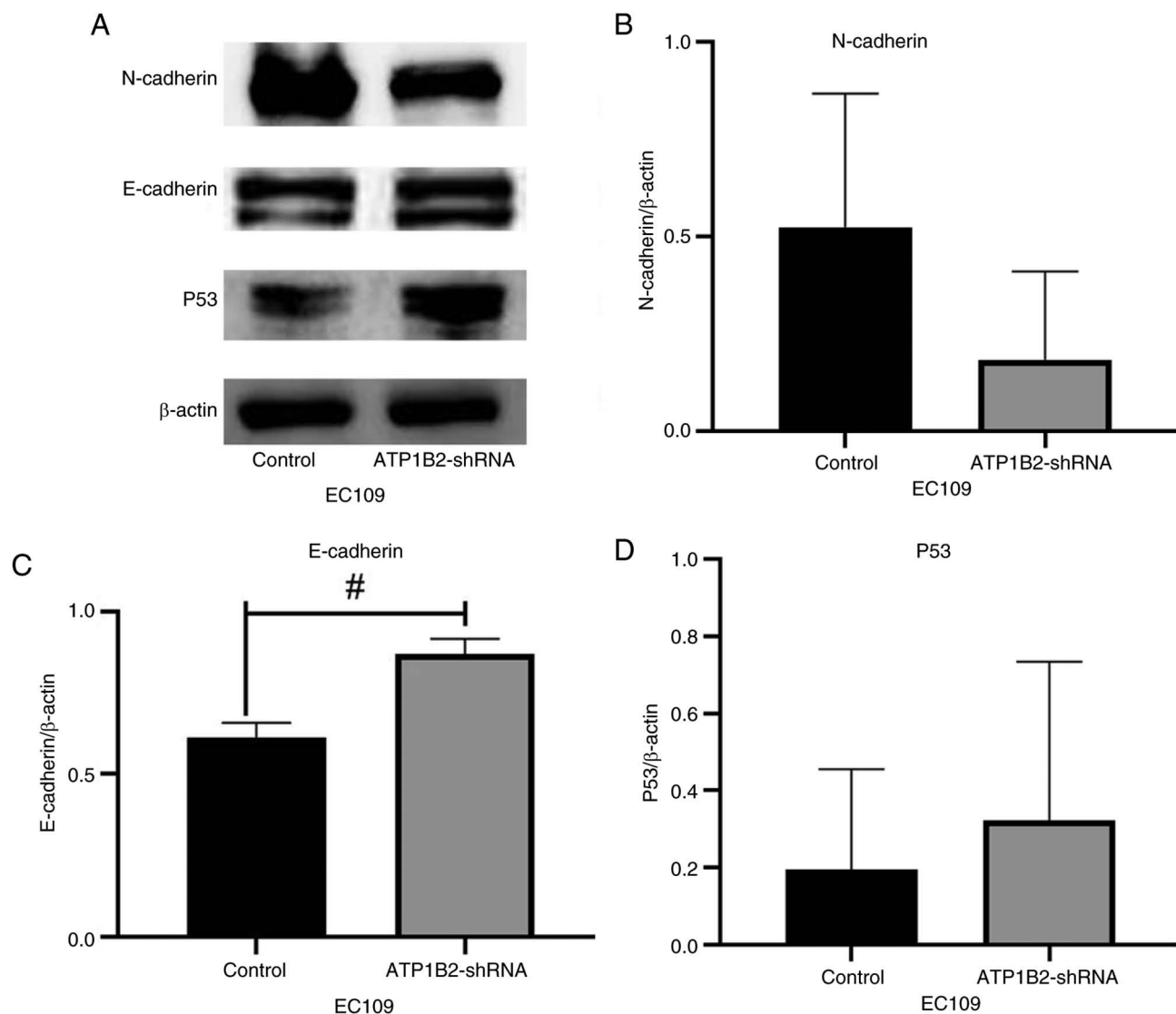


Figure 6. Expression of epithelial-mesenchymal transformation-related proteins was detected by western blotting. (A) Western blotting of various proteins in EC109 cells and EC109-ATP1B2-shRNA cells. (B-D) Densitometric analysis of expression levels in EC109 cells and EC109-ATP1B2-shRNA cells of (B) N-cadherin/ β -actin; (C) E-cadherin/ β -actin and (D) P53/ β -actin $^{\#}P<0.05$. shRNA, short hairpin RNA.

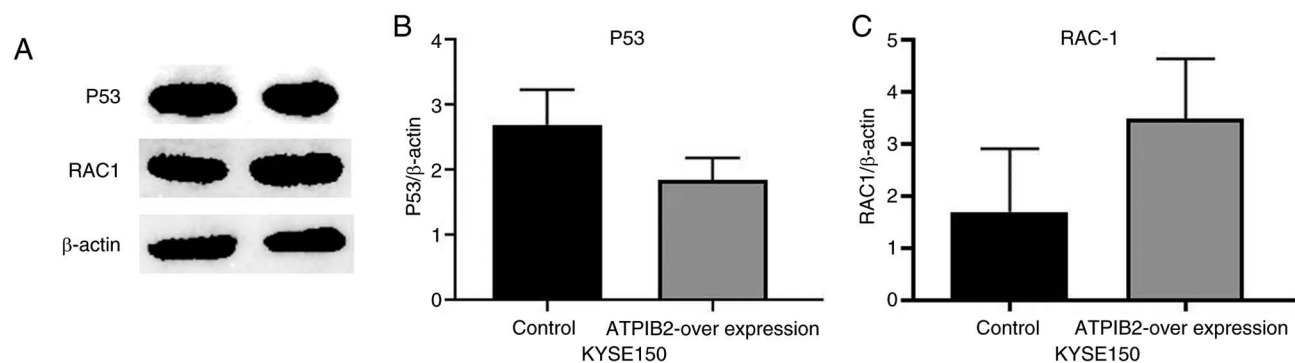


Figure 7. Protein expression levels of P53 and RAC1 in ATP1B2-overexpressing cells. (A) Western blotting of P53 and RAC1 proteins in KYSE150 cells and EC109-ATP1B2-overexpressing cells. (B and C) Densitometric analysis in KYSE150 cells and EC109-ATP1B2-overexpressing cells of expression levels of (B) P53/ β -actin and (C) RAC1/ β -actin.

High ATP1B2 expression is closely associated with poor prognosis in patients with ESCC and with nerve, vessel and lymph node metastasis.

Change of ion flux can influence cell signal transduction and cytoskeleton remodeling and support cell migration (18). The activity of Na^+/K^+ -ATPase in tumor cells

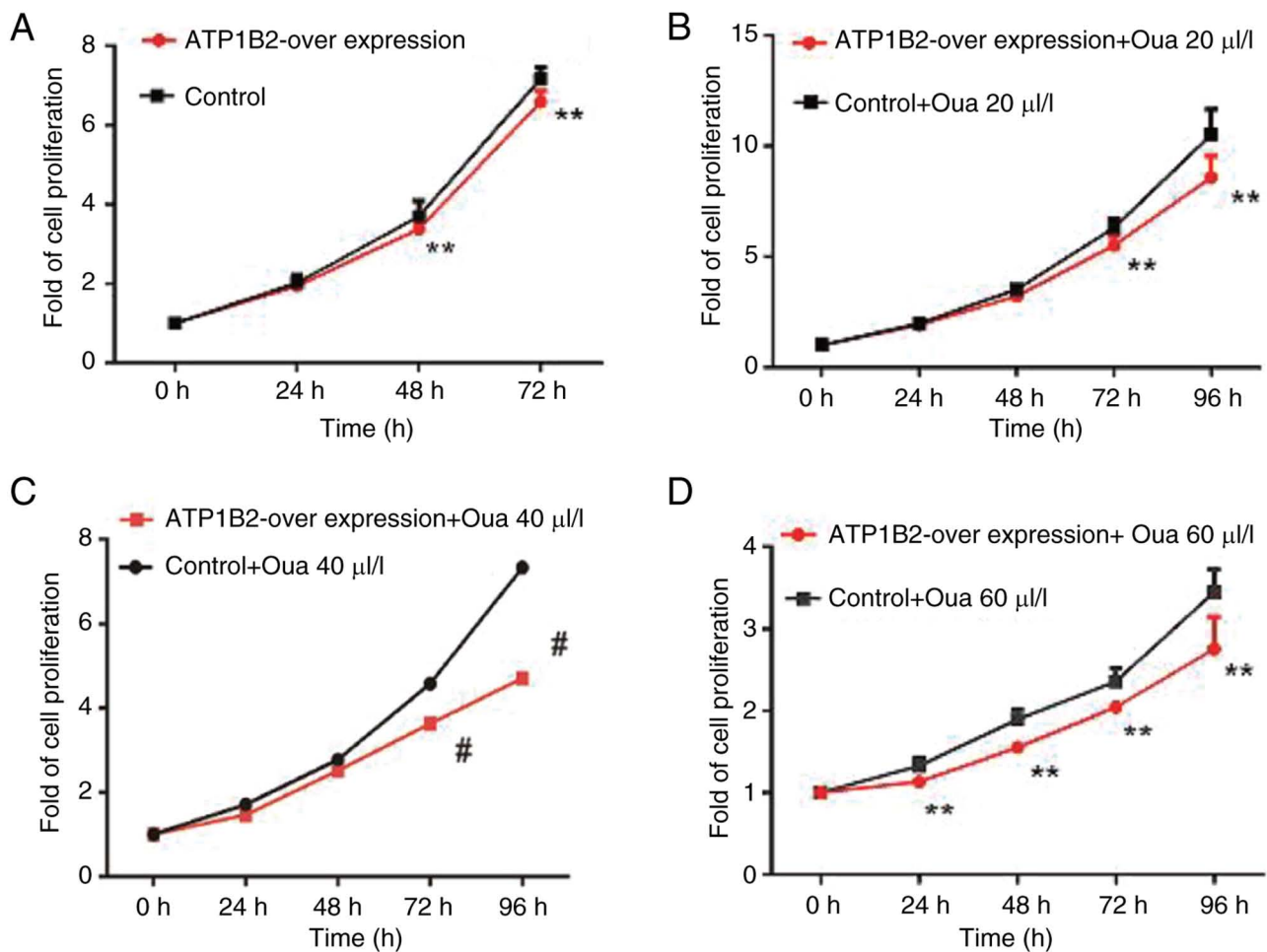


Figure 8. Effect of different concentrations of ouabain on proliferation of esophageal cancer cells. MTT test confirmed that compared with the empty-vector control group, the inhibitory effect of ouabain on the proliferation of esophageal cancer cells overexpressing ATP1B2 was significantly enhanced with the prolongation of the concentration and time of action. (A) MTT detection of cancer cell proliferation in the empty-vector control group. (B-D) Compared with the empty-vector control group, the inhibitory effect on the proliferation of esophageal cancer cells in the overexpressing ATP1B2 group of (B) 20 μ l/l ouabain; (C) of 40 μ l/l ouabain and (D) 60 μ l/l ouabain. # P <0.05 and ** P <0.01. MTT, methylcyclopentadienyl manganese tricarbonyl.

is greater than that in normal cells, altering the concentration of ions inside and outside the cells and promote cancer progression (19,20). Cardiac glycosides can inhibit the activity of indoleamine pyrrole 2',3'-dioxygenase 1 in cancer cells by inhibiting the activity of Na^+/K^+ ATPase to activate STAT1. These findings provide insights into the immune mechanism of cancer (21). The Na^+/K^+ -ATPase may serve as a potential target for tumor therapy. ATP1B2 is overexpressed in glioma cell lines, and knockdown of ATP1B2 inhibits cell migration and induces apoptosis (17). Furthermore, patients with high ATP1B2 expression in mononuclear cell lines are more sensitive to the toxicity of marine polyether marsh (22). In the present study, ATP1B2 was highly expressed in the ESCC cells, and ATP1B2 knockdown inhibited cancer cell migration and induced apoptosis. Conversely, ATP1B2 overexpression promoted cell migration and inhibited apoptosis (10). These findings align with the observation that patients with ESCC with high ATP1B2 expression are prone to lymph node metastasis and have a worse prognosis. Therefore, ATP1B2 played a pivotal role in the invasion and metastasis of ESCC.

The invasion and migration of cancer involve a multifactor, multistep cascade reaction. Matrix-degrading protease 7

(MMP7) plays a crucial role in the degradation of the extracellular matrix. Its expression is highly upregulated in ESCC tissue and enhances cell migration (23). Similarly, MMP10 is overexpressed in ESCC tissue (24). In the present study, the expression of MMP7 and MMP10 in the experimental group overexpressing ATP1B2 was higher than that in the empty-vector control group. These results suggest that ATP1B2 may promote cell migration by upregulating EMT-related genes. Wild-type P53 regulates the cell cycle through the transcription of multiple factors, induces apoptosis of cancer cells, and prevents initiation of cancer. P53 is a tumor suppressor gene, but the mutation rate in ESCC cells can reach 96%, and its overexpression promotes cancer progression with poor prognosis (25,26). Dey *et al* (27) used immunohistochemistry to detect the expression level of P53 in cancer tissue and adjacent tissues of patients with ESCC. The results showed that the expression level of P53 in squamous cell carcinoma tissue was significantly higher than that in adjacent tissues, and the expression level of P53 was directly proportional to the size of the tumor. In the present study, it was found that overexpression of ATP1B2 can inhibit the expression of P53 gene; subsequent protein expression assays were also consistent with this finding, which suggests that cell proliferation may be inhibited by altering the expression of transcription

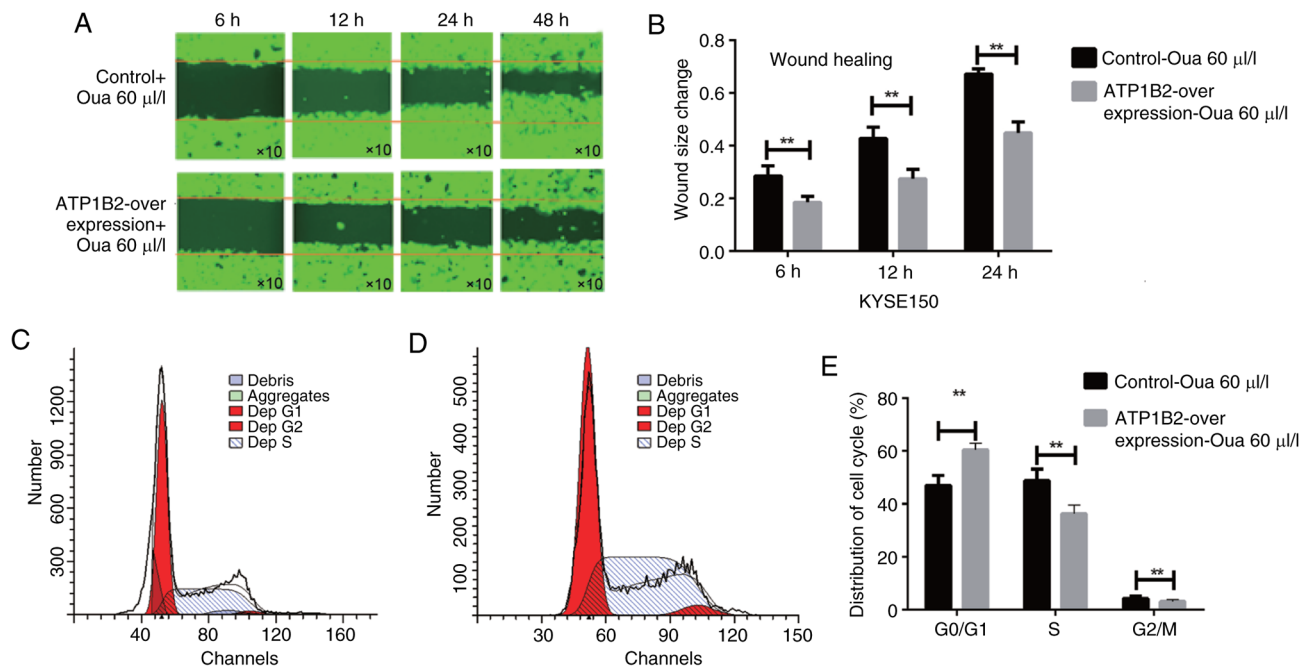


Figure 9. Effect of ouabain on the migration ability and cell cycle of esophageal cancer cells after overexpression of ATP1B2. (A) Scratch test showed that the inhibitory effect of ouabain on the migration ability of overexpression ATP1B2 cells was significantly enhanced by adding 60 µl/l of ouabain. (B) Compared with the empty-vector control group, the inhibitory effect of ouabain on the proliferation of esophageal cancer cells overexpressing ATP1B2 was significantly enhanced with the prolongation of the action concentration and time. (C and D) The cell cycle experiment revealed that ouabain significantly enhanced the inhibition of cell cycle arrest in G₁/S phase of esophageal cancer cells with overexpression of ATP1B2; (C) experimental group over-expressing ATP1B2 with ouabain; (D) control group with ouabain. (E) Inhibitory effect of E ouabain on cell cycle arrest in G₁/S phase of esophageal cancer cells overexpressing ATP1B2 was significantly enhanced. **P<0.01.

genes in P53. After knocking down ATP1B2, the expression of E-cadherin and P53 in cancer cells increased, while the expression of N-cadherin decreased, indicating that ATP1B2 may play a role in the signaling pathway through EMT and P53. However, the detailed mechanisms involved have not yet been thoroughly studied due to energy and financial constraints.

As a member of the RAS family, RAC1 encodes GTPs. When combined with GTP, ATP1B2 activates the downstream pathway of RAC1 and plays an important role in regulating cell invasion, migration and cytoskeleton (28). In gastric cancer, RAC1 activation regulates tumor cell invasion and migration (29). Overexpression of RAC1 in ESCC is positively correlated with lymph node metastasis and prognosis of patients with ESCC (30). In the current experiment, the results of RT-qPCR further demonstrated that the overexpression of ATP1B2 promoted the expression of the migration gene RAC1. Furthermore, the corresponding protein expression levels were consistent with this trend. These findings were consistent with the scratch test results, in which ATP1B2 overexpression enhanced cell migration.

The proliferation and migration effects of most genes in tumor cells are the same. For instance, ATP1B3 knockdown inhibited the proliferation and migration of gastric cancer cells (31), and the knockdown of ATP1B2 inhibited the proliferation and migration of glioma cells and promoted their apoptosis (32). However, there are also cases where the opposite occurs. For example, FXB022 promoted cancer cell proliferation in primary breast cancer but targeted SNAIL, a crucial regulator of EMT and breast cancer metastasis, via glycogen synthase kinase 3β (33). Its migration was inhibited

through ubiquitin-mediated proteasome degradation in a phosphorylation-dependent manner. Cheng *et al* (34) reported that the overexpression of miR-210 inhibits proliferation in the ESCC EC109 cell line but promotes cell migration and induced apoptosis. Hezova *et al* (35) found that overexpressing miR-205 in esophageal adenocarcinoma cells could restrict proliferation and migration and induce apoptosis through EMT process. However, the knockdown of miR-205 in ESCC inhibits cell proliferation and migration and induces cell apoptosis by regulating metalloproteinase 10.

In the present study, it was observed that the overexpression of ATP1B2 enhanced cell migration and inhibited apoptosis. Conversely, ATP1B2 knockdown decreased cell migration and induced apoptosis, indicating that patients with ESCC are susceptible to lymph node metastasis. However, in the proliferation experiment, ATP1B2 knockdown promoted the proliferation of well-differentiated ESCC EC109 cells, while ATP1B2 overexpression inhibited the proliferation of poorly differentiated ESCC KYSE150 cells. Consequently, ATP1B2 exhibited varying proliferative capacities in different ESCC cells. This result was further confirmed through a plate cloning experiment, which aligned with the MTT results.

ATP1B2 had different effects on cell proliferation in highly differentiated ESCC cell line EC109 and the poorly differentiated ESCC cell line KYSE150. First, only *in vitro* cell experiments have been completed; the specific characteristics of the two ESCC cells have not been fully elucidated. The cell characteristics and involvement of other genes may have contributed to the differential proliferative rates of ATP1B2 in the ESCC cells EC109 and KYSE150.

However, the exact underlying mechanism remains to be further explored. Second, gene transfer regulates numerous signal pathways post-translationally. The activation factors differ in the ESCC cells with varying degrees of differentiation and exert diverse effects on proliferation. Moreover, these dual effects illustrate the diversity of multifunctional transfer factors involved in regulating the onset and progression of cancer, potentially explaining the complexity of ESCC diagnosis and treatment.

The focus of the present study was to investigate the potential therapeutic effects of ouabain, a specific inhibitor of Na^+/K^+ -ATPase, on various medical conditions, including coronary heart disease, hypertension, neuritis, viral infections and cancer (36,37). Previous findings revealed that ouabain can inhibit the proliferation of multiple tumor cells, such as those of breast cancer, lung cancer, prostate cancer, colon cancer, glioma and leukemia (38,39). Research at Moscow State University observed that the combination of ouabain with Na^+/K^+ -ATPase could inhibit cell function and cause cell death (40). Additionally, low-concentration ouabain combined with Na^+/K^+ -ATPase has no effect on cell survival (41) but could change the conformation of subunits of Na^+/K^+ -ATPase and activate multiple signal pathways (37).

Furthermore, it was found that ouabain in combination with Na^+/K^+ -ATPase enhanced the phosphorylation of Tyr10 stimulated by EGF in adrenal epithelial cells. It also increased the ADP:ATP ratio while inhibiting the production of lactic acid and the oxygen consumption rate in a dose- and time-dependent manner (42). Ouabain is a specific inhibitor of Na^+/K^+ -ATPase, which exerts cardiotoxic effect by inhibiting the efflux of Na^+ and indirectly increasing the concentration of intracellular Ca^{2+} . Ouabain is a Na^+/K^+ -ATPase inhibitor but not a specific inhibitor of ATP1B2. Ouabain was shown to reduce the viability of human osteosarcoma cancer cells, induce cell chromosome aggregation, cause DNA fragmentation damage, and promote cell apoptosis (43). Specifically, in the context of study, our research demonstrated that ouabain significantly enhanced the inhibitory effect on the proliferation of ESCC cell lines overexpressing ATP1B2. Flow cytometry revealed that at a concentration of $60 \mu\text{l/l}$, ouabain promoted cell cycle arrest and G_1/S phase. Therefore, ouabain could inhibit cell proliferation and migration and promote cell cycle arrest in ESCC cells overexpressing ATP1B2. However, the impact of ATP1B2 on the invasive ability of ESCC cells in the present study was indirectly validated through experiments, and no *in vivo* experiments were conducted. The detailed mechanism is currently unclear and further research is needed.

A significant association was also discovered between the overexpression of ATP1B2 in ESCC tissues and cell lines, lymph node metastasis and prognosis. The overexpression of ATP1B2 promoted cell migration while inhibiting cell apoptosis. Conversely, ATP1B2 knockdown inhibited cell migration and promoted cell apoptosis. Interestingly, ouabain significantly inhibit the proliferation and migration of ESCC cells overexpressing ATP1B2 and induced cell cycle arrest at the G_1/S phase. These findings may contribute to the development of novel ATP1B2-targeted anticancer drugs for ESCC.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Health Commission of Hubei scientific research project (grant nos. WJ2021M046 and WJ2023Q022), the Shiyan City Science and Technology Bureau Guiding Research Project (grant no. 21Y19), the Foundation of Taihe Hospital (grant no. 2020JJXM032) and the Free Exploration Fund Project of Hubei University of Medicine (grant no. FDFR201904).

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

QT and XBL conceived and designed the project. FFL and HW conducted the majority of the experiments. FFL and HW confirm the authenticity of all the raw data. FFL, SBL and SJ were responsible for the animal experiments and part of molecular biology experiment. ZYG contributed to data extraction and data analysis. FFL and XBL wrote the manuscript, SBL, XBL and QT approved and submitted the final manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

It was decided to start a cohort study on esophageal cancer in the northwest region of Hubei in 2016. When applying for this project, it had already been approved by the Ethics Committee of Taihe Hospital (approval no. 2016KS001;). Since then, our research center has been collecting esophageal samples and gradually conducting related research. The study protocol was reviewed and approved by the Taihe Hospital Ethics Committee (approval no. 2022KS038), and all patients received information concerning their participation in the study and provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Chen L, Jiang P, Li J, Xie Z, Xu Y, Qu W, Feng F and Liu W: Periplocin promotes wound healing through the activation of Src/ERK and PI3K/Akt pathways mediated by Na^+/K^+ -ATPase. *Phytomedicine* 57: 72-83, 2019.
2. Felipe Goncalves-De-Albuquerque C, Ribeiro Silva A, Ignacio Da Silva C, Caire Castro-Faria-Neto H and Burth P: Na^+/K^+ pump and beyond: Na^+/K^+ -ATPase as a modulator of apoptosis and autophagy. *Molecules* 22: 578, 2017.

3. Bejček J, Spiwok V, Kmoníčková E and Rimpelová S: Na⁺/K⁺-ATPase revisited: On its mechanism of action, role in cancer, and activity modulation. *Molecules* 26: 1905, 2021.
4. Hilbers F, Kopec W, Isaksen TJ, Holm TH, Lykke-Hartmann K, Nissen P, Khandeliah H and Poulsen H: Tuning of the Na₂K-ATPase by the beta subunit. *Sci Rep* 6: 20442, 2016.
5. Rotoli D, Cejas MM, Maeso MD, Pérez-Rodríguez ND, Morales M, Ávila J, Mobasheri A and Martín-Vasallo P: The Na₂K-ATPase β -subunit isoforms expression in glioblastoma multi-forme: Moonlighting roles. *Int J Mol Sci* 18: 2369, 2017.
6. Banerjee M, Cui X, Li Z, Yu H, Cai L, Jia X, He D, Wang C, Gao T and Xie Z: Na₂K-ATPase Y260 Phosphorylation-mediated src regulation in control of aerobic glycolysis and tumor growth. *Sci Rep* 8: 12322, 2018.
7. Busonero C, Leone S, Bianchi F, Maspero E, Fiocchetti M, Palumbo O, Cipolletti M, Bartoloni S and Acconcia F: Ouabain and digoxin activate the proteasome and the degradation of the ER α in cells modeling primary and metastatic breast cancer. *Cancers (Basel)* 12: 3840, 2020.
8. Du J, Jiang L, Chen F, Hu H and Zhou M: Cardiac glycoside ouabain exerts anticancer activity via downregulation of STAT3. *Front Oncol* 11: 684316, 2021.
9. Wang Y, Wu C, Qin Y, Liu S and Zhang R: Multi-angle investigation of the fractal characteristics of nanoscale pores in the lower cambrian niutitang shale and their implications for CH₄ adsorption. *J Nanosci Nanotechnol* 21: 156-167, 2021.
10. Li S, Dai Z, Yang D, Li W, Dai H, Sun B, Liu X, Xie X, Xu R and Zhao X: Targeting β 2 subunit of Na⁺/K⁺-ATPase induces glioblastoma cell apoptosis through elevation of intracellular Ca²⁺. *Am J Cancer Res* 9: 1293-1308, 2019.
11. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
12. Pfaffl MW: A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29: e45, 2001.
13. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A and Bray F: Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 71: 209-249, 2021.
14. Li J: Digestive cancer incidence and mortality among young adults worldwide in 2020: A population-based study. *World J Gastrointest Oncol* 14: 278-294, 2022.
15. Wu SG, Zhang WW, Sun JY, Li FY, Lin Q and He ZY: Patterns of distant metastasis between histological types in esophageal cancer. *Front Oncol* 8: 302, 2018.
16. Wu C, Wang Z, Song X, Feng XS, Abnet CC, He J, Hu N, Zuo XB, Tan W, Zhan Q, *et al.*: Joint analysis of three genome-wide association studies of esophageal squamous cell carcinoma in Chinese populations. *Nat Genet* 46: 1001-1006, 2014.
17. Singhi AD, Wood LD, Parks E, Torbenson MS, Felsenstein M, Hruban RH, Nikiforova MN, Wald AI, Kaya C, Nikiforov YE, *et al.*: Recurrent rearrangements in PRKACA and PRKACB in intraductal oncocytic papillary neoplasms of the pancreas and bile duct. *Gastroenterology* 158: 573-582.e2, 2020.
18. Yang M, James AD, Suman R, Kasprovicz R, Nelson M, O'Toole PJ and Brackenbury WJ: Voltage-dependent activation of Rac1 by Nav 1.5 channels promotes cell migration. *J Cell Physiol* 235: 3950-3972, 2020.
19. Leslie TK, James AD, Zaccagna F, Grist JT, Deen S, Kennerley A, Riemer F, Kaggie JD, Gallagher FA, Gilbert FJ and Brackenbury WJ: Sodium homeostasis in the tumour microenvironment. *Biochim Biophys Acta Rev Cancer* 1872: 188304, 2019.
20. Capatina AL, Lagos D and Brackenbury WJ: Targeting ion channels for cancer treatment: Current progress and future challenges. *Rev Physiol Biochem Pharmacol* 183: 1-43, 2023.
21. Shandell MA, Capatina AL, Lawrence SM, Brackenbury WJ and Lagos D: Inhibition of the Na⁺/K⁺-ATPase by cardiac glycosides suppresses expression of the IDO1 immune checkpoint in cancer cells by reducing STAT1 activation. *J Biol Chem* 298: 101707, 2022.
22. Pelin M, Stocco G, Florio C, Sosa S and Tubaro A: In vitro cell sensitivity to palytoxin correlates with high gene expression of the Na⁺/K⁺-ATPase β 2 subunit isoform. *Int J Mol Sci* 21: 5833, 2020.
23. Xu YW, Peng YH, Chen B, Wu ZY, Wu JY, Shen JH, Zheng CP, Wang SH, Guo HP, Li EM and Xu LY: Autoantibodies as potential biomarkers for the early detection of esophageal squamous cell carcinoma. *Am J Gastroenterol* 109: 36-45, 2014.
24. Ardalan Khaled S, Abbaszadegan MR, Majd A and Forghanifard MM: TWIST1 upregulates matrix metalloproteinase (MMP) genes family in esophageal squamous carcinoma cells. *Gene Expr Patterns* 37: 119127, 2020.
25. Yao L, Zhong X, Huang G, Ma Q, Xu L, Xiao H and Guo X: Investigation on the potential correlation between TP53 and esophageal cancer. *Front Cell Dev Biol* 9: 730337, 2021.
26. Zhang N, Shi J, Shi X, Chen W and Liu J: Mutational characterization and potential prognostic biomarkers of Chinese patients with esophageal squamous cell carcinoma. *OncoTargets Ther* 13: 12797-12809, 2020.
27. Dey B, Raphael V, Khonglah Y and Lynrah KG: Immunohistochemical analysis of P53 and PRB in esophageal squamous cell carcinoma. *J Clin Diagn Res* 8: FC01-FC03, 2014.
28. Wang C, Yan G, Zhang Y, Jia X and Bu P: Long non-coding RNA MEG3 suppresses migration and invasion of thyroid carcinoma by targeting of Rac1. *Neoplasma* 62: 541-549, 2015.
29. Kim HJ, Ryu KJ, Kim M, Kim T, Kim SH, Han H, Kim H, Hong KS, Song CY, Choi Y, *et al.*: RhoGDI2-mediated Rac1 recruitment to filamin A enhances Rac1 activity and promotes invasive abilities of gastric cancer cells. *Cancers (Basel)* 14: 255, 2022.
30. Yang Q, Luo GY, Li Y, Shan HB, Wang HY and Xu GL: Expression of Rac-1 related to tumor depth, lymph node metastasis and patient prognosis in esophageal squamous cell carcinoma. *Med Oncol* 30: 689, 2013.
31. Li L, Feng R, Xu Q, Zhang F, Liu T, Cao J and Fei S: Expression of the β 3 subunit of Na⁺/K⁺-ATPase is increased in gastric cancer and regulates gastric cancer cell progression and prognosis via the PI3/AKT pathway. *Oncotarget* 8: 84285-84299, 2017.
32. Sun MZ, Kim JM, Oh MC, Safaei M, Kaur G, Clark AJ, Bloch O, Ivan ME, Kaur R, Oh T, *et al.*: Na⁺/K⁺-ATPase β 2-subunit (AMOG) expression abrogates invasion of glioblastoma-derived brain tumor-initiating cells. *Neuro Oncol* 15: 1518-1531, 2013.
33. Sun R, Xie HY, Qian JX, Huang YN, Yang F, Zhang FL, Shao ZM and Li DQ: FBXO22 possesses both protumorigenic and antimetastatic roles in breast cancer progression. *Cancer Res* 78: 5274-5286, 2018.
34. Cheng Z, Geng H, Cheng Y, Dong N, Ning F, Yu Z, Jian J and Chen S: Effects of MiR-210 on proliferation, apoptosis and invasion abilities of esophageal cancer cells. *J BUON* 23: 814-819, 2018.
35. Hezova R, Kovarikova A, Srovnal J, Zemanova M, Harustiak T, Ehrmann J, Hajdich M, Sachlova M, Svoboda M and Slaby O: MiR-205 functions as a tumor suppressor in adenocarcinoma and an oncogene in squamous cell carcinoma of esophagus. *Tumour Biol* 37: 8007-8018, 2016.
36. Botelho AFM, Pierezan F, Soto-Blanco B and Melo MM: A review of cardiac glycosides: Structure, toxicokinetics, clinical signs, diagnosis and antineoplastic potential. *Toxicol* 158: 63-68, 2019.
37. Tverskoi AM, Poluektov YM, Klimanova EA, Mitkevich VA, Makarov AA, Orlov SN, Petrushanko IY and Lopina OD: Depth of the steroid core location determines the mode of Na₂K-ATPase inhibition by cardiotonic steroids. *Int J Mol Sci* 22: 13268, 2021.
38. Rupaimoole R, Yoon B, Zhang WC, Adams BD and Slack FJ: A High-throughput small molecule screen identifies ouabain as synergistic with miR-34a in killing lung cancer cells. *iScience* 23: 100878, 2020.
39. Chang YM, Shih YL, Chen CP, Liu KL, Lee MH, Lee MZ, Hou HT, Huang HC, Lu HF, Peng SF, *et al.*: Ouabain induces apoptotic cell death in human prostate DU 145 cancer cells through DNA damage and TRAIL pathways. *Environ Toxicol* 34: 1329-1339, 2019.
40. Klimanova EA, Fedorov DA, Sidorenko SV, Abramicheva PA, Lopina OD and Orlov SN: Ouabain and marinobufagenin: Physiological effects on human epithelial and endothelial cells. *Biochemistry (Mosc)* 85: 507-515, 2020.
41. Akimova OA, Tverskoi AM, Smolyaninova LV, Mongin AA, Lopina OD, La J, Dulin NO and Orlov SN: Critical role of the α 1-Na(+), K(+)-ATPase subunit in insensitivity of rodent cells to cytotoxic action of ouabain. *Apoptosis* 20: 1200-1210, 2015.
42. Petrič M, Vidović A, Dolinar K, Miš K, Chibalin AV and Pirkmajer S: Phosphorylation of Na⁺/K⁺-ATPase at Tyr10 of the α 1-Subunit is suppressed by AMPK and enhanced by ouabain in cultured kidney cells. *J Membr Biol* 254: 531-548, 2021.
43. Yang JL, Yang MD, Chen JC, Lu KW, Huang YP, Peng SF, Chueh FS, Liu KC, Lin TS, Chen PY and Chen WJ: Ouabain induces DNA damage in human osteosarcoma U-2 OS cells and alters the expression of DNA damage and DNA Repair-associated proteins. *In Vivo* 35: 2687-2696, 2021.

