AT-101 induces G_1/G_0 phase arrest via the β-catenin/cyclin D1 signaling pathway in human esophageal cancer cells

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Abstract. AT-101, an orally available and well-tolerated natural pan-Bcl-2 family protein inhibitor, has been reported to be effective against a variety of cancers. However, the mechanisms whereby AT-101 exhibits anticancer activity have not been fully elucidated. In this study, we demonstrated that AT-101 reduced the cell viability of human esophageal cancer cells by inducing G_1/G_0 phase arrest and apoptosis. Apoptotic cell death occurred later than cell cycle arrest, as evidenced by an increase in the proportion of Annexin V-positive cells and cleaved caspase-3, -9 and PARP protein levels. AT-101 markedly downregulated the protein levels of phospho-retinoblastoma (Ser 780) and cyclin D1, whereas it elevated protein levels of p53 and p21$^{Waf1/Cip1}$, contributing to the inhibition of cell cycle progression. Moreover, AT-101 substantially reduced β-catenin expression. XAV-939, a small molecule that inhibits the Wnt/β-catenin signaling pathway by facilitating β-catenin degradation, lowered β-catenin and cyclin D1 protein expression to an extent similar to AT-101. XAV-939 alone resulted in G_1/G_0 phase arrest and further induced cell cycle arrest in combination with AT-101, suggesting that the β-catenin/cyclin D1 signaling pathway mediated, at least in part, the cell cycle arrest induced by AT-101. The present study may shed new light on the anticancer activity of AT-101 in relation to cell cycle arrest as well as apoptosis in human esophageal cancer cells.

Introduction

Esophageal cancer is the eighth most commonly diagnosed type of cancer and results in more than 400,000 deaths worldwide annually (1,2). The prognosis of this malignancy remains poor due to the absence of targeted therapeutics and resistance to conventional DNA-damaging agents. Evading apoptosis, a hallmark of cancer, is a major cause of tumor progression, resistance to chemotherapy and treatment failure (3). Many signaling pathways converge on Bcl-2 family members to regulate the induction of apoptosis. In cancer, initiation of apoptosis is hard to achieve due to the disturbed balance between anti-apoptotic members (Bcl-2/Bcl-xL/Mcl-1/A1/Bcl-w) and pro-apoptotic members that include multidomain Bak, Bax and the BH3-only proteins (Bad/Bim/Bid/Puma/Noxa) (4,5). The development of BH3 mimetics, which inhibit anti-apoptotic Bcl-2 family members, appears to be an attractive strategy for restoring the apoptotic response.

High Bcl-2 expression has been reported to be correlated with a low apoptotic index and is associated with the histopathological grade in human esophageal carcinomas (6). By contrast, low Bax expression was found to be negatively associated with the survival of patients with locally advanced esophageal cancer (7). Furthermore, a recent study demonstrated that the Bcl-2 protein level was abnormally elevated with the progression of cancer in carcinogen-induced esophageal cancer in rats (8). These findings suggest that the inhibition of anti-apoptotic Bcl-2 proteins holds promise as a therapeutic strategy for esophageal cancer.

AT-101, the R-(-) enantiomer of gossypol acetic acid, is a polyphenolic compound derived from the cottonseed plant (9). AT-101 is capable of inhibiting the anti-apoptotic

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function of Bcl-2, Bcl-xL, Mcl-1 and Bcl-w by operating as a BH3 mimetic (10). The therapeutic potential against multiple cancers of AT-101 alone or in combination with standard anticancer therapies has been widely reported preclinically (11-16). Notably, the preliminary activity of AT-101 was also observed in patients with esophageal cancer in a phase I study (17). However, the mechanisms underlying the anticancer activity of AT-101 in esophageal cancer remain poorly understood. Our study aimed to investigate the effects of AT-101 on cell viability in relation to cell cycle progression and apoptosis in esophageal cancer cells, and explore the signaling pathways underlying the effects of AT-101 on cell cycle progression. We hope that this study will shed new light on the anticancer mechanism of AT-101.

Materials and methods

Reagents. AT-101 was purchased from Selleck Chemicals (Houston, TX, USA). XAV-939 (a small-molecule inhibitor of the Wnt/β-catenin pathway) and WAY-262611 (a WNT pathway agonist) were purchased from Target Mol (Beijing, China). Antibodies for caspase-9 (cat. no. 9502), caspase-3 (cat. no. 9662), PARP (cat. no. 9532), β-actin (cat. no. 4970), p21$^{Waf1/Cip1}$ (cat. no. 2947), p27^{kip1} (cat. no. 3686), phospho (p)-β-catenin (cat. no. 9561), β-catenin (cat. no. 4581), cyclin D1 (cat. no. 2978), cyclin-dependent kinase (CDK)2 (cat. no. 2546), p-retinoblastoma (Rb. cat. no. 9307) and Rb (cat. no. 9309) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA) and used at the appropriate dilution of 1:1,000. Antibodies for p-p53 (cat. no. sc-101762), p53 (cat. no. SC-126), CDK4 (cat. no. SC-601) and CDK6 (cat. no. SC-7961) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA) and used at the appropriate dilution and diluent as recommended in the product datasheet. The membranes were then incubated for 1 h at room temperature with secondary peroxidase-conjugated antibodies (1:2,000) (anti-rabbit antibodies; Cell Signaling Technology, Inc.; cat. no. 7074; anti-mouse antibodies, Cell Signaling Technology, Inc.; cat. no. 7076). Chemiluminescent signals were then developed with Lumiglo reagent (Cell Signaling Technology, Inc.; cat. no. 7003) and exposed to X-ray film (Fujifilm Europe GmbH, Dusseldorf, Germany).

Cell culture and cell viability assay. EC109 and CaES-17 are human esophageal squamous cell carcinoma cell lines. EC109 and CaES-17 cells originate from squamous cell carcinoma in the middle part of the human esophagus. They both are epithelioid adherent cells. EC109 cells were obtained from the Cancer Institute of the Chinese Academy of Medical Sciences (Beijing, China), while CaES-17 cells were obtained from the China Center for Type Culture Collection (Wuhan, China). EC109 cells were routinely maintained in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C with 5% CO_2, while CaES-17 cells were grown in MEM (Corning Cellgro, Corning, NY, USA). Both media were supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin G and 100 μg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.). Cell viability was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany).

EC109 and CaES-17 cells were plated into 96-well plates and allowed to adhere overnight. Cells were treated with various concentrations of AT-101 for 48 h. After 48 h, MTT solution was added at a final concentration of 0.5 mg/ml, and the cells were incubated for a further 4 h. The absorbance of the colored formazan product was determined at 570 nm using a multiwall plate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Cell cycle analysis. After AT-101 treatment, EC109 and CaES-17 cells were collected, washed twice with phosphate-buffered saline (PBS) and fixed with ice-cold 70% ethanol in PBS for at least 18 h. Cells were then stained with 50 μg/ml propidium iodide (PI; Sigma-Aldrich; Merck KGaA) and 0.5 μg/ml RNase-A (Sigma-Aldrich; Merck KGaA) at 37°C for 30 min and analyzed by BD FACSCanto™ II flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA). Cell cycle was analyzed based on the DNA contents using FlowJo 7.6 software (Treestar, Inc., Ashland, OR, USA).

Apoptosis assay. After AT-101 treatment, adherent cells were harvested through trypsinization (Invitrogen; Thermo Fisher Scientific, Inc.) and then combined with unattached cells. Apoptosis was determined using the Fluorescein Isothiocyanate (FITC) Annexin V Apoptosis Detection Kit (BD Biosciences) according to the manufacturer's instructions. The extent of apoptosis was quantified as the proportion of Annexin V-positive cells.

Western blot analysis. Cells were lysed in radioimmunoprecipitation buffer containing protease inhibitors and phosphatase inhibitor cocktail (Sigma-Aldrich; Merck KGaA). Equal amounts of protein were subjected to SDS-PAGE and then transferred to polyvinylidene fluoride membranes (Roche Diagnostics, Indianapolis, IN, USA). The membranes were blocked with 5% skimmed milk solution for 1 h and then probed with the indicated primary antibodies overnight at 4°C and secondary horseradish peroxidase-conjugated antibodies for 1 h at room temperature. Chemiluminescent signals were then developed with Lumiglo reagent (Cell Signaling Technology, Inc.) and exposed to X-ray film (Fujifilm Europe GmbH). Quantification of protein in luminescent bands was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Inhibition and activation assays. EC109 and CaES-17 cells (1x10^5/dish) were plated into 6-well plates and allowed to adhere overnight. Cells were treated with AT-101 (2 μM) alone or in combination with the β-catenin inhibitor XAV-939 (5 μM) or β-catenin activator WAY-262611 (5 μM) for 48 h. The untreated cells were used as negative control. After drug treatment, cells were collected for conducting western blot analysis or subjected to cell cycle analysis. The gray level of the band from western blot was analyzed by ImageJ software (National Institutes of Health).

Statistical analysis. All the experiments described were independently repeated at least three times. The data are representative of multiple experiments and expressed as the mean $\pm$ SD. The statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. P-values were defined as *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and NS, not significant (as shown in the figures).
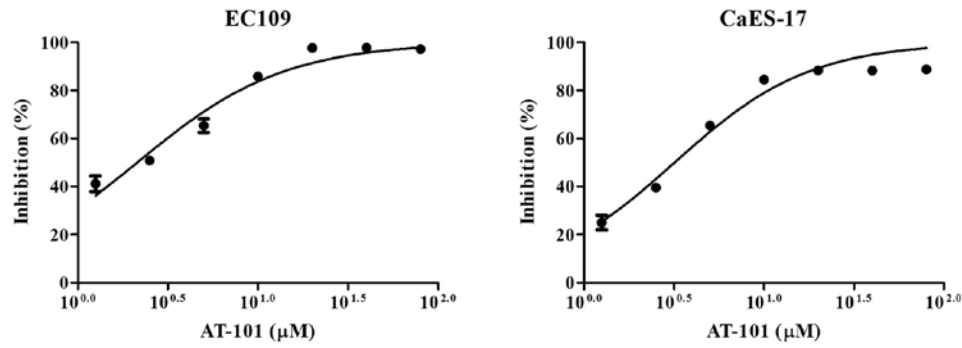
Figure 1. AT-101 reduces the cell viability of human esophageal cancer cells. Human esophageal cancer cells EC109 and CaES-17 were exposed to increasing concentrations of AT-101 for 48 h. Then the cell viability was measured by an MTT assay. Data are presented as the mean ± SD from three independent experiments.

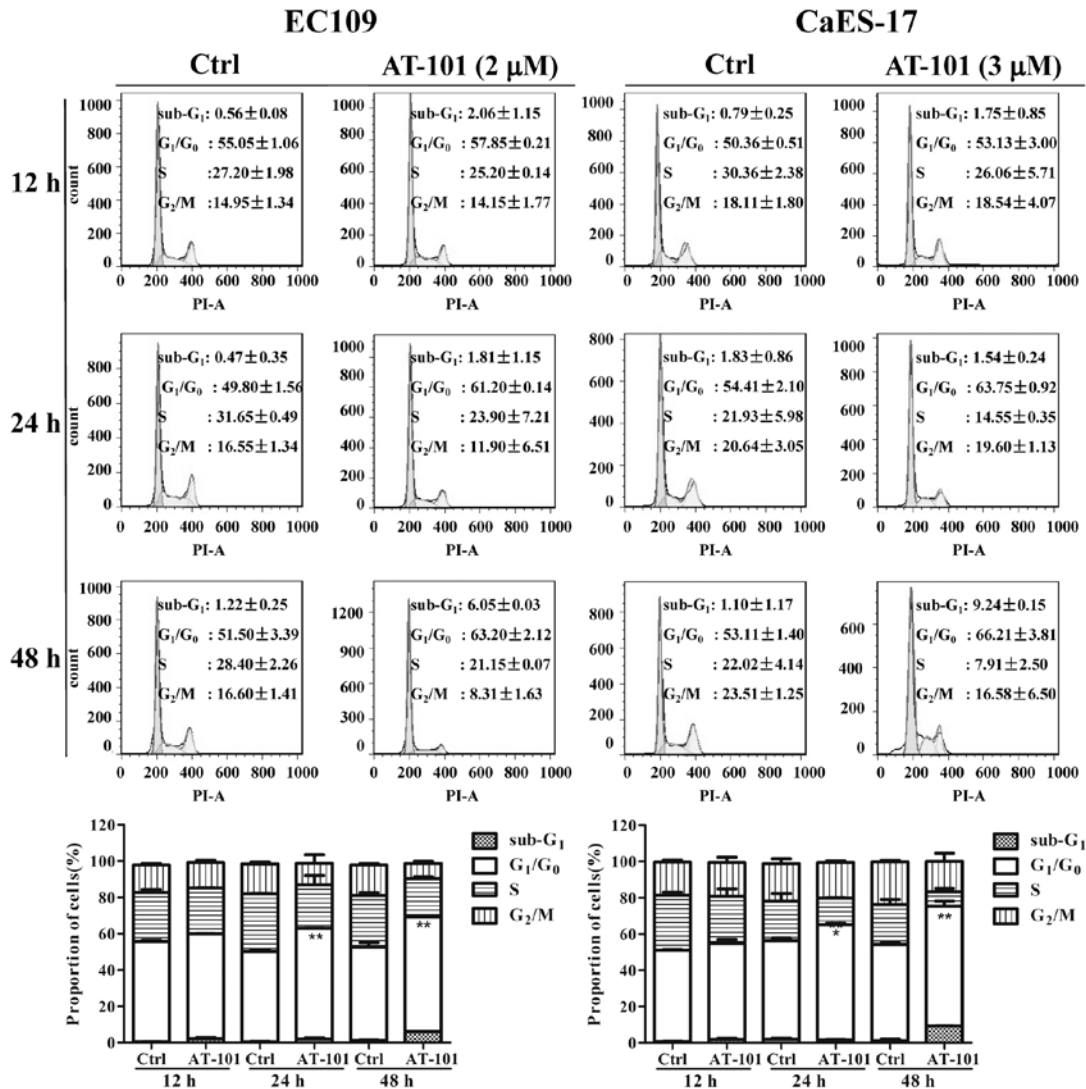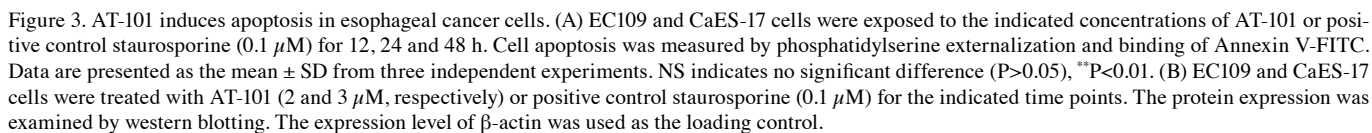Figure 2. AT-101 induces G_1/G_0 phase arrest in esophageal cancer cells. EC109 and CaES-17 cells were treated with the indicated concentrations of AT-101 for 12, 24 and 48 h, and their DNA contents were measured by flow cytometric analysis. The accumulation of G_1/G_0 phase cells induced by AT-101 is showed in the DNA histogram. Data are presented as the mean ± SD from three independent experiments. $^*P<0.05$, $^{**}P<0.01$ compared with the control.

Results

AT-101 reduces the cell viability of human esophageal cancer cells. To study the effects of AT-101 on cell viability, IC_{50} values of AT-101 were measured in two human esophageal carcinoma cell lines (EC109 and CaES-17 cells) by an MTT assay. As shown in Fig. 1, AT-101 significantly reduced the cell viability of EC109 and CaES-17 cells in a dose-dependent manner, with IC_{50} values of 2.1±0.1 and 3.2±0.1 μM, respectively.

Figure 3. AT-101 induces apoptosis in esophageal cancer cells. (A) EC109 and CaES-17 cells were exposed to the indicated concentrations of AT-101 or positive control staurosporine (0.1 μM) for 12, 24 and 48 h. Cell apoptosis was measured by phosphatidylserine externalization and binding of Annexin V-FITC. Data are presented as the mean ± SD from three independent experiments. NS indicates no significant difference (P>0.05), **P<0.01. (B) EC109 and CaES-17 cells were treated with AT-101 (2 and 3 μM, respectively) or positive control staurosporine (0.1 μM) for the indicated time points. The protein expression was examined by western blotting. The expression level of β-actin was used as the loading control.

AT-101 induces G_1/G_0 phase arrest. The results from the flow cytometry-based cell cycle analysis showed that a 24- or 48-h treatment with AT-101 (2 μM) induced a substantial accumulation of EC109 cells in the G_1/G_0 phase. A reciprocal reduction in the proportion of cells in the S phase and G_2/M phase was also observed in the AT-101-treated EC109 cells. The G_1-arresting effect of AT-101 was confirmed in CaES-17 cells. In addition, exposure to AT-101 for 48 h increased the number of cells in the sub-G_1 phase in both cell lines, which is indicative of apoptotic cells (Fig. 2).

AT-101 induces apoptosis. In order to further confirm the pro-apoptotic action of AT-101, flow cytometry-based FITC Annexin V/PI double staining was performed. As shown in Fig. 3A, a 12- or 24-h treatment of AT-101 (2 μM) did not significantly increase the proportion of Annexin V-positive cells. However, when exposure time was prolonged to 48 h, AT-101 significantly elevated the cell population with Annexin V-positive staining. The pro-apoptotic effect of AT-101 was also observed in CaES-17 cells. Staurosporine (0.1 μM) was used as a positive control for the induction of apoptosis (Fig. 3A). Moreover, AT-101 exposure for 48 h substantially induced cleavage of caspase-3, caspase-9 and PARP in EC109 and CaES-17 cells, whereas a 12- or 24-h treatment did not exert such an effect (Fig. 3B).
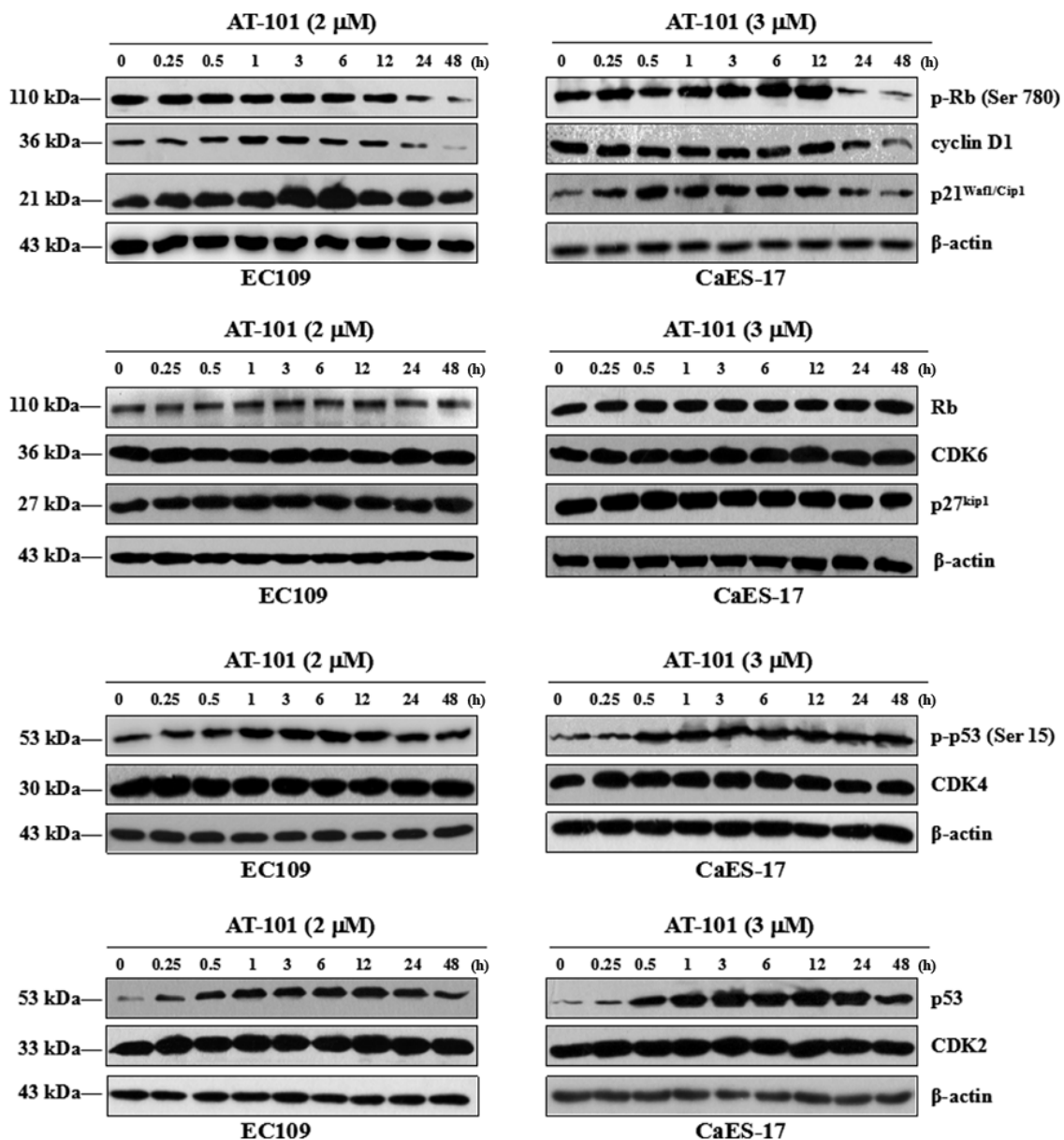
Figure 4. AT-101 regulates the expression of cell cycle regulatory proteins. EC109 and CaES-17 cells were treated with AT-101 (2 and 3 μM, respectively) for the indicated time points. The protein expression was measured by western blotting. The expression level of β-actin was used as the loading control.

AT-101 regulates the expression of cell cycle regulators.
Given the ability of AT-101 to arrest cells in the G_1/G_0-phase, we further examined the expression level of the regulatory proteins involved in G_1-to-S transition in EC109 and CaES-17 cells treated with AT-101. In this respect, there was no significant change in the expression of CDKs, including CDK2, CDK4 and CDK6, whereas cyclin D1 expression was substantially decreased after AT-101 treatment. AT-101 obviously increased the phosphorylation of p53 at Ser15 as well as the p53 protein level. Although the expression of the CDK inhibitor p27^{kip1} was not affected in response to AT-101 exposure, the protein level of another CDK inhibitor p21$^{Waf1/Cip1}$ was substantially elevated within 12 h and went back to basal level after 24 h treatment. Moreover, AT-101 did not alter the total Rb protein levels, whereas it substantially reduced the phosphorylation of Rb at Ser780 (Fig. 4).

AT-101 induces G_1/G_0 phase arrest via β-catenin/cyclin D1 signaling. Cyclin D1 plays a critical role in G_1-to-S transition. Thus, we further determined the mechanisms whereby AT-101 decreases the protein level of cyclin D1. In this scenario, AT-101 dramatically decreased the protein expression of β-catenin, with a concomitant increase in phosphorylated β-catenin at Ser33, Ser37 and Thr41, which facilitated its degradation by GSK-3β (Fig. 5A). Notably, XAV-939, a small molecule that inhibits the Wnt/β-catenin signaling pathway by stimulating β-catenin degradation, decreased β-catenin as well as cyclin D1 protein expression to an extent analogous to AT-101 (Fig. 5B). Moreover, both AT-101 and XAV-939 significantly arrested cells in the G_1/G_0 phase. The combination of these two compounds further induced G_1/G_0 phase arrest (Fig. 5C). On the contrary, the selective β-catenin agonist WAY-262611 recovered the decreased expression levels of β-catenin and cyclin D1 as well as G_1/G_0 phase arrest caused
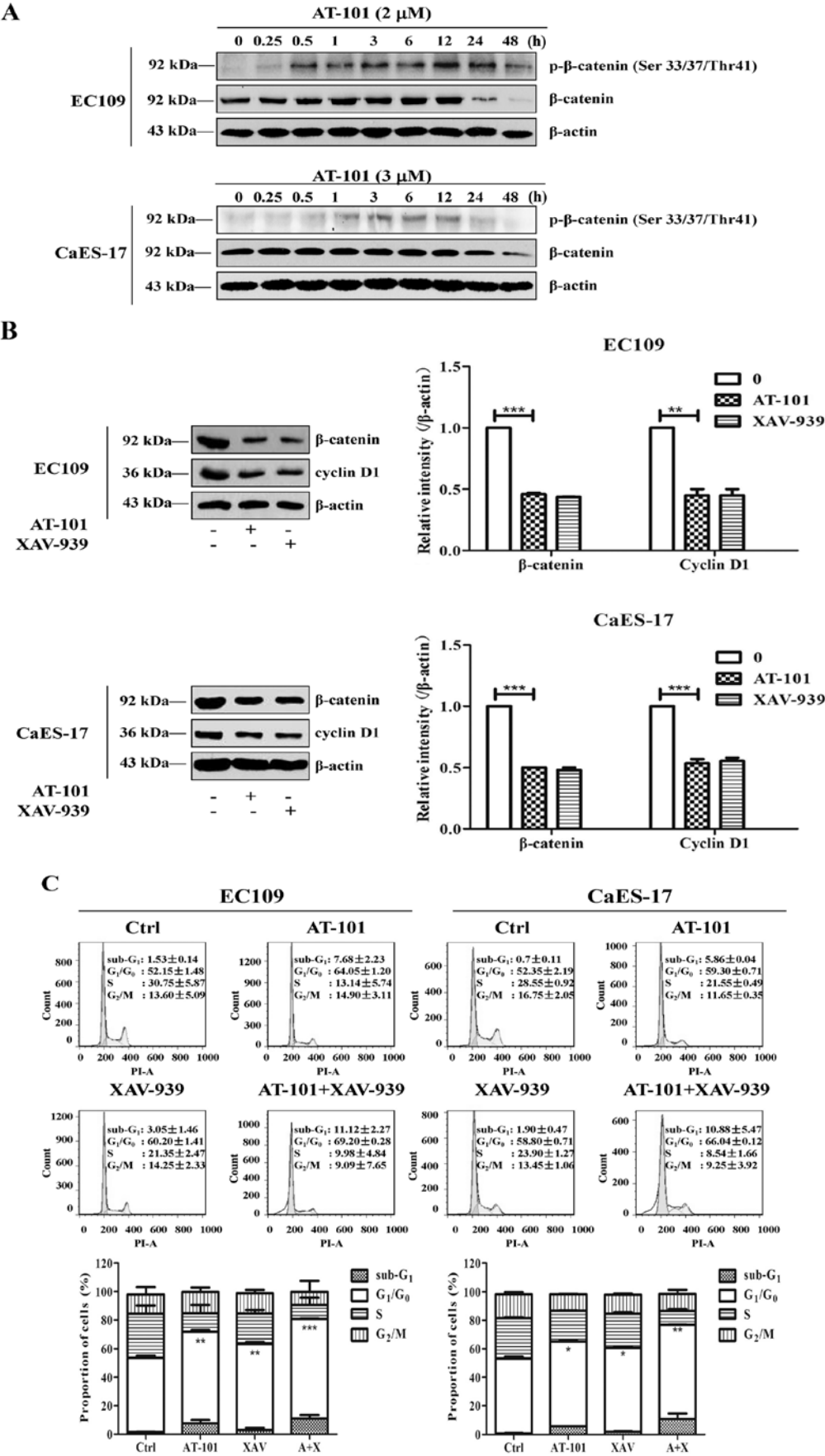
Figure 5. AT-101 induces G_1/G_0 phase arrest via the β-catenin/cyclin D1 signaling pathway. (A) EC109 and CaES-17 cells were treated with AT-101 (2 and 3 µM, respectively) for the indicated time points. (B) EC109 and CaES-17 cells were treated with AT-101 (2 and 3 µM, respectively) alone or in combination with the β-catenin inhibitor XAV-939 (5 µM) for 48 h. The protein expression was measured by western blotting. β-actin was used as the loading control. (C) EC109 and CaES-17 cells were treated with AT-101 (2 and 3 µM, respectively) alone or in combination with the β-catenin inhibitor XAV-939 (5 µM) for 48 h. Their DNA contents were measured by flow cytometric analysis.
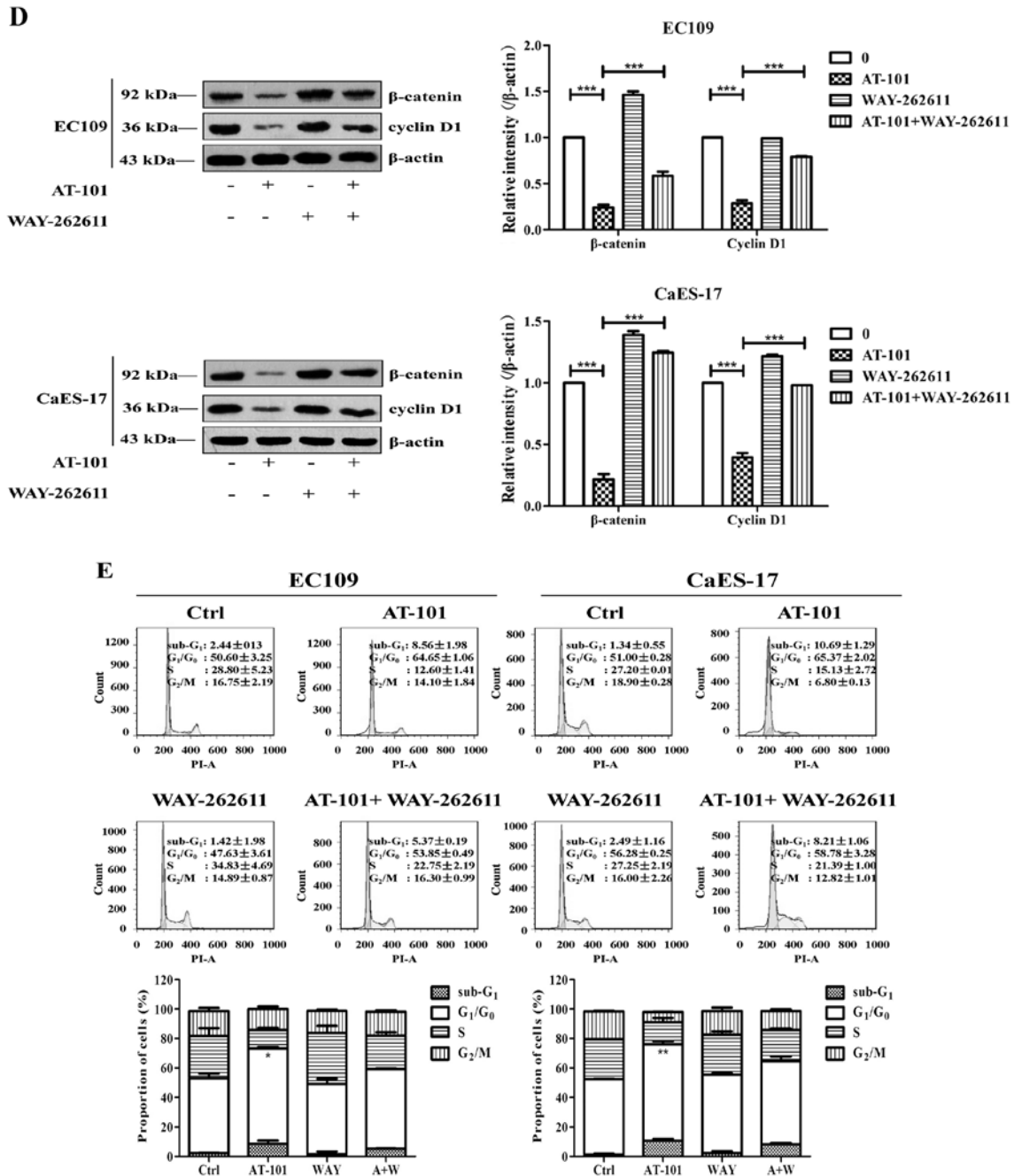
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Figure 5. Continued. (D) EC109 and CaES-17 cells were treated with AT-101 (2 and 3 μM, respectively) alone or in combination with the selective β-catenin agonist WAY-262611 (5 μM) for 48 h. The protein expression was measured by western blotting. β-actin was used as the loading control. (E) EC109 and CaES-17 cells were treated with AT-101 (2 and 3 μM, respectively) alone or in combination with WAY-262611 (5 μM) (A+W) for 48 h. Their DNA contents were measured by flow cytometric analysis. Data are presented as the mean ± SD from three independent experiments. NS indicates no significant difference (P>0.05), *P<0.05, **P<0.01, ***P<0.001 compared with the control.

by AT-101 (Fig. 5D and E). These findings suggest that AT-101 may induce G_1/G_0 phase arrest through β-catenin/cyclin D1 signaling.

Discussion

Esophageal cancer is a lethal disease with a dismal prognosis. The therapeutic benefits for patients with esophageal cancer are still limited after decades of research. New therapies to improve patient outcome are urgently needed. In this study, we demonstrated that the Bcl-2 inhibitor AT-101 strongly induced

G_1/G_0 phase arrest followed by apoptosis in human esophageal cancer cell lines. Furthermore, the β-catenin/cyclin D1 pathway contributed to its cell cycle arresting activity.

There is accumulating evidence showing that the antitumor activity of Bcl-2 inhibitors is not limited to the initiation of apoptosis, despite the fact that Bcl-2 inhibitors were originally designed to induce apoptosis. In this regard, Bcl-2 inhibitors have been reported to block cell cycle progression (18,19), regulate autophagy (20-22) and inhibit tumor stemness (23). It has been showed that AT-101 significantly suppressed the hedgehog (Hh) signaling pathway and suppressed the growth

of Hh-driven cancer *in vitro* and *in vivo* (24). Moreover, it was reported that AT-101 induced autophagy in apoptosis-resistant prostate cancer by modulating Bcl-2-Beclin1 interaction in the endoplasmic reticulum (25). In addition, gossypol induced apoptosis via a reactive oxygen species-independent mitochondrial pathway (26). Moon *et al* (27) reported that gossypol suppressed NF-κB activity and induced apoptosis in human leukemia U937 cells. Our data showed that AT-101 reduced cell viability in esophageal cancer cells by inducing G_1/G_0 phase arrest and apoptosis. AT-101 significantly increased the G_1/G_0 cell population following a 24-h treatment. However, AT-101 induced obvious apoptosis after 48 h treatment, evidenced by an increase in Annexin V-positive cells as well as cleavage of caspase-3, caspase-9 and PARP. Notably, AT-101 obviously decreased the expression of Mcl-1 and Bcl-2 both in EC109 and CaES-17 cells after a 48-h exposure, which may also contribute to its pro-apoptotic action (data not shown). Although cell cycle progression blockage may activate signaling pathways leading to apoptosis (28), whether G_1/G_0 phase arrest induced by AT-101 has a potential connection with apoptosis occurring later, warrants further investigation. On the basis of the data presented in this study, AT-101-induced cell cycle arrest results in its anticancer activity and occurs prior to apoptosis.

In view of the G_1/G_0 phase arrest being induced by AT-101, we further explored the effect of AT-101 on the molecular pathways mediating G_1 to S phase transition. Activity of CDKs, critical master regulators of the cell cycle, is regulated by the abundance of their cyclin partners and by association with endogenous CDK inhibitors. The active CDK-cyclin complex targets the Rb protein for phosphorylation, which is followed by the release of E2F transcription factors that activate G_1/S-phase gene expression (29-31). In our study, AT-101 treatment did not affect the CDK2/4/6 protein levels but significantly decreased cyclin D1 expression. Furthermore, AT-101 substantially increased the CDK inhibitor p21[Waf1/Cip1] protein expression, although it did not change the expression of another CDK inhibitor p27[kip1]. In parallel with the decrease in cyclin D1 and the increase in p21[Waf1/Cip1], p-Rb (Ser780) expression was downregulated after cells were treated with AT-101, whereas the protein level of total Rb remained quite stable. Notably, we also observed an obvious increase in the protein level of p53 and p-p53 (Ser15) in response to AT-101 treatment. Given the fact that p53 Ser15 phosphorylation has been reported to promote both the accumulation and activation of p53 (32), we proposed that AT-101-induced phosphorylation of p53 could contribute to the increased protein level of p53. The tumor suppressor protein p53 plays a key role in both cell cycle arrest and apoptosis. In addition, p21[Waf1/Cip1] is a direct transcription target of p53 (33). These findings suggest that elevated protein expression of p53 in response to AT-101 exposure could take part in both apoptosis and G_1/G_0 phase arrest by increasing the p21[Waf1/Cip1] protein level.

The cyclin D1 gene has been demonstrated to be a direct target for transactivation via the β-catenin/LEF-1 pathway through a LEF-1 binding site in the cyclin D1 promoter (34). Thus, we determined whether the increased cyclin D1 protein level in response to AT-101 treatment was regulated by β-catenin. In this respect, AT-101 substantially lowered protein expression of β-catenin, which could be due to the

acceleration of its degradation, evidenced by the increase of p-β-catenin at Ser33, Ser37 and Thr41. Moreover, the β-catenin inhibitor XAV-939 suppressed the protein level of β-catenin and cyclin D1, mimicking the effect of AT-101. Similarly, XAV-939 treatment induced cell cycle arrest in G_1/G_0 phase alone and further arrested cells in combination with AT-101. These findings suggest that the suppression of β-catenin by AT-101 could contribute to the downregulation of cyclin D1, leading to G_1/G_0 cell cycle phase arrest.

In conclusion, our study showed that the Bcl-2 inhibitor AT-101 induces G_1/G_0 phase arrest prior to apoptosis in human esophageal cancer cells. Furthermore, AT-101 blocks cell cycle progression by regulating a variety of proteins that mediate G_1 to S phase transition. Of note, our previous studies have demonstrated that other Bcl-2 inhibitors with different chemical structures from AT-101 exhibited a similar pattern on cell cycle progression as well as apoptosis (18,20). Thus, we propose that G_1/G_0 phase arrest might not be a unique cellular response to AT-101 but instead a common response to Bcl-2 inhibitors, suggesting a potential link between Bcl-2 family members and cell cycle progression. In summary, our findings may shed new light on the anticancer activity of AT-101 and its potential application for patients with esophageal cancer.

Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

SL, YL and LY designed the research; FQ, LD, DZ, QL and XZ conducted the experiments and analyzed the data; FQ and LY drafted the manuscript; SL, YL and LY revised the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors state that they have no competing interests.

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