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E2F1 downregulation by arsenic trioxide in lung adenocarcinoma

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Abstract. Lung cancer is one of the most common cancers worldwide. Arsenic trioxide (ATO) has been approved by the US Food and Drug Administration for the treatment of acute promyelocytic leukemia. Nonetheless preliminary data have suggested potential activity of ATO in solid tumors including lung cancer. This study aimed to examine the underlying mechanisms of ATO in the treatment of lung adenocarcinoma. Using a panel of 7 lung adenocarcinoma cell lines, the effects of ATO treatment on cell viability, expression of E2F1 and its downstream targets, phosphatidylserine externalization, mitochondrial membrane depolarization and alteration of apoptotic/anti-apoptotic factors were studied. Tumor growth inhibition *in vivo* was investigated using a nude mouse xenograft model. ATO decreased cell viability with clinically achievable concentrations (8 μ M) in all cell lines investigated. This was accompanied by reduced expression of E2F1, cyclin A2, skp2, c-myc, thymidine kinase and ribonucleotide reductase M1, while p-c-Jun was upregulated. Cell viability was significantly decreased with E2F1 knockdown. Treatment with ATO resulted in phosphatidylserine externalization in H23 cells and mitochondrial membrane depolarization in all cell lines, associated with truncation of Bid, downregulation of Bcl-2, upregulation of Bax and Bak, caspase-9 and -3 activation and PARP cleavage. Using the H358 xenograft model, the tumor growth was suppressed in the ATO treatment group during 8 days of treatment, associated with downregulation of E2F1 and upregulation of truncated Bid and cleaved caspase-3. In conclusion, ATO has potent *in vitro* and *in vivo* activity in lung adenocarcinoma, partially mediated through E2F1 downregulation and apoptosis.

Introduction

Based on the updated GLOBOCAN project of the World Health Organization in 2012, breast, prostate and lung remain

the three most common global cancers (<http://globocan.iarc.fr/>). The incidence and mortality rates of lung cancer have increased from 12.7 to 16.7% and 18.2 to 23.2% of all cancers respectively since 2008. Lung cancer is histologically classified as non-small cell (NSCLC) or small cell carcinoma (SCLC), and is associated with distinct treatment implications. The majority (85%) of lung cancer cases are NSCLC, comprised mostly of adenocarcinoma. Notably, tobacco smoking, pre-existing lung disease, diet, occupational exposure, exposure to estrogen, and genetic predisposition are the major causes of lung cancer (1).

Systemic chemotherapy remains the cornerstone treatment for advanced or metastatic NSCLC. First-line platinum doublets with newer agents (docetaxel, gemcitabine, paclitaxel, pemetrexed or vinorelbine) and salvage monotherapy with docetaxel or pemetrexed have conferred only a modest survival benefit with 5-year overall survival <5% (2,3). Emerging molecularly-targeted therapy against epidermal growth factor receptor or anaplastic lymphoma kinase has provided a superior treatment option to systemic chemotherapy in patients with NSCLC driven by actionable targets. Nonetheless development of acquired drug resistance ~1 year following targeted therapy is almost inevitable (4). Thus novel effective treatment for NSCLC is urgently needed.

Arsenic trioxide (ATO), which is now a standard treatment for acute promyelocytic leukemia, has demonstrated promising activity in solid tumors including lung cancer (5-8). Nonetheless the exact mechanisms of action of ATO in NSCLC have not been fully elucidated. We have recently reported the role of ATO-induced suppression of thymidylate synthase (TYMS) in 4 lung adenocarcinoma cell lines with basal expression (9), while ATO might have other effects in cell lines not expressing TYMS. The role of E2F1 is still not fully elucidated, therefore, a panel of 7 lung adenocarcinoma cell lines with basal E2F1 expression was studied. E2F1 is a transcription factor that controls cell fate including apoptosis (10) and DNA synthesis (11). Depending on specific cancer types, the E2F1 gene can serve as an oncogene (12) with a prognostic role (13) or a tumor suppressor gene (14). This study aimed to investigate the action of ATO in lung adenocarcinoma, with an emphasis on E2F1-mediated pathways and apoptosis.

Materials and methods

Cell lines and reagents. A panel of 7 lung adenocarcinoma cell lines was obtained from the American Type Culture Collection

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(Manassas, VA, USA). Cells were incubated in RPMI-1640 culture medium (Gibco®, Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) (Gibco) in a humidified atmosphere at 37°C with 5% CO₂. ATO was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Assay of cell viability. Cell viability following ATO treatment was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (9).

Western blot analysis of cell lysates. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis were carried out as described (15). Primary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). β -actin (Sigma-Aldrich) was used as a house-keeping protein.

E2F1 siRNA knockdown. Cells were cultured for 6 h with a mixture of transfection reagent and control (sc-37007) or E2F1 (sc-29297) siRNA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) in RPMI-1640 medium. The transfected cells were maintained in 1% FBS-containing medium for 2 days. Cell viability and E2F1 expression were assessed by MTT assay and western blot analysis, respectively (9).

Phycoerythrin (PE)-conjugated Annexin V and 7-(aminoactinomycin D) AAD staining. Phosphatidylserine externalization (PS) (loss of membrane asymmetry) was examined using the PE-conjugated Annexin V and 7-AAD staining method as previously described (15).

Measurement of mitochondrial membrane potential by JC-1 staining. The fluorescent dye JC-1 was employed for the determination of mitochondrial transmembrane potential. ATO-treated cells were harvested and re-suspended for 15 min at 37°C in darkness with RPMI medium containing 2.5 μ g/ml JC-1 (Sigma-Aldrich). Flow analysis was performed and signals were detected by FL-1 (525 nm) and FL-2 (575 nm) channels (Beckman FC500).

Tumor growth inhibition in vivo. Tumor xenograft was established by subcutaneous injection of 10 million H358 cells in PBS into the back of nude mice (female, 4-week-old, 10-12 g, BALB/cAnN-nu, Charles River Laboratories, Wilmington, MA, USA). Tumors were allowed to grow for 5 days before mice were randomised to two groups. ATO at 5 mg/kg (n=8) or PBS as control (n=7), was daily administered intraperitoneally. Tumor growth was measured using standard calipers and body weight of mice was recorded on alternate days. Tumor volume (V) was calculated [$V = (\text{length} \times \text{width} \times \text{width})/2$] (16). Mice were sacrificed following completion of ATO treatment. Tumor xenografts were collected and homogenized to obtain protein lysates for western blot analysis. The *in vivo* study was approved by the Committee on the Use of Live Animals in Teaching and Research (CULATR) of the University of Hong Kong (CULATR reference no. 2510-11).

Statistical analysis. Data from three individual experiments are shown as mean \pm standard deviation (SD). Comparison between groups was performed using Student's two-tailed

t-test by Prism (GraphPad Software, La Jolla, CA, USA). A p-value <0.05 was considered statistically significant.

Results

***In vitro* activity of ATO in lung adenocarcinoma.** Incubation with ATO for 48 h reduced cell viability in different lung adenocarcinoma cell lines, with IC₅₀ values ranging from 1.8 to 16.5 μ M (H23, H358, HCC827, H1650, H1975, HCC2935 and HCC4006 cells: 1.8, 16.1, 2.0, 3.8, 2.6, 12.1 and 9.0 μ M, respectively). After 72 h of ATO treatment, IC₅₀ values were further decreased (H23, H358, HCC827, H1650, H1975, HCC2935 and HCC4006 cells: 0.5, 7.4, 0.08, 4.0, 1.5, 5.7 and 4.0 μ M, respectively).

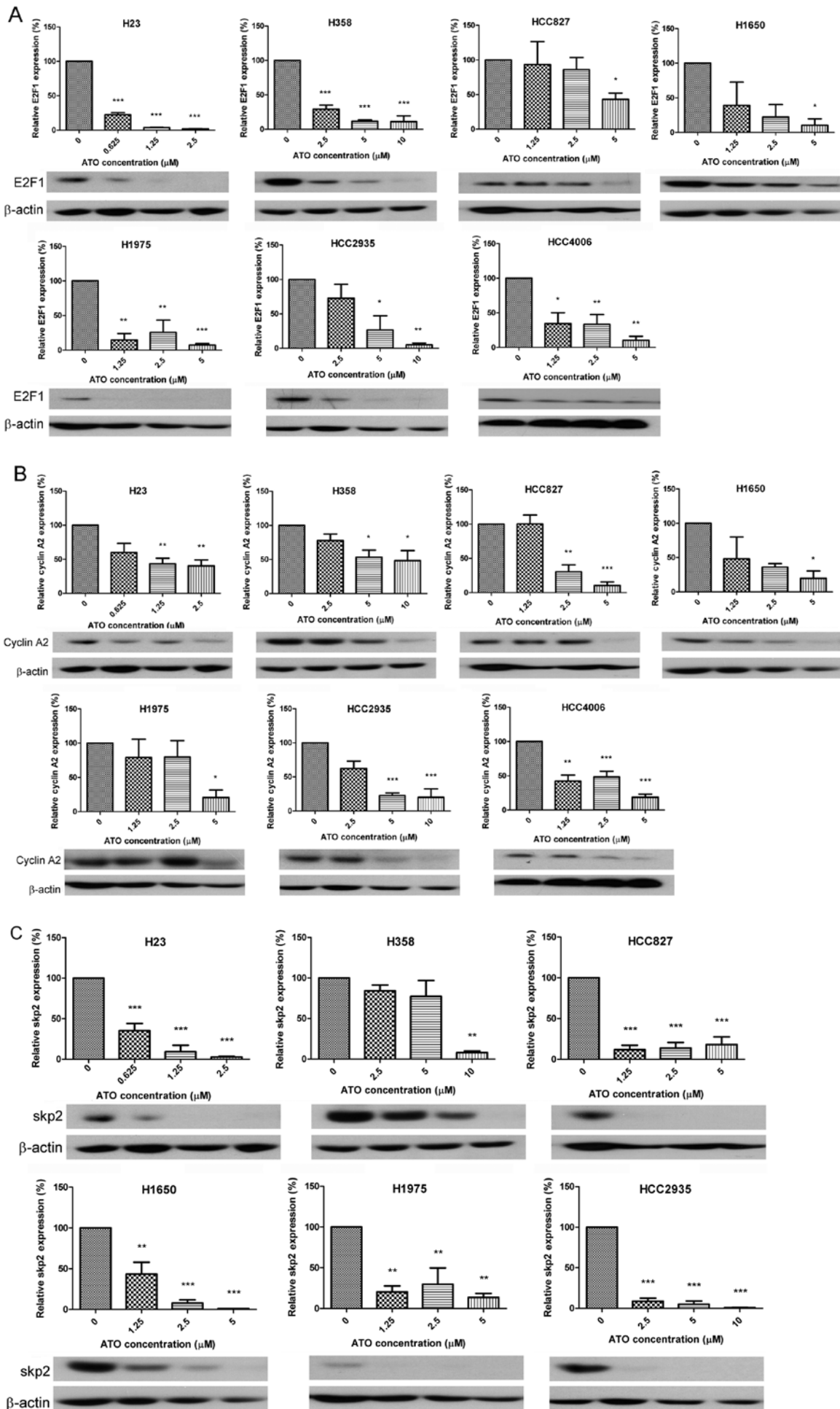
Downregulation of E2F1 and alteration of related downstream proteins. ATO reduced expression of E2F1 (Fig. 1A) in a dose-dependent manner, thus downstream targets of E2F1 were also investigated. Expression of cyclin A2 (Fig. 1B) was consistently downregulated by ATO in all cell lines. ATO also decreased the expression of *skp2* (all cell lines except HCC4006 cells) (Fig. 1C), *c-myc* (H23 and H1975 cells) (Fig. 1D), thymidine kinase (TK) (H358, H1650, HCC2935 and HCC4006 cells) (Fig. 1E) and ribonucleotide reductase M1 (RRM1) (all cell lines except HCC827 and H1975 cells) (Fig. 1F). Nevertheless ATO upregulated p-c-Jun in H23, H358, HCC827 and H1975 cells (Fig. 1G). Representative western blots are shown in Fig. 1.

E2F1 downregulation reduced cell viability. The role of E2F1 in lung adenocarcinoma was studied using siRNA knockdown. With E2F1 protein expression decreased by 50-80% compared with control siRNA treatment, cell viability was significantly decreased by 60-88% (Fig. 2).

Phosphatidylserine (PS) externalization and mitochondrial membrane depolarization induced by ATO. ATO caused PS externalization in H23 cells only (Fig. 3A). Nonetheless ATO aggravated mitochondrial membrane depolarization in all cell lines in a dose-dependent manner (Fig. 3B).

Alteration of apoptosis-related factors by ATO. Truncated BID was detected in H358, H1650 and HCC2935 cells (Fig. 4A) following treatment with ATO. In contrast, there was a dose-dependent downregulation of Bcl-2 in all cell lines (Fig. 4B), and upregulation of Bax in H23 cells (Fig. 4C). There was also a dose-dependent increase in expression of Bak in all cell lines (Fig. 4D). Expression of cleaved caspase-9 was elevated in H827 cells (Fig. 4E). On the other hand, cleaved caspase-3 (CC3) was activated in H23, HCC827, H1975 and HCC4006 cells, but unaltered in H1650 and downregulated in HCC2935 cells. The expression of CC3 in H358 cells was first elevated when exposed to 5 μ M ATO, then suppressed with 10 μ M ATO (Fig. 4F). Caspase-3 expression was decreased in H358 and H2935 cells upon treatment with ATO (Fig. 4G). The expression of cleaved PARP was also augmented in H23, H358 and H1975 cells (Fig. 4H). Representative western blots are shown in Fig. 4.

In vivo effect of ATO on tumor xenografts. Tumor growth was observed by day 5 following implantation of H358 cells. Mice



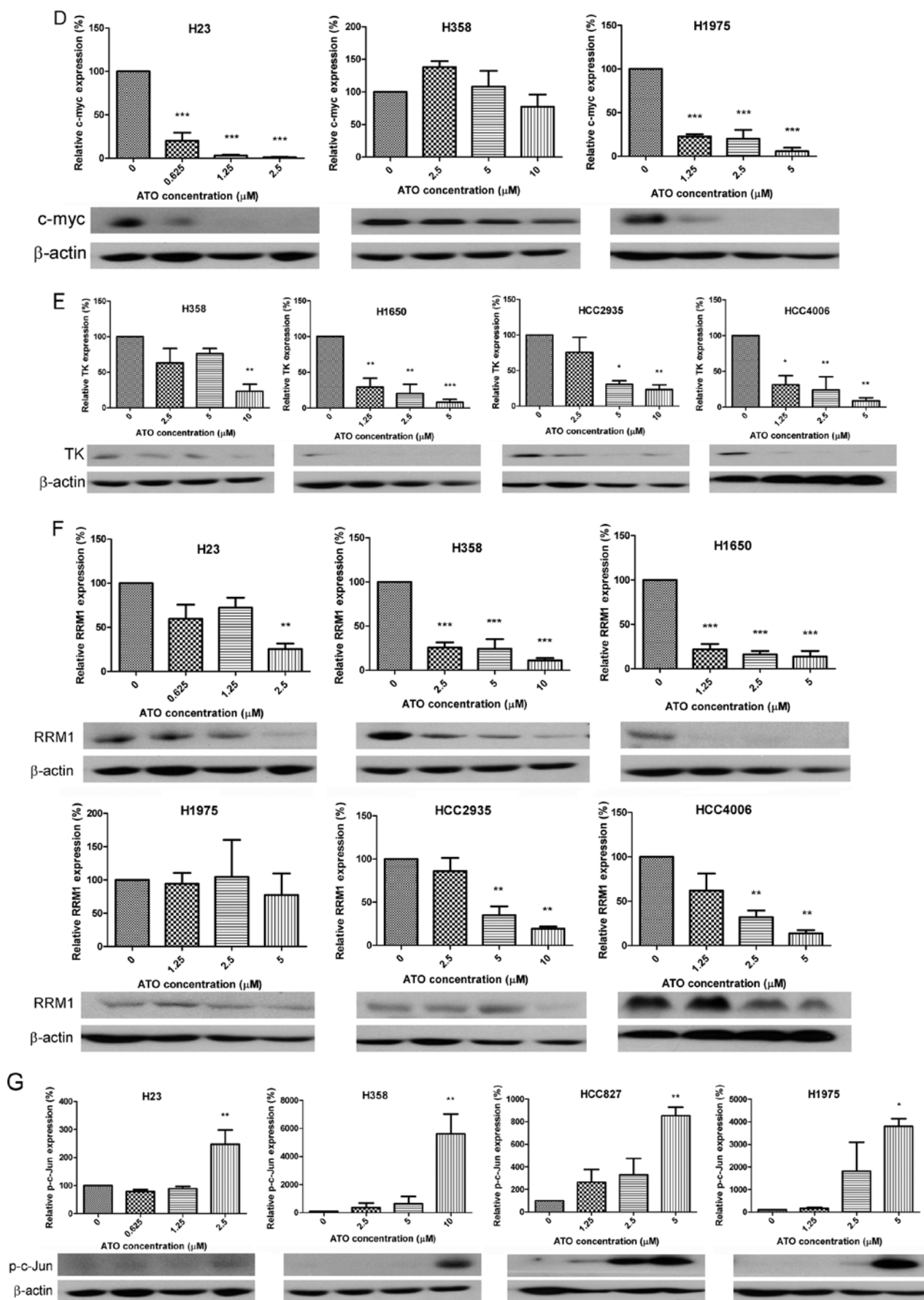


Figure 1. Downregulation of E2F1 and its downstream targets by ATO in different lung adenocarcinoma cell lines. ATO reduced expression of E2F1 (A), cyclin A2 (B), *skp2* (C), *c-myc* (D), TK (E) and RRM1 (F), while expression of p-c-Jun (G) was increased. β -actin was used as an internal control. A representative western blot is shown for each, except for those with undetectable basal expression. Statistical significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) is indicated for comparison with control.

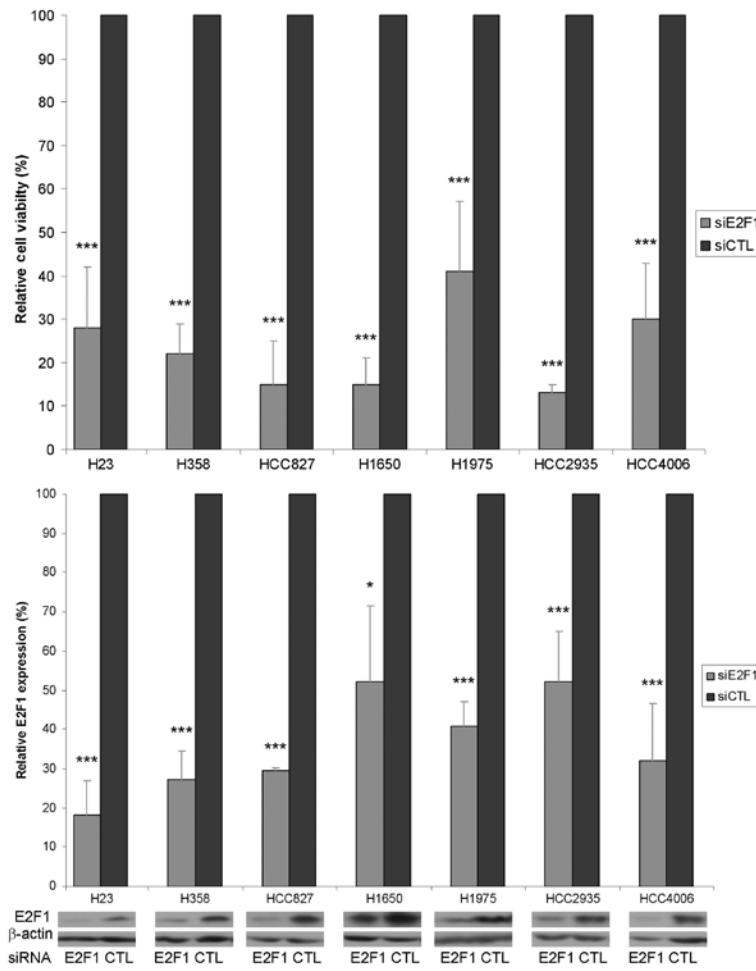


Figure 2. Effect of E2F1 knockdown on cell viability in cells treated with E2F1-targeted siRNA. With E2F1 knockdown, cell viability was significantly decreased in different cell lines. Statistical significance (*p<0.05, **p<0.01, ***p<0.001) is indicated for comparison with control.

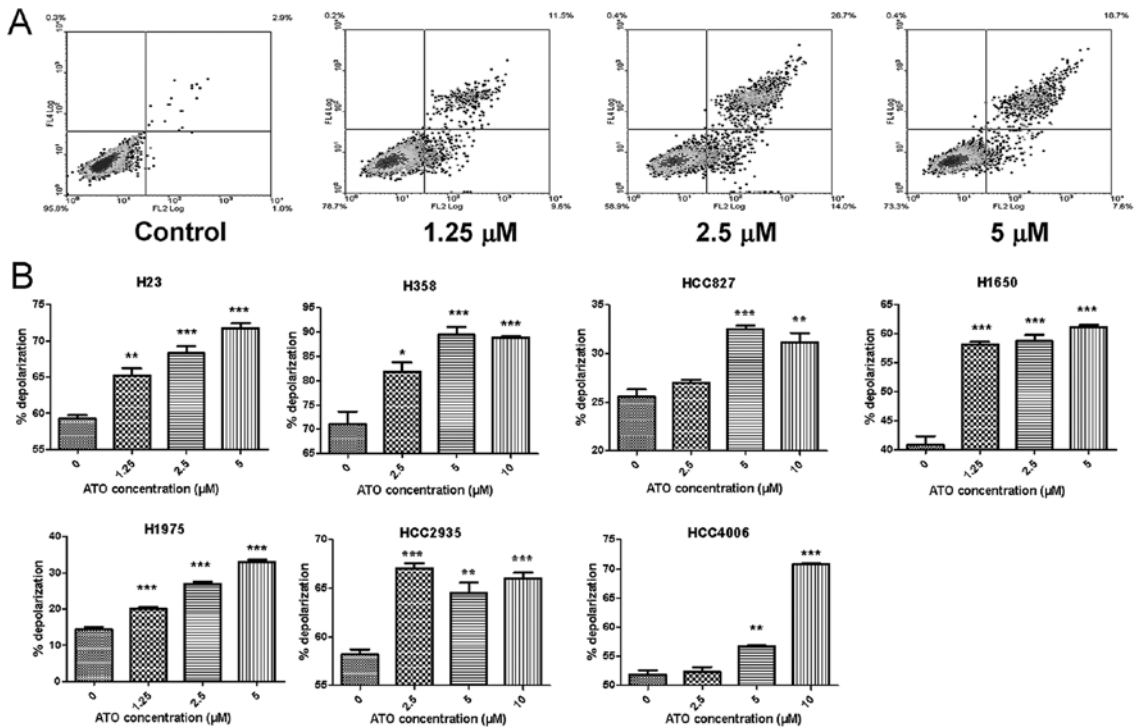


Figure 3. PE-conjugated Annexin V/7-AAD staining and JC-1 staining of ATO-treated lung adenocarcinoma cells. (A) Phosphatidylserine externalization was observed in ATO-treated H23 cells as evidenced by an increased percentage of cells stained with Annexin V. (B) Cells with depolarized mitochondrial membrane were elevated in all cell lines after incubation with ATO. Statistical significance (*p<0.05, **p<0.01, ***p<0.001) is indicated for comparison with control.

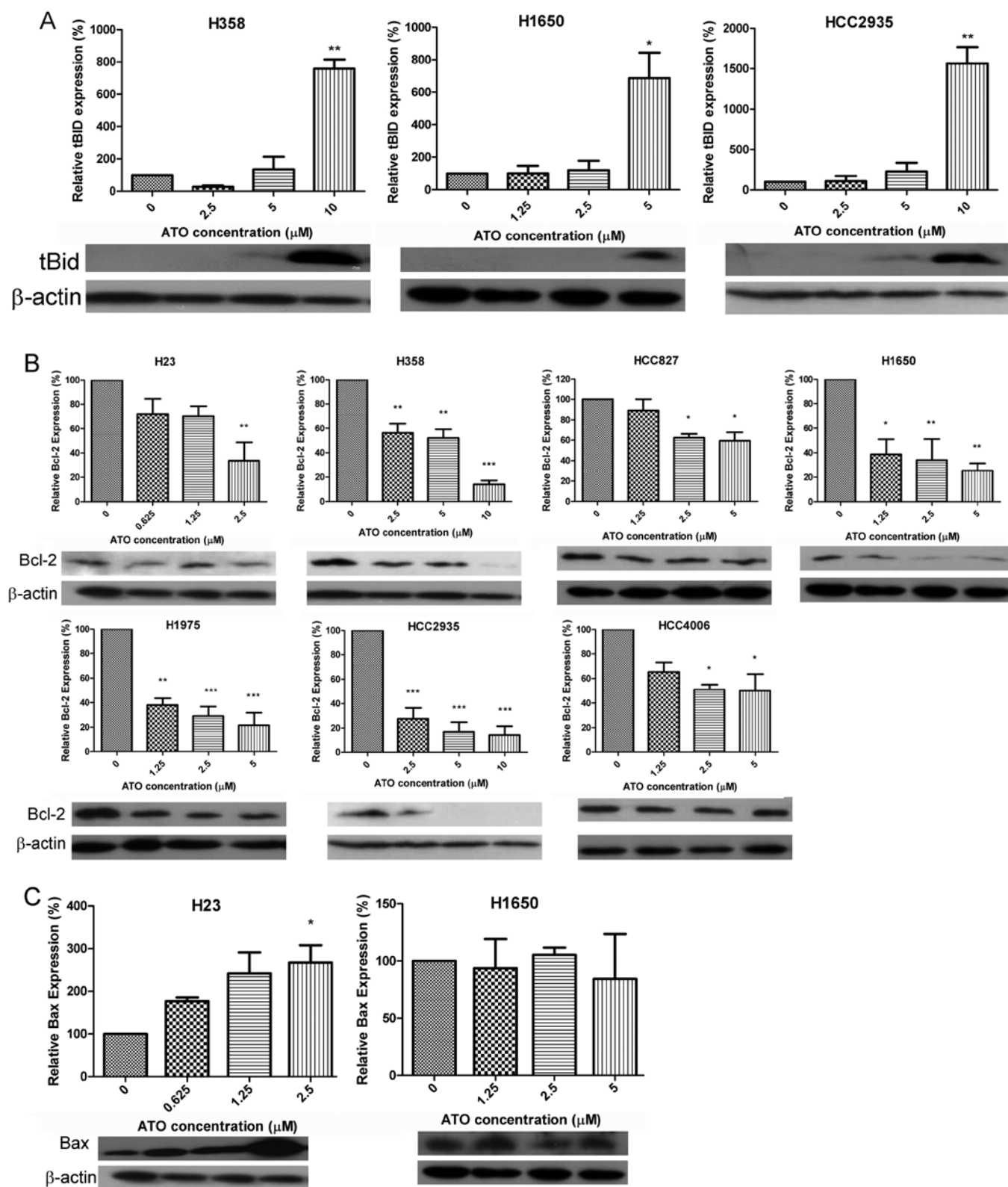


Figure 4. Alteration of apoptotic factors in lung adenocarcinoma cell lines by ATO. Truncation of BID was observed in H358, H1650 and HCC2935 cells (A). Bcl-2 was downregulated in all cell lines (B). Upregulated Bax was found in H23 cells (C).

were then randomly assigned to two treatment groups with no significant difference in baseline tumor volume. Tumor growth was significantly suppressed in the ATO treatment group compared with controls during 8 days of treatment (Fig. 5A). As the tumor size had reached the humane endpoint (a width

of 17 mm) in control group, mice were sacrificed after 8 days of treatment. The relative tumor volume in the ATO treatment arm was 32% that of the control group at the end of treatment ($p=0.0072$). No obvious toxic effect due to ATO treatment was noted and all the mice were alive following 8 days of treatment.

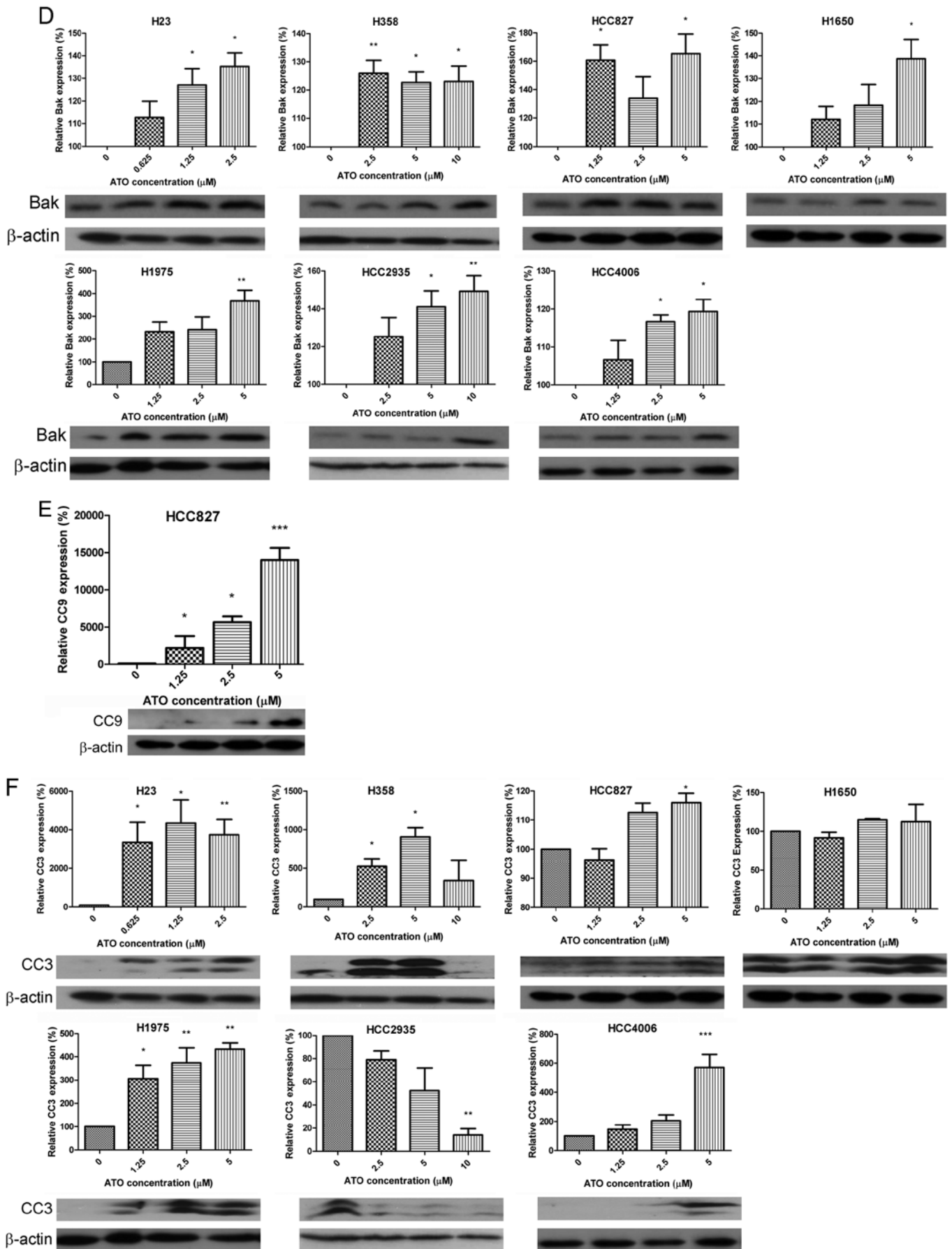


Figure 4. Continued. The expression of Bak was elevated in all cell lines (D). Caspase-9 was activated in HCC827 cells (E). Cleaved caspase-3 (CC3) was upregulated by ATO in H23, H358, HCC827, H1975 and HCC4006 cells. The expression of CC3 was unchanged in H1650 cells. CC3 expression was decreased in H358 and HCC2935 cells with 10 μ M ATO (F).

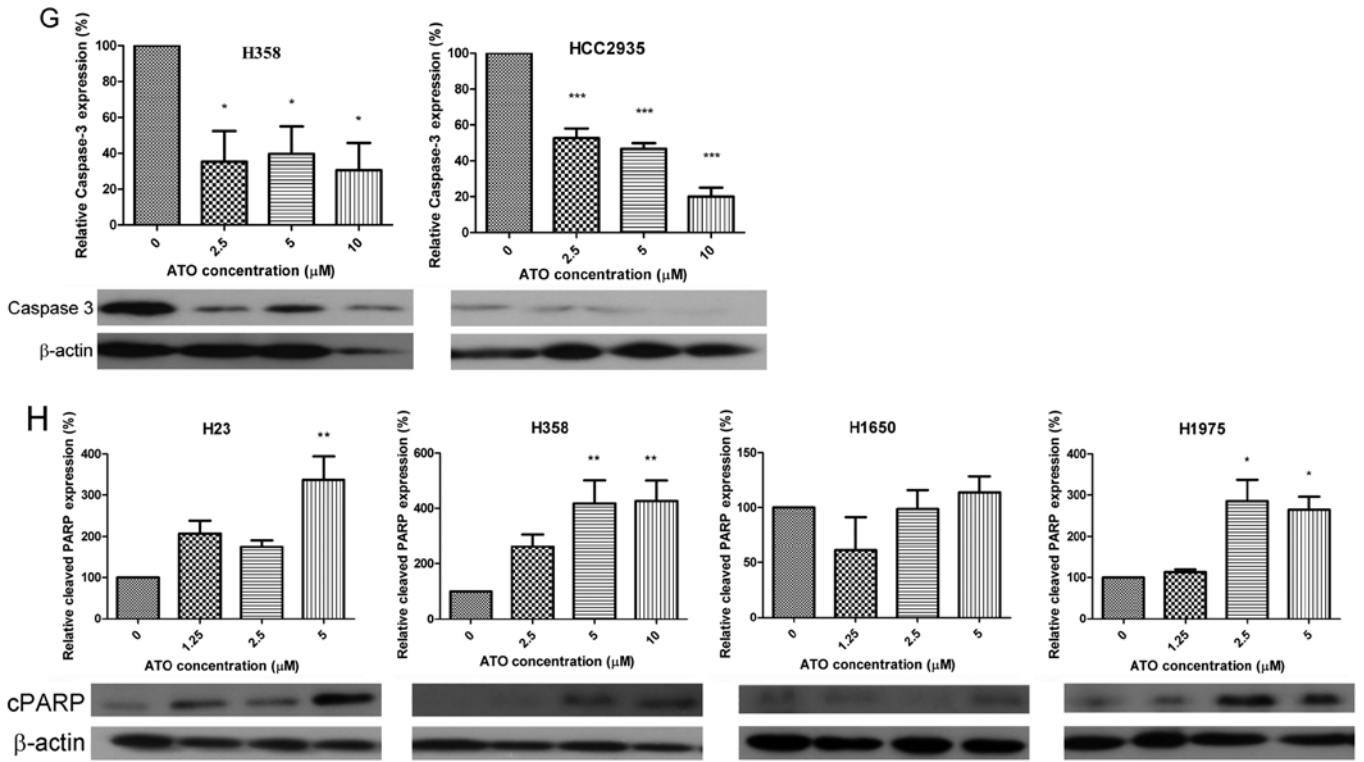


Figure 4. Continued. Caspase-3 was downregulated in H358 and HCC2935 cells (G). Cleavage of PARP was observed in H23, H358 and H1975 cells (H). A representative western blot was shown except for those with undetectable basal expression. β -actin was used as a housekeeping protein. Statistical significance ($^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$) is indicated for comparison with control.

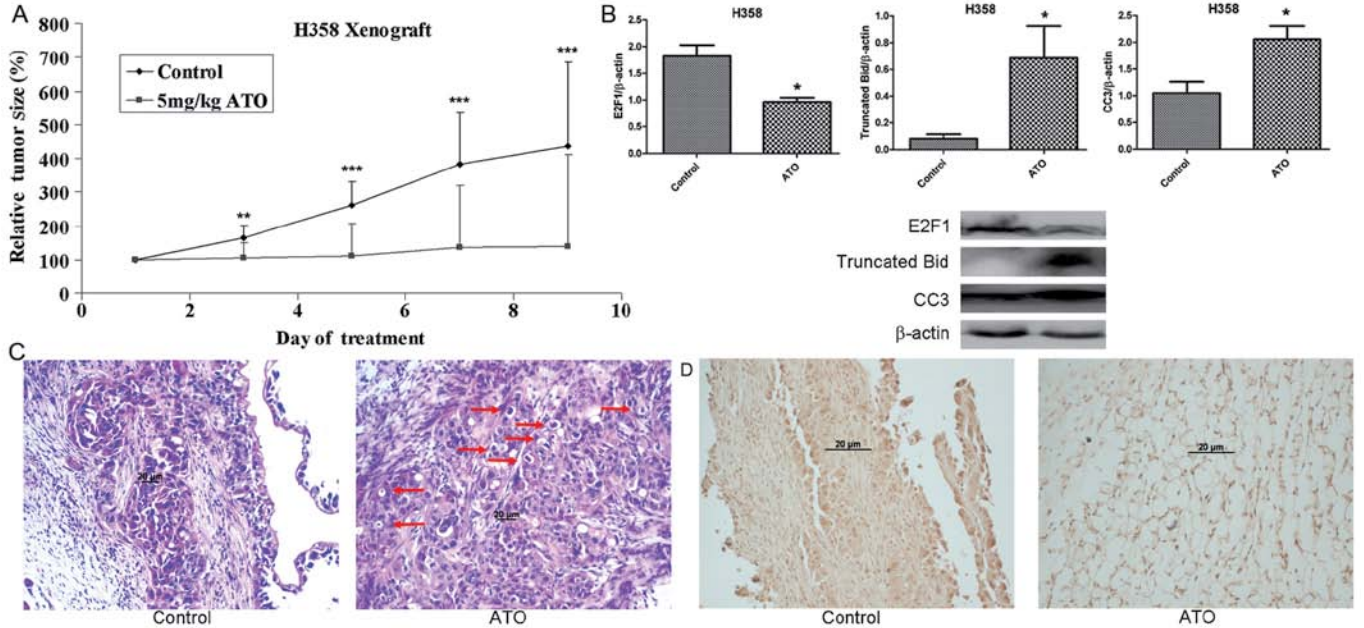


Figure 5. Tumor growth *in vivo* was inhibited by ATO in H358 xenograft model. ATO treatment was associated with (A) suppression of tumor growth and (B) downregulation of E2F1 as well as upregulation of truncated BID and CC3 expression in the ATO treatment group compared with control group in H358 xenograft model. Statistical significance ($^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$) was indicated for comparison with control. (C) Tumor sections were examined histologically with H&E staining, showing prominent apoptosis (examples indicated by arrows) among the ATO treatment group. Representative histological sections of tumor xenografts are shown (x200). (D) Immunostaining for CC3 showed nuclear localization in ATO-treated xenografts, while a lack of compartmentalization was evident in the control.

The body weight of mice in the ATO treatment group and control group was similar during treatment. Based on western blotting, E2F1 protein was downregulated and truncated BID

and cleaved caspase-3 were upregulated with ATO treatment (Fig. 5B). Histological examination (H&E staining) of tumor sections revealed prominent apoptosis (formation of apoptosis

bodies) with ATO treatment (Fig. 5C). Immunohistochemical staining demonstrated nuclear localization of cleaved caspase-3 in the ATO treatment group (Fig. 5D).

Discussion

In our cell line and xenograft models, ATO has demonstrated anti-proliferative and cytotoxic activity in lung adenocarcinoma at least partially mediated via downregulation of E2F1 and apoptosis. The concentrations of ATO corresponding to the *in vitro* IC₅₀ values were within a clinically reachable plasma level (8.3 μM) (17). The regulatory role of E2F1 in cellular proliferation in lung adenocarcinoma cell lines was confirmed using E2F1 siRNA knockdown experiment.

Pi Shuang is notoriously poisonous and has been paradoxically used in traditional Chinese medicine to treat various conditions, including cancers. The active ingredient of *Pi Shuang* is now known to be arsenic trioxide (As₂O₃ or ATO). ATO has been shown to induce apoptosis (at 0.5-2 μM) and promote cellular differentiation (at 0.1-0.5 μM) in acute promyelocytic leukemia (APL) cells (18). Its mechanisms of action in leukemia have been extensively investigated in the past decade, and involve alteration or activation of Bcl-2, cytochrome *c*, caspase-9, -3 and reactive oxygen species (19), p73, XIAP, cIAP2, Bcl-xL and survivin (20), DNA mutation and apoptosis (21), tubulin assembly disarrangement and microtubule depolymerization (22), survivin and telomerase (7). An intravenous formulation of ATO has received approval from the US Food and Drug Administration in the treatment of APL. In recent years, our institution has developed an oral liquid form of ATO that is more convenient for clinical use with a better safety profile (5). The role of ATO in the treatment of NSCLC has been less well-defined, though some preclinical data have suggested potential activity. We therefore aimed to further investigate the activity and mechanisms of action of ATO in preclinical models of lung adenocarcinoma.

The role of E2F1 in cancer appears to be a double-edged sword, with oncogenic or tumor suppressive properties, depending on the specific cancer type. In human breast cancer: E2F1 mRNA expression was lower with more advanced tumor stage in malignant breast tissue (23), nonetheless E2F1 was shown to promote proliferation in breast cancer cells (24). In NSCLC, E2F1 was reported to be oncogenic (12) and associated with an adverse prognosis (13) that is also observed in thyroid (25), liver (26) and pancreatic (27) cancers.

E2F1 consists of a cyclin A binding domain, DNA binding domain, pocket protein binding domain, nuclear export signal and nuclear localization signal (11). It is a transcription factor that controls apoptosis, cell cycle, senescence and tumor growth (10), as well as DNA damage, repair, synthesis and replication (11). The E2F1 pathway is frequently deregulated in cancers. As a consequence, amplification of cyclin A2 (28), c-myc (29), thymidylate synthase (TYMS) (30) and skp2 (31) is commonly found in various tumors, serving as important cell cycle regulators that are essential for cell proliferation. Thymidine kinase (TK) overexpression is associated with a higher incidence of clinical disease recurrence and mortality in breast cancer (32), while a lower level of expression of ribonucleotide reductase M1 (RRM1) predicts a longer time to progression in lung cancer with chemotherapy treatment

(33). Activation of c-Jun is correlated with CHOP upregulation and induction of apoptosis by AW00178 in human H1299 lung carcinoma cells (34) and apoptosis activation by 6-(7-nitro-2,1,3-benzoxadiazol-4-ylthio)hexanol in multidrug-resistant small cell lung cancer H69AR cells (35). These important molecular signals are ultimately controlled by E2F1. In this study, ATO has been shown to suppress E2F1 expression with alteration of its downstream targets. Notably TYMS (9), TK and RRM1 were downregulated, leading to inhibition of DNA synthesis. In addition, decreased expression of other proliferation factors (cyclin A2, c-myc, skp2) may also have contributed to the observed antiproliferative effect of ATO.

While E2F1 has been reported as an oncogene (12), its functional role in lung adenocarcinoma was demonstrated by specific E2F1 siRNA knockdown in our cell line model. Upon E2F1 knockdown by 50-80%, cell viability was significantly reduced by 60-88%, in support of its critical role in cell survival. The same phenomenon was recently reported in other lung cancer cell lines (36), nonetheless neither downstream targets of E2F1 nor other possible mechanisms were studied. In our study, E2F1 and its downstream targets were downregulated with ATO treatment, while the pro-apoptotic factor p-c-Jun was upregulated. As an executioner of apoptosis, expression of cleaved caspase-3 (CC3) after E2F1 knockdown was investigated. By simply knocking down E2F1, expression of CC3 was increased in HCC2935 cells only (data not shown), suggesting that E2F1 is mainly responsible for cell proliferation rather than apoptosis.

Although the induction of cell death by ATO has been investigated extensively in different cancer models, only a few reports have shown ATO-induced PS externalization (37-41). To our knowledge, this is the first report of PS externalization in an ATO-treated lung cancer cell line (H23). Nonetheless flow analysis did reveal that more cells became susceptible to mitochondrial membrane depolarization across different cell lines in our model with treatment of increasing ATO concentration, similar to previous reports in both lung cancer (8,42,43) and other cancer cell lines (44-46).

Theoretically, truncation of BID can increase the expression of Bax and Bak. Together with reduction in the expression of Bcl-2, an anti-apoptotic factor, truncated BID can direct the activation of caspase-9 and -3. The activation of caspase-3 may then cleave PARP leading to apoptosis. Thus the key apoptotic factors related to mitochondrial pathway were investigated in our lung adenocarcinoma cell line model with ATO treatment.

The expression of Bcl-2 was frequently inhibited by ATO in other lung cancer cell lines (8,47,48). In accordance with previous reports, we have demonstrated downregulation of Bcl-2 expression in our panel of ATO-treated lung adenocarcinoma cell lines. Upregulation of Bax was induced by ATO in H23 cells, while a similar phenomenon was only reported in small cell lung carcinoma (49). Nonetheless expression of Bak was elevated across different lung adenocarcinoma cell lines with ATO treatment. This is the first report to date of BID truncation and Bak upregulation in ATO-treated lung cancer cell lines.

Although there are reports of cleaved caspase-9 upregulation by ATO in other cancer cell lines (50,51), our similar observation in HCC827 cells is the first report in a lung cancer model. Caspase-3 activation was shown in ATO-treated A549 cells (52), Calu-6 cells (8) and SCLC cell lines (49). This study

has reinforced these findings in a panel of lung adenocarcinoma cell lines. Interestingly, the expression of CC3 in H358 cells was first increased when exposed to 5 μ M ATO and then decreased with 10 μ M ATO, whereas, CC3 expression decreased in a dose-dependent manner in HCC2935 cells when incubated with ATO. A similar observation was reported with prolonged incubation of ATO in lymphocytic leukemia cells (53). This paradoxical result was due to the direct suppression of caspase-3 expression by ATO in H358 and HCC2935 cells, and has been previously reported (53). ATO-induced cleavage of PARP has been reported in the H1355 NSCLC cell line (54) and in SCLC cell lines (49). We have provided further evidence of PARP cleavage in lung adenocarcinoma cell lines with ATO treatment.

Apart from promising *in vitro* activity in our lung adenocarcinoma model, the *in vivo* effect of ATO was confirmed using a nude mouse xenograft model. E2F1 downregulation was observed in tumor xenografts in keeping with the antiproliferative effect of ATO. Moreover, formation of apoptotic bodies and upregulation of truncated Bid and CC3 were also observed in treated tumor xenografts. Translocation of CC3 from the cytoplasm to the nucleus was shown by IHC staining. Pro-caspase-3 is located predominantly in the cytoplasm of cells. Caspase-3 is activated by upstream caspases and its active form (CC3) is then translocated into the nucleus. The substrates in the nucleus, e.g., PARP, are then cleaved. Eventually, chromatin condensation, DNA fragmentation and nuclear disruption occur and cells are directed to apoptosis (55). Our findings have provided evidence that apoptosis is induced by ATO in a lung adenocarcinoma xenograft model.

In conclusion, the anticancer effect of ATO was demonstrated through antiproliferation (E2F1 downregulation) and cell death (apoptosis) in both *in vitro* and *in vivo* lung adenocarcinoma models. Our novel finding of E2F1 suppression by ATO provides an additional mechanism to explain the activity of ATO in lung adenocarcinoma. Future potential clinical applications of ATO in lung adenocarcinoma treatment should be explored.

Acknowledgements

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