Downregulation of thymidylate synthase and E2F1 by arsenic trioxide in mesothelioma

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Abstract. Malignant pleural mesothelioma is a global health issue. Arsenic trioxide (ATO) has been shown to suppress thymidylate synthase (TYMS) in lung adenocarcinoma and colorectal cancer, and induce apoptosis in acute promyelocytic leukemia. With TYMS as a putative therapeutic target, the effect of ATO in mesothelioma was therefore studied. A panel of 5 mesothelioma cell lines was used to study the effect of ATO on cell viability, protein expression, mRNA expression and TYMS activity by MTT assay, western blot, qPCR and tritium-release assay, respectively. The knockdown of TYMS and E2F1 was performed with a specific siRNA. Phosphatidylserine externalization and mitochondrial membrane depolarization were measured by Annexin V and JC-1 staining respectively. The *in vivo* effect of ATO was studied using a nude mouse xenograft model. Application of ATO demonstrated anticancer effects in the cell line model with clinically achievable concentrations. Downregulation of TYMS protein (except H226 cells and 1.25 μM ATO in H2052 cells) and mRNA expression (H28 cells), pRB1 (H28 cells) and E2F1 and TYMS activity (except H226 cells) were also evident. E2F1 knockdown decreased cell viability more significantly than TYMS knockdown. In general, thymidine kinase 1, ribonucleotide reductase M1, c-myc and skp2 were downregulated by ATO. p-c-Jun was downregulated in H28 cells while upregulated in 211H cells. Phosphatidylserine externalization, mitochondrial membrane depolarization, downregulation of Bcl-2 and Bcl-xL, and upregulation of Bak and cleaved caspase-3 were observed. In the H226 xenograft model, the relative tumor growth was aborted, and E2F1 was downregulated while cleaved caspase-3 was elevated and localized to the nucleus in the ATO treatment group. ATO has potent antiproliferative and cytotoxic effects in mesothelioma *in vitro* and *in vivo*, partially mediated through E2F1 targeting (less effect through TYMS targeting). There is sound scientific evidence to support the clinical application of ATO in treatment of mesothelioma.

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Introduction

Malignant pleural mesothelioma (MPM) arises from exposure to asbestos fibers with a long latency period and has been a global health problem for the past few decades. Its peak incidence is yet to be reached. Mortality and morbidity remain extremely high, and treatment is rarely curative. Surgery alone or in combination with radiotherapy and chemotherapy (1) remains the standard treatment for early-stage disease. Combination chemotherapy for advanced disease has modest survival benefit (2). Thymidylate synthase (TYMS) converts 2'-deoxyuridine-monophosphate (dUMP) to deoxythymidine-5'-monophosphate (dTMP) in DNA synthesis (3). Pemetrexed is an antifolate that mainly inhibits TYMS to abort DNA synthesis and cell division: overexpression of TYMS has resulted in pemetrexed resistance (4,5). Notably, TYMS is a key pharmacological target in MPM though its role is still controversial (6,7). Alternative treatment options are very limited, especially for patients with a poor response to previous chemotherapy.

Most anticancer drugs work through inhibition of cellular proliferation or reactivation of apoptosis. The mechanisms of apoptosis in mesothelioma are less well-known, although it is frequently impeded (8). Thus development of anticancer drugs with novel targets that can inhibit cell proliferation and induce apoptosis is urgently needed to improve the overall outcome for patients with mesothelioma.

Arsenic trioxide (As_2O_3 or ATO) is the active component of a traditional Chinese medicine called *Pi Shuang*. ATO has demonstrated significant clinical activity in acute promyelocytic leukemia (APL): an intravenous formulation was approved by the US Food and Drug Administration over a decade ago. Our institution has subsequently pioneered the development of an oral preparation of ATO for clinical use with a much better safety profile (9). Interestingly, ATO has recently been shown to decrease TYMS expression in lung adenocarcinoma (10) and colorectal cancer (11), suggesting a possible role as a TYMS inhibitor. Nonetheless, the therapeutic role and targets of ATO in mesothelioma are less well-known and only two publications are available: ATO induced apop-

tosis through JNK and ERK (12) and repressed Hedgehog signal transduction pathway (13). Specifically the upstream transcription factor of TYMS, E2F1, which is responsible for controlling proliferation and apoptosis (14), has not been explored as a therapeutic target in mesothelioma. We therefore aimed to investigate the anticancer effect and target of ATO in mesothelioma *in vitro* and *in vivo*, and provide a scientific basis for the future clinical development of ATO as a treatment for MPM.

Materials and methods

Cell lines and reagents. A panel of five mesothelioma cell lines [NCI-H28 (sarcomatoid), MSTO-211H (biphasic), NCI-H226 (epithelioid), NCI-H2052 (sarcomatoid) and NCI-H2452 (epithelioid)] was purchased (American Type Culture Collection, Manassas, VA, USA). Cells were incubated in RPMI-1640 medium (Gibco®, Life Technologies, Carlsbad, CA, USA) enriched with 10% fetal bovine serum (FBS) (Gibco) in a humidified atmosphere of 5% CO_2 at 37˚C. ATO (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in sodium hydroxide (1.65 M) with pH adjusted to 7.4 by 6 M hydrochloric acid.

Cell viability assay. Briefly, cells (5,000/well) were incubated with different concentrations of ATO. Cells incubated with medium only served as a negative control. Following incubation for 48 or 72 h, cells were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 2 h, followed by addition of DMSO after removal of all medium. Absorbance (595 nm) was measured using a microplate reader Fluo Star Optima (Bmg Labtec GmbH, Ortenberg, Germany (10).

Western blotting study of whole-cell lysate. Western blotting was performed as previously described (15). Specific primary antibodies [mouse monoclonal anti-human β-actin (Sigma-Aldrich), anti-c-Myc, anti-E2F1, anti-p-c-Jun, anti-pRB1, anti-TYMS, anti-thymidine kinase 1, anti-ribonucleotide reductase M1, anti-skp2, anti-Bcl-2, anti-Bcl-xL, anti-Bak and anti-cleaved caspase-3 (Cell Signaling Technology, Danvers, MA, USA) antibodies] and corresponding horseradish peroxidase (HRP)-conjugated secondary antibody (Cell Signaling Technology) were purchased. An enhanced chemiluminescence (ECL) kit (GE Healthcare) was used to detect protein expression. β-actin was selected as reference protein.

Quantification of TYMS mRNA. A standard TRIzol/chloroform method was used to extract total cellular RNA. Reverse transcription and quantitative polymerase chain reaction (qPCR) were performed using the *Power*SYBR® Green Cells-to-C_T™ kit (Life Technologies) and standard protocol in StepOnePlus Real-Time PCR System (Applied Biosystems, CA, USA). The sequence of TYMS forward and reverse primers were TCAAGGGATCCACAAATGCT and TCTG TCGTCAGGGTTGGTTT, respectively. GAPDH served as internal control. Relative expression was calculated (10).

TYMS activity using tritium-release assay. Cells were incubated for 2 h in fresh medium containing 3 μl [5-^{3}H]-dUMP (American Radiolabeled Chemical, MO, USA) per well. The medium was collected, and mixed with charcoal and trichloroacetic acid before centrifugation. One milliliter scintillation fluid was added to the supernatant and read using a scintillation counter (16). TYMS activity = total scintillation count of sample/total scintillation count of control.

TYMS and E2F1 siRNA knockdown. TYMS (sc-44978), E2F1 (sc-29297) or control (sc-37007) siRNA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was allowed to transfect cells for 6 h using a transfection reagent (Santa Cruz Biotechnology) in RPMI-1640 medium, followed by replenishment with new medium containing 1% FBS for 3 days (10). Cell viability, TYMS and E2F1 protein expression were measured.

Measurement of phosphatidylserine externalization. Cells were stained at room temperature for 20 min with phycoerythrin (PE)-conjugated Annexin V and 7-amino-actinomycin (AAD) (BD Phamingen, CA, USA) in binding buffer and incubated in darkness. Cells were then analyzed by flow cytometry using FL-2 (575 nm) and FL-4 channel (675 nm) with Beckman FC500 (Beckman Coulter, Brea, CA, USA) (15).

Measurement of mitochondrial membrane potential. In short, cells were incubated for 15 min at 37˚C in 500 μl RPMI medium containing 5,5',6,6'-tetrachloro-1,1',3,3'tetraethylbenz imidazolylcarbocyanine iodide (JC-1) (Sigma-Aldrich) in the dark. Cells were then analyzed using flow cytometry FL-1 (525 nm) and FL-2 (575 nm) channel (Beckman FC500).

Tumor growth inhibition in vivo. The H226 xenograft model was created by subcutaneous injection of 10^7 cells in phosphate-buffered saline (PBS) into the upper back of 18 nude mice (female, 4-6-week-old, 10-14 g, BALB/cAnN-nu, Charles River Laboratories, Wilmington, MA, USA). Mice were randomized into 3 groups after tumor growth was established. PBS (served as control) or ATO (3.75 and 7.5 mg/kg) was administered daily intraperitoneally. Tumor dimension (using standard calipers) and body weight of mice were measured on alternate days and tumor volume calculated [volume = length x width x width)/2] (17). For humane reasons, mice were sacrificed when tumor size reached 17 mm in diameter. Part of the tumor xenografts were collected and lysed for western blotting. The remainder was fixed in paraffin block and cut into sections for immunohistochemical (IHC) staining according to standard procedures. The study protocol was approved by the institutional Animal Ethics Committee (approval reference no. CULATR 2510-11), and standard humane endpoints for animal research were applied.

Statistical analysis. Experiments were repeated at least 3 times and data analysed (mean ± standard deviation). The difference between groups was analyzed using Student's two-tailed t-test by Prism (GraphPad Software, La Jolla, CA, USA). A p-value <0.05 defined statistical significance ($^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ in the figures).

Results

In vitro activity of ATO in mesothelioma. Treatment with ATO induced a dose- and time-dependent antiproliferative effect in

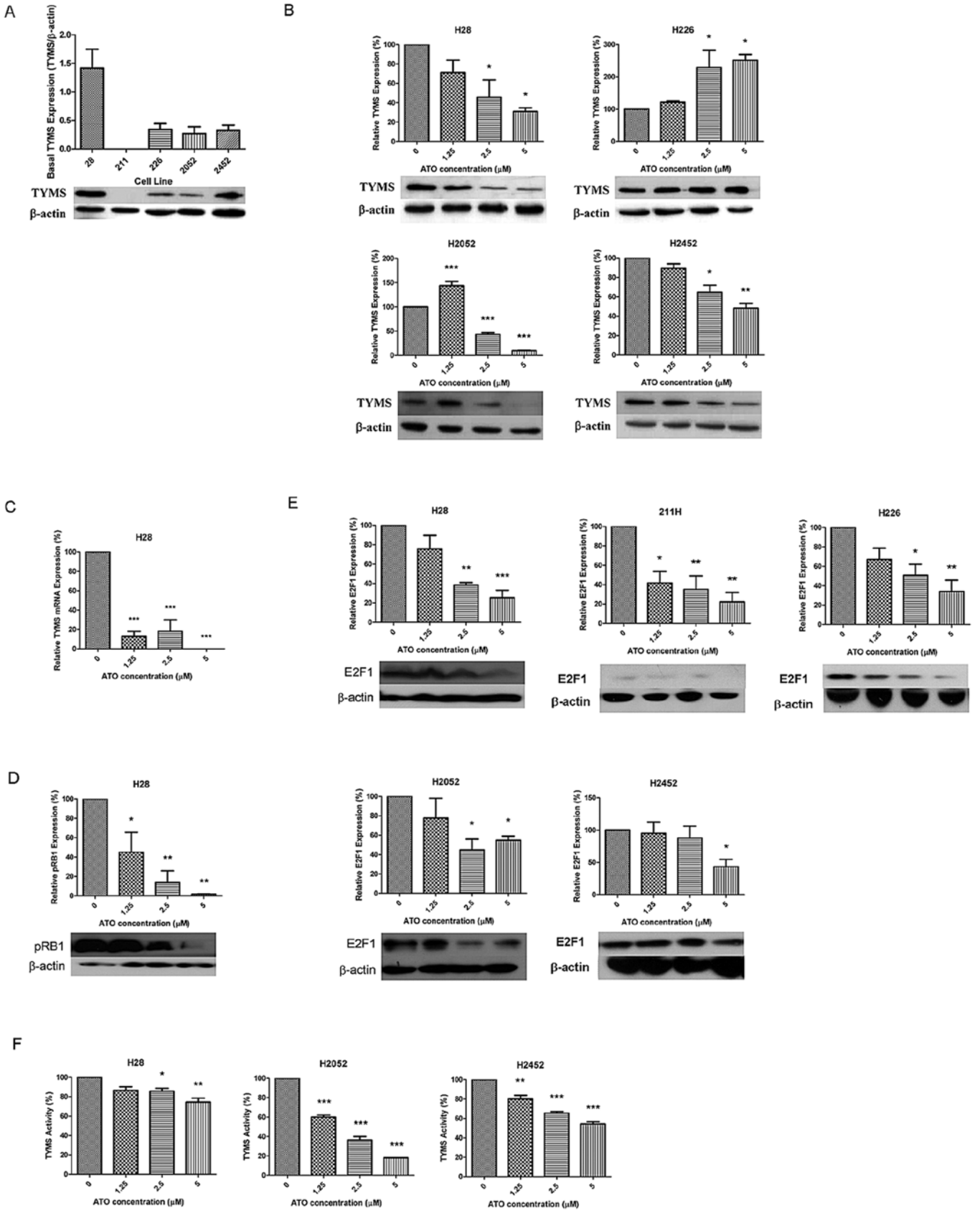

Figure 1. (A) Basal TYMS protein expression in different mesothelioma cell lines. H28 cells showed the highest expression level while 211H cells did not display any basal expression. Alternation of TYMS (B) protein and (C) mRNA (H28 cells) by incubation with ATO. ATO downregulated TYMS protein expression except H226 cells and at low concentration (1.25 μM ATO) in H2052 cells. (D) ATO induced a decrease in protein expression of (D) pRB1 in H28 cells and (E) E2F1 in all cell lines in a dose-dependent manner. (F) TYMS activity of different mesothelioma cells after exposure to ATO for 48 h. TYMS activity was inhibited after incubation of ATO in H28, H2052 and H2452 cells while increased in H226 cells. Results were measured in triplicate experiments. A representative result of western blotting is displayed. Statistical significance ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$) indicates comparison with control.

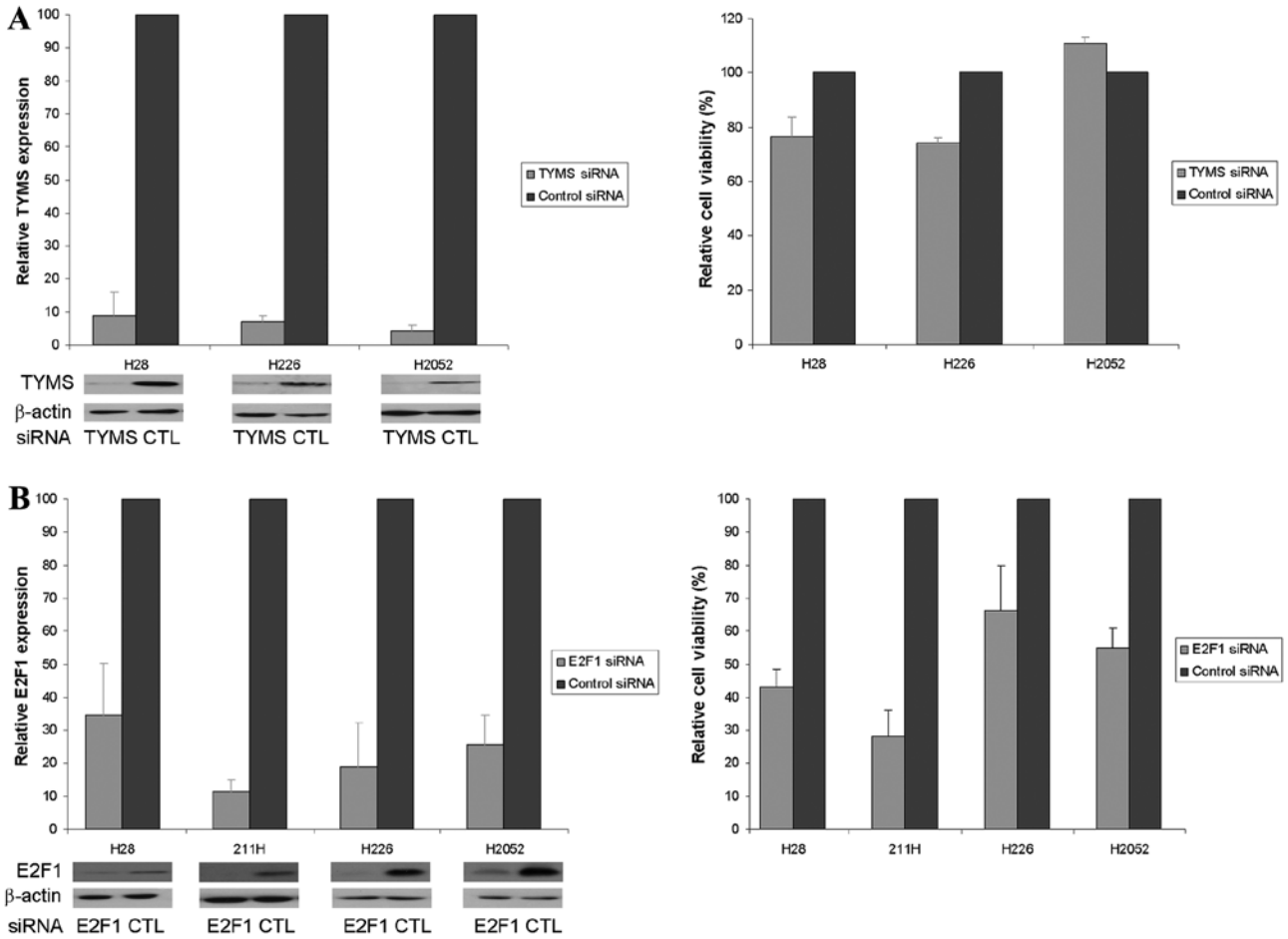

Figure 2. Effect of TYMS-targeted and E2F1-targeted siRNA on TYMS and E2F1 protein expression and cell viability in different cell lines. Since 211H cells did not express TYMS, and H2452 cells could not survive in 1% FBS condition, only H28, H226 and H2052 cells were used in TYMS knockdown experiment. (A) TYMS siRNA reduced the relative TYMS protein expression when compared with control siRNA arm and associated with different degrees of reduction in cell viability in H28 and H226 cells. TYMS knockdown had no effect on cell viability in H2052 cells. (B) H28, 211H, H226 and H2052 cells were used in E2F1 knockdown experiment. E2F1 siRNA decreased the relative E2F1 protein expression when compared with control siRNA group and was associated with decrease in cell viability in all cell lines. Results were measured in triplicate experiments. A representative result of western blotting is shown. Statistical significance ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$) indicates comparison with control.

all mesothelioma cell lines. The IC_{50} values in different cell lines were 4.7-7.8 μM (H28, 211H, H226, H2052 and H2452 cells: 6.9±1.6, 6.6±1.1, 7.8±1.1, 4.7±2.1 and 5.6±2.6 μM respectively) and 1.7-7 μM (H28, 211H, H226, H2052 and H2452 cells: 2.1±0.7, 7.0±0.6, 6.6±1.0, 1.7±0.5 and 2.8±0.8 μM respectively) after 48- and 72-h treatment, respectively.

Effects of ATO on TYMS protein and mRNA expression, pRB1 and E2F1 protein expression and TYMS activity. Basal TYMS protein expression is shown in Fig. 1A. H28 cells displayed the highest TYMS protein expression. The TYMS expression levels in H226, H2052 and H2452 cells were similar, but undetectable in 211H cells. Following a 48-h incubation with ATO, TYMS protein expression was decreased in H28 and H2452 cells, and increased in H226 cells in a dose-dependent manner. The TYMS protein expression was first increased (1.25 μM ATO), then decreased at higher concentrations of ATO in H2052 cells (Fig. 1B). TYMS mRNA expression was significantly decreased in H28 cells (Fig. 1C), while basal TYMS mRNA expression was undetectable in other cells. Basal pRB1 was highly expressed and downregulated in H28 cells in a dose-dependent manner after ATO treatment (Fig. 1D), but undetectable in other cells. Upon ATO treatment, expression of E2F1 protein was decreased significantly in all cell lines (Fig. 1E). ATO inhibited TYMS activity in H28, H2052 and H2452 cells, but increased TYMS activity in H226 cells (Fig. 1F).

Decreased cell viability after knockdown of TYMS and E2F1. H28, H226, H2052 and H2452 cells were used in TYMS silencing experiment, however, H2452 cells could not survive in 1% FBS condition. TYMS siRNA reduced the relative TYMS protein expression to <10% that of the control siRNA and was associated with a 25% reduction in cell viability in H28 and H226 cells but unaltered viability in H2052 cells (Fig. 2A). E2F1 siRNA decreased the relative E2F1 protein expression to ~35, 12, 19 and 26% that of the control siRNA and was associated with 57, 72, 37 and 45% reduction in cell viability in H28, 211H, H226 and H2052 cells, respectively (Fig. 2B).

Alteration of E2F1 downstream targets by ATO. ATO decreased expression of thymidine kinase 1 (TK) in H28 cells, but upregulated TK expression initially at 1.25 μM and then downregulated with higher concentrations in H2052 cells.

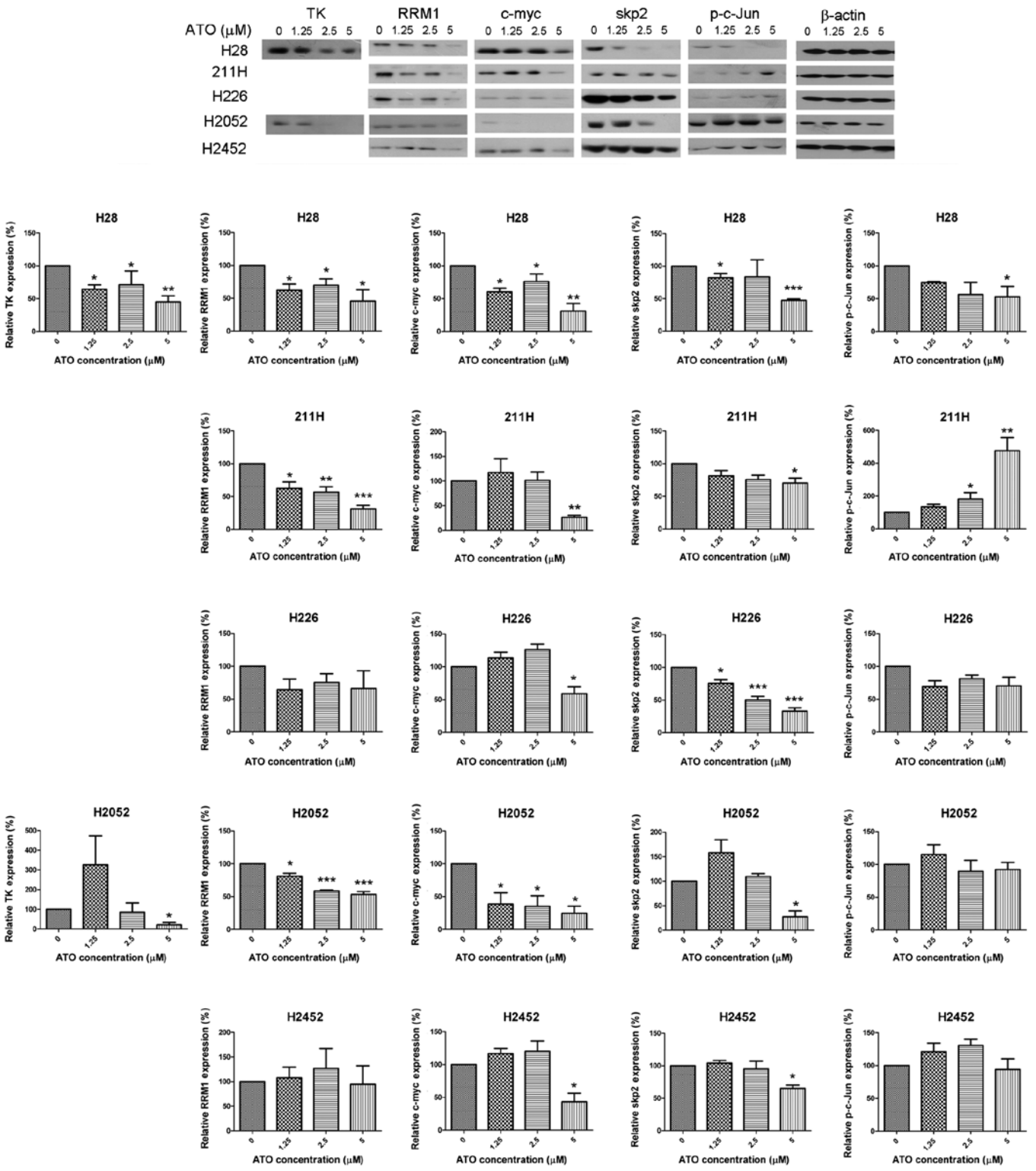

Figure 3. Alteration in expression of TK, RRM1, c-myc, skp2 and p-c-Jun in different cell lines upon ATO treatment. Basal expression of TK in 211H, H226 and H2452 cells was undetectable. ATO downregulated the expression of TK (H28 and H2052 cells), RRM1 (H28, 211H and H2052 cells), c-myc and skp2. p-c-Jun expression was decreased in H28 cells and increased in 211H cells. A representative result of western blotting is shown. Statistical significance ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$) indicates comparison with control.

The expression of ribonucleotide reductase M1 (RRM1) was suppressed in H28, 211H and H2052 cells. ATO downregulated the expression of c-myc and skp2 in all cell lines. The expression of p-c-Jun was decreased in H28 cells while upregulated in 211H cells (Fig. 3).

Phosphatidylserine (PS) externalization and mitochondrial membrane depolarization induced by ATO. PS externalization induced by ATO was observed in H2452 cells (Fig. 4A) although ATO enhanced mitochondrial membrane depolarization in all cell lines in a dose-dependent manner (Fig. 4B).

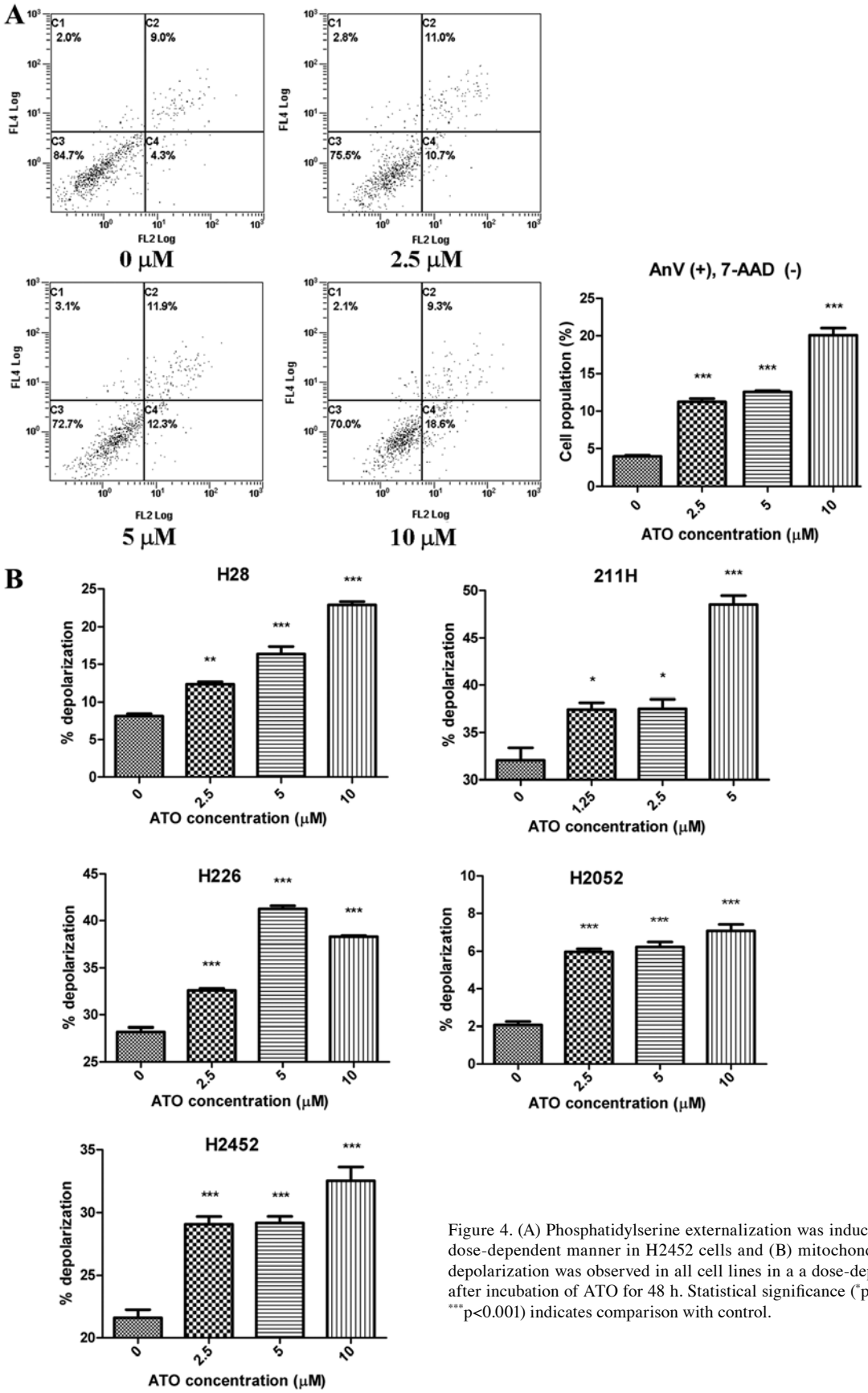

Figure 4. (A) Phosphatidylserine externalization was induced by ATO in a dose-dependent manner in H2452 cells and (B) mitochondrial membrane depolarization was observed in all cell lines in a a dose-dependent manner after incubation of ATO for 48 h. Statistical significance ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$) indicates comparison with control.

Alteration of apoptosis-related factors by ATO. The expression of anti-apoptotic factor Bcl-2 was downregulated in a dose-dependent manner with ATO treatment in all cell lines except H28 cells (Fig. 5A). The anti-apoptotic factor Bcl-xL was suppressed in H2052 cells (Fig. 5B). The expression of the downstream Bak in 211H, H226 and H2052 cells was upregu-

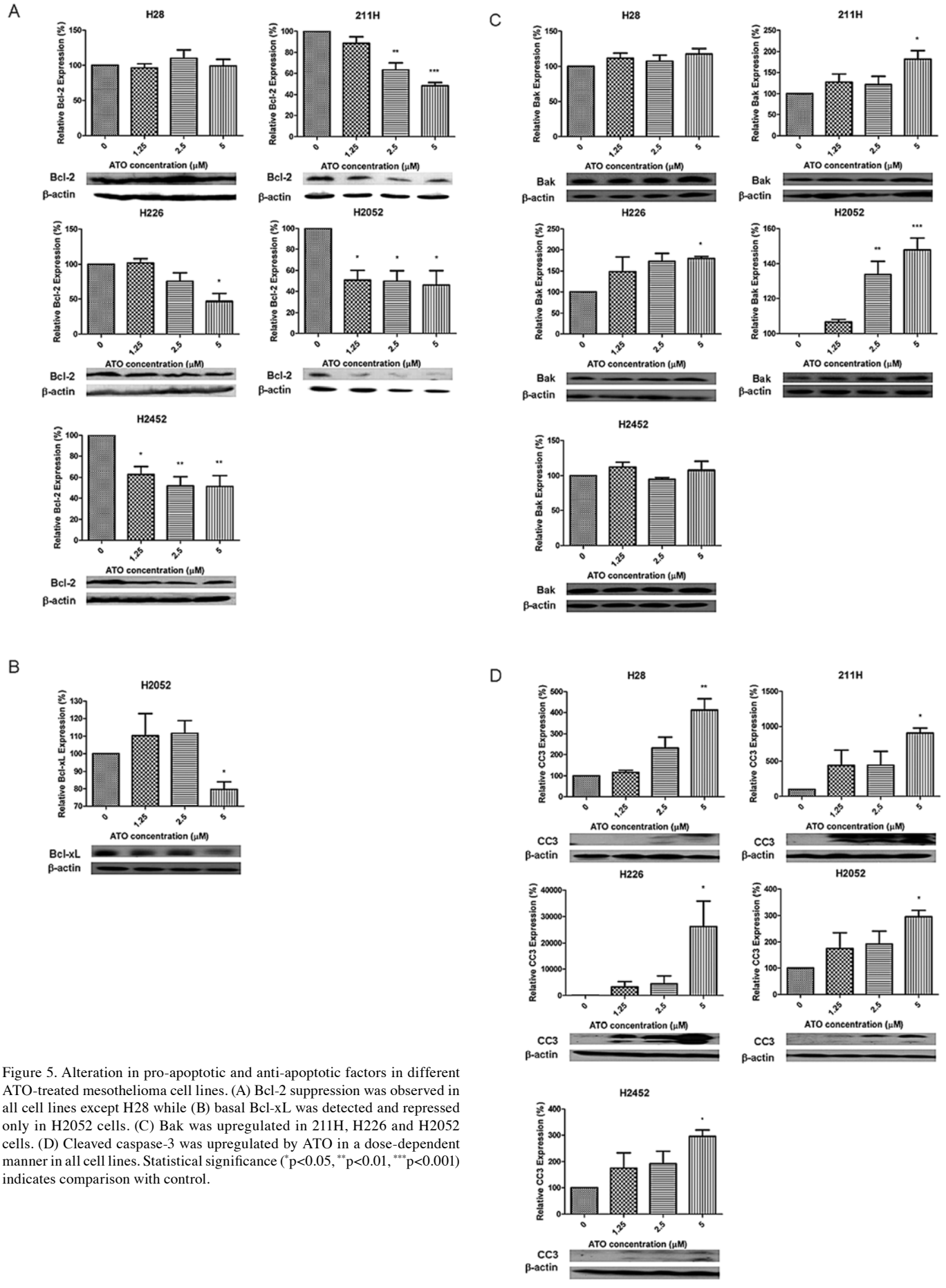

Figure 5. Alteration in pro-apoptotic and anti-apoptotic factors in different ATO-treated mesothelioma cell lines. (A) Bcl-2 suppression was observed in all cell lines except H28 while (B) basal Bcl-xL was detected and repressed only in H2052 cells. (C) Bak was upregulated in 211H, H226 and H2052 cells. (D) Cleaved caspase-3 was upregulated by ATO in a dose-dependent manner in all cell lines. Statistical significance ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$) indicates comparison with control.

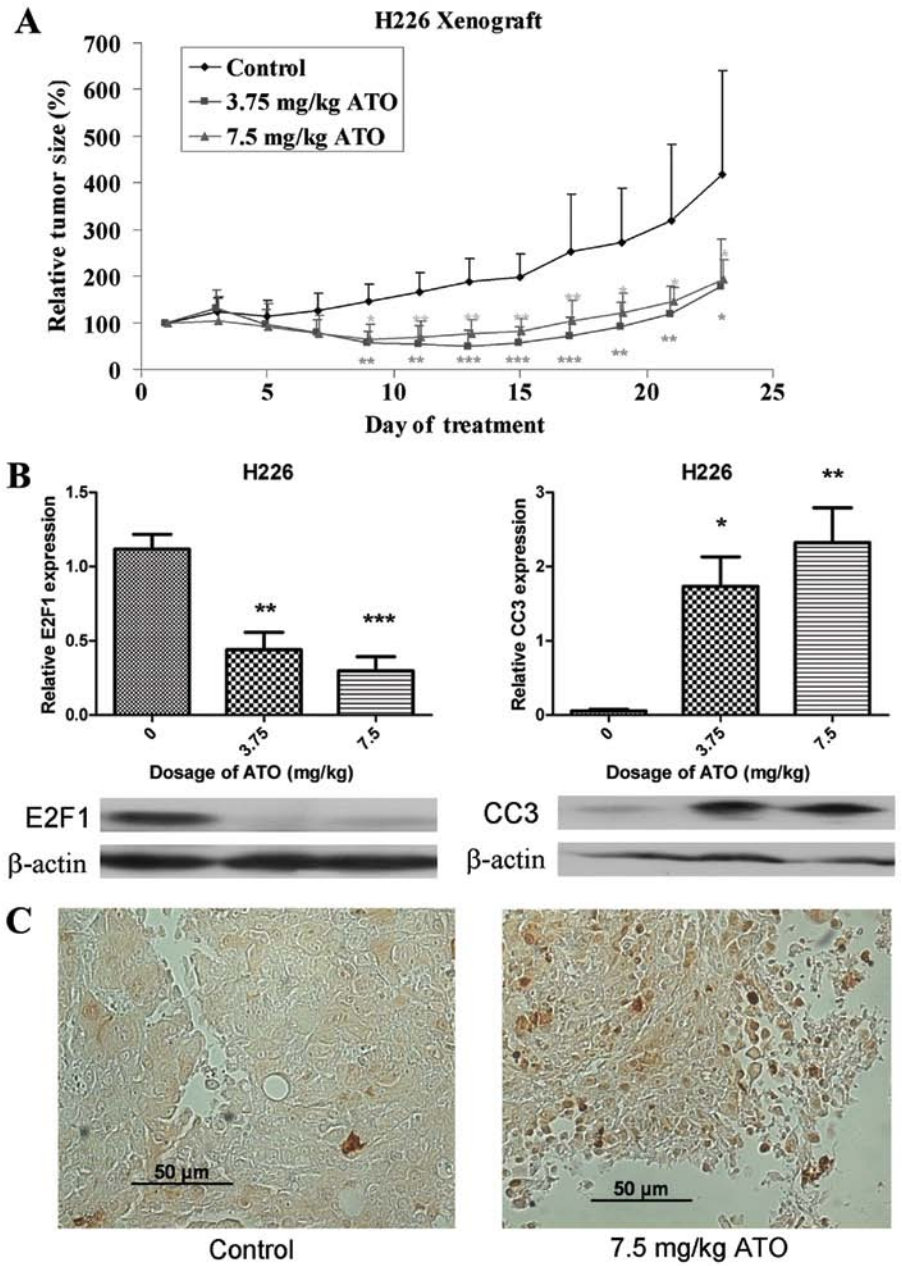

Figure 6. ATO treatment was associated with (A) stabilization of relative tumor size, (B) downregulation of E2F1 as well as upregulation of cleaved caspase-3 expression and (C) localization of cleaved caspase-3 in the nucleus in the ATO treatment group compared with control group in H226 xenograft model. Results were measured in triplicate experiments. Statistical significance ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$) indicates comparison with control.

lated when the concentration of ATO increased (Fig. 5C). Expression of cleaved caspase-3 (CC3) was increased by ATO in all cell lines (Fig. 5D).

In vivo effect of ATO on tumor xenografts. After inoculation of H226 cells for 7 days, obvious tumors were established. There was no significant difference in baseline tumor size amongst different groups of mice. The relative tumor size in the control and ATO treatment groups during treatment was determined (Fig. 6A). After 23 days of ATO treatment, the relative tumor volume in the 3.75 and 7.5 mg/kg treatment groups were 42.9 and 46.5% that of the control group respectively ($p<0.05$). There was no difference in body weight between different groups ($p>0.05$) and no pathological changes (H&E staining) were observed in the liver of mice in the ATO treatment arms. E2F1 was suppressed and CC3 was upregulated in the ATO treatment arm (Fig. 6B), with IHC showing localization of CC3 to the nucleus (Fig. 6C).

Discussion

ATO showed antiproliferative effects as demonstrated by MTT cell viability assay with clinically achievable concentrations (18) in our cell line model accompanied by downregulation of TYMS (except H226 cells) and E2F1. The significance of TYMS in cell viability was demonstrated *in vitro* with TYMS knockdown, nonetheless with rather modest effect. In contrast, knockdown of the upstream target E2F1 resulted in more significant inhibition of cell viability. Our findings of downregulation of E2F1 and its various downstream signals would

support E2F1 as a significant target of ATO leading to its antiproliferative effect. Upon treatment with ATO, apoptosis was also observed. In the H226 xenograft model, tumor growth was suppressed, E2F1 was downregulated while cleaved caspase-3 was upregulated and translocated to the nucleus in ATO treated mice.

It is evident that arsenic contamination in drinking water and food is related to liver, skin, kidney, bladder and lung cancers (19,20). Contrary to this though, ATO is being used for treatment of APL, mediated through SUMOylation of retinoic acid receptor α oncoprotein (21). ATO is also known to exert its anti-leukemic action through reactive oxygen species induction, mitochondrial membrane destruction, cytochrome *c* release, caspase activation and finally apoptosis (22-27). The US Food and Drug Administration has approved APL as an indication for treatment with ATO. Since TYMS is a key target in mesothelioma and ATO has recently been shown to suppress TYMS expression in lung adenocarcinoma (10) and colorectal cancer (11), we postulated that ATO would exert a TYMS inhibitory effect in mesothelioma cell lines.

In order to understand the significance of reduced protein expression of TYMS and TYMS activity by ATO in the 3 mesothelioma cell lines, the pivotal role of TYMS in cellular proliferation was studied using a TYMS knockdown technique. However, the impact of TYMS knockdown on cell viability was unexpectedly low. The role of TYMS in mesothelioma is controversial which has been shown to be non-essential (6,7). Notably, the expression of E2F1 has demonstrated a strong correlation with cancer cell proliferation (28,29), leading to our further exploration of a new actionable target the E2F1.

E2F1 is a well-known transcription factor which is involved in proliferation, apoptosis, cell cycle, tumor growth and senescence. It is released and activated upon phosphorylation of retinoblastoma tumor suppressor protein (RB) (14). However, the role of E2F1 in mesothelioma has never been elucidated. Thus, E2F1-targeted siRNA experiment was performed to investigate the functional role of E2F1. Decrease in cell viability upon E2F1 knockdown was more profound when compared with TYMS, in support of a more important role of E2F1 than TYMS on cancer cell proliferation. Moreover, cleaved caspase-3 was elevated only in 211H cells (data not shown) after E2F1 knockdown showing that the relationship between E2F1 and apoptosis is cell line-specific. As such, the downstream targets of E2F1 related to cell proliferation and apoptosis were also investigated.

TYMS, TK and RRM1 are three key enzymes that take part in DNA synthesis and thus important in cell proliferation. TK activity in sarcomatoid type was reported to be higher than epithelioid type mesothelioma (30), which is in line with our observed relatively higher TK expression in sarcomatoid cells (H28 and H2052 cells). RRM1 polymorphisms and haplotypes were related to efficacy of gemcitabine treatment in mesothelioma (31). Interestingly, RRM1 downregulation by ATO was only observed in sarcomatoid mesothelioma cell lines in this study, suggesting a differential effect of ATO in different histological types of mesothelioma. Degradation of tumor suppressor proteins, e.g., p21 and p27, are induced by skp2 so as to accelerate cell cycle (32). Nonetheless, the role of skp2 in mesothelioma has so far not been described. Our findings have provided the first evidence of TK, RRM1 and skp2 downregulation by ATO in mesothelioma.

c-myc is a transcription factor which governs cell proliferation and metastasis. Suppression of c-myc has been shown to have antiproliferative (33) and apoptotic (34) effect in mesothelioma. In addition, growth inhibition in APL by ATO was partially mediated through suppression of c-myc (35).

JNK-c-JUN pathway regulates cell proliferation and upregulation of p-c-Jun mediates cell apoptosis (36). Apoptosis induced by ATO was reported only in one mesothelioma cell line (H2052) (12). Hence, we further studied the apoptotic effects of ATO in our 5 mesothelioma cell lines.

Phosphatidylserine (PS) externalization (37) and mitochondrial membrane depolarization (38) are well-known hallmarks of apoptosis. In addition, our findings of upregulated pro-apoptotic (Bak and cleaved caspase-3) and downregulated anti-apoptotic (Bcl-2 and Bcl-xL) proteins have provided supportive evidence that apoptosis was induced by ATO in mesothelioma cell lines.

The expression of cleaved caspase-3 in all mesothelioma cell lines increased in a dose-dependent manner with ATO, substantiated in our H226 xenograft model. However, H28, 211H, H2052 and H2452 xenograft models could not be generated despite multiple attempts. Based on IHC in H226 tumor xenografts, cytoplasmic cleaved caspase-3 was translocated to the nucleus, leading to subsequent chromatin condensation, DNA fragmentation and/or nuclear disruption with eventual apoptosis (39). In addition, downregulation of E2F1 expression in H226 xenograft model with ATO treatment was observed. Our findings provide strong evidence for the *in vitro* and *in vivo* antiproliferative and pro-apoptotic effect of ATO in mesothelioma which is similar in lung adenocarcinoma recently reported (40).

In conclusion, ATO has demonstrated an antiproliferative effect at least partially mediated through downregulation of E2F1, as well as a cytotoxic effect through apoptosis in both *in vitro* and *in vivo* mesothelioma models. Moreover, we propose the E2F1 as a new actionable target in MPM. Our findings provide the scientific basis for future exploration of the clinical application of ATO in treatment of MPM. This is particularly feasible with the recent development of an oral-ATO preparation by our institution for clinical use.

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