Acetylbritannilactone suppresses growth via upregulation of krüppel-like transcription factor 4 expression in HT-29 colorectal cancer cells

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Abstract. Acetylbritannilactone (ABL) is a new active compound isolated from *Inula Britannica* L, a traditional Chinese medicinal herb. It has been reported that ABL can inhibit the proliferation of vascular smooth muscle cells (VSMCs) and neointima formation after balloon injury in rats. ABL also shows chemopreventive properties by inducing cell apoptosis in breast and ovarian cancers, but the antitumor activity and the molecular targets of ABL in colon cancer cells have not been determined. In this study, we showed that ABL inhibits the growth in dose- and time-dependent manners by inducing cell cycle arrest in G0/G1 phase of HT-29 human colon cancer cells. This suppression was accompanied by a strong decrease of cyclin E and CDK4 protein levels, and an increase in p21 protein expression in HT-29 cells. We also show that ABL-induced growth inhibition is associated with the upregulation of KLF4 expression. The overexpression of KLF4 by infection with pAd-KLF4 resulted in growth inhibition, with decrease in the protein levels of cyclin E and CDK4, and increase in the expression of p21, similarly to the effects of ABL. Conversely, knockdown of KLF4 using a specific siRNA impaired the ABL-induced growth inhibition in HT-29 cells. These results suggest that KLF4 as an important cellular target of ABL mediates the growth inhibition of HT-29 cells induced by ABL via upregulation of p21 expression.

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Introduction

Colorectal cancer is one of the leading causes of cancer death among both men and women in the world (1). Chronic or recurrent inflammation and uncontrolled cell proliferation have been strongly correlated with colorectal cancer development (2). However, limited chances for cure by chemotherapy are a major contributing factor to this situation. Despite much progress in recent years, a key problem in tumor therapy with established cytostatic compounds is the development of drug resistance and threatening side effects. Most established drugs suffer from insufficient specificity toward tumor cells. Hence, the identification of the new compounds which exert improved antitumor activity and have less toxicity is especially interest in phytochemicals and is urgently needed (3). In this regard, Chinese herbal medicine that has been practiced for thousands of years offers some unique advantages and provides a vast source of pharmaceutical material for the development of effective anticancer drugs with multiple targeting properties.

Acetylbritannilactone (ABL), a new active extract isolated from a traditional Chinese medicinal herb *Inula Britannica* L, is a kind of sesquiterpene (Fig. 1) and has been shown to possess anti-inflammatory and anticancer activities (4-8). Moreover, ABL has been shown to inhibit the expression of cyclooxygenase-2 (COX-2) that plays a significant role in colon carcinogenesis (9,10). Our recent study revealed that one mechanism for the inhibitory effects of ABL-N, a derivative of ABL, on breast cancer is through the activation of caspases and JNK signaling pathways, and increase in the expression of pro-apoptotic members (Bax and Bad) with a concomitant decrease in Bcl-2, subsequently inducing cell apoptosis (11). Taken together, the results suggest that ABL is an anticancer lead compound of multiple targeting molecules, and chemotherapeutics with ABL may provide a superior therapeutic strategy in the clinical setting for treatment of refractory tumors.

Krüppel-like factor 4 (KLF4; formerly known as gut-enriched Krüppel-like factor) is a zinc finger transcription factor that is highly expressed in the gastrointestinal tract (12). It has been proposed to function as a tumor suppressor and an oncoprotein, depending on cellular context. For example, over-expression of KLF4 has been linked to reduced tumorigenicity of colonic and gastric cancer cells *in vivo* (13). In addition, specific ablation of KLF4 in the gastric epithelium of mice results in premalignant changes, suggesting that it may be a tumor suppressor (14). Conversely, loss of KLF4 causes altered proliferation, differentiation, and precancerous changes in the adult blood vessel and stomach (14,15). Reduced expression of KLF4 has been reported in human gastric cancer (13,16) and restoration of KLF4 expression can induce growth arrest in vascular smooth muscle and colon cancer cells or apoptosis in gastric cancer cells (13,17,18). Accumulating clinical evidence also suggests that KLF4 functions as a tumor suppressor; genetic and epigenetic alterations of the KLF4 gene have been found in colorectal cancers (13,19,20). However, a role for KLF4 as an oncogene has been also supported by the induction of squamous epithelial dysplasia by ectopic KLF4 expression in mice. This paradox was partially resolved by a recent study showing that $p21^{WAF1/Cip1}$ (p21) status may be a switch that determines the tumor suppressor or oncoprotein function of KLF4 (21).

In the present study, we investigated the expression and molecular function of KLF4 in ABL-induced growth suppression in HT-29 human colon cancer cells. Our results showed that ABL induces p21 expression and inhibits growth of HT-29 cells via upregulating KLF4 expression.

Materials and methods

Reagents. ABL was isolated by Silica gel column chromatography from *Inula Britannica* L grown in Shan-xi Province in China. ABL was characterized by nuclear magnetic resonance and mass spectroscopy. The purified ABL were dissolved in DMSO at 1,000-fold final concentration and then used to treat cells.

Adenoviral constructs. Full-length cDNA of mouse KLF4 was cloned into the replication-defective adenovirus pAd/CMV/V5-DEST Gateway Vector (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol, to obtain pAd-KLF4 that was confirmed by sequencing. An adenoviral vector (pAd-null) was used as a control virus. The resulting constructs were packaged in A293 cell (ATCC) by transfection with Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Culture supernatants from individual A293 cells were used to infect HT-29 cells.

Cell culture and treatment. The human HT-29 colon carcinoma cells were obtained from American Type Culture Collection and were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 100 U/ml penicillin, and 100 Ag/ml streptomycin. For stimulation, the cells were at 70-80% confluence and then were treated with ABL in series concentrates or infected with pAd-KLF4 at a titre of 50 pfu/cell.

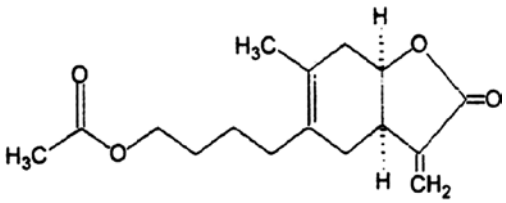

Figure 1. Chemical structure of 1-O-acetylbritannilactone.

Cell proliferation assay. HT-29 cells were seeded onto 96-well plates ($2x10^3$ cells per well) and treated with various concentrations of ABL for 24 h. Proliferation was measured using MTT assay and counting cell number, respectively. The data are mean values from three different experiments.

Fluorescence activated cell sorting (FACS) analysis. HT-29 cells were collected after 24 h of treatment with or without of various concentrations of ABL, fixed in 70% ethanol, washed twice with PBS, and stained with a 50 μg/ml propidium iodide solution containing 0.5% Triton X-100, 0.1 mM EDTA, and 25 μg/ml RNase A. Fluorescence was measured and analyzed using a FACS Calibur Flow Cytometer (Becton Dickinson Immunocytometry Systems).

Western blot analysis. The protein expression for cyclinA, cyclinD1, cyclinE, CDK2, CDK4, CDK6, KLF4, p21, β-actin in HT-29 cells were examined by Western blot analysis as previously described (22). The blots were detected with the enhanced chemiluminescence detection system (Santa Cruz Biotechnology). All experiments were repeated at least three times and yielded similar results.

Immunofluorescent. Cells were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 at room temperature for 20 min. Thereafter, cells were incubated with anti-KLF4 antibody and further stained with appropriate TRITC-conjugated secondary antibody (Santa Cruz, CA). Confocal microscopy was performed with the Confocal Laser Scanning Microscope Systems (Leica).

Small interfering RNA (siRNA) transfection. The siRNAs targeting KLF4 (KLF4 siRNA) (sc-35480) and control siRNA were purchased from Santa Cruz, and then were transiently transfected into HT-29 cells using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. KLF4 protein levels were assessed by Western blotting to confirm adequate silencing of KLF4.

Data analysis. All of the experiments were repeated at least three times with a similar pattern of results. Data are expressed as the mean ± SE, and the effects of ABL treatment were analyzed by Student's t-test using SPSS 13.0 software (SPSS Inc., Chicago, IL) and multiple comparisons using ANOVA. $p<0.05$ was considered to be statistically significant.

Results

ABL inhibits the growth of HT-29 cells in a time- and dose-dependent manner. Previous studies have demonstrated that

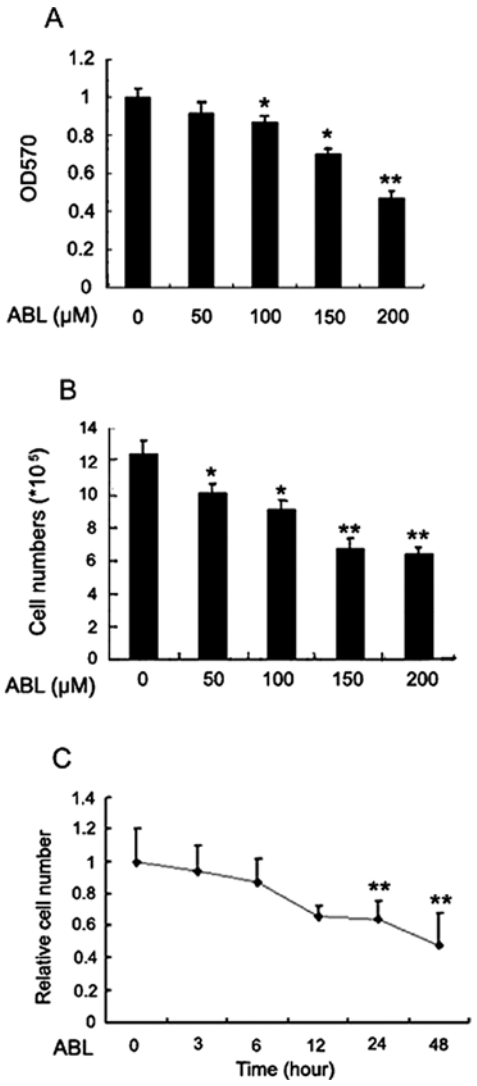

Figure 2. ABL inhibits HT-29 cells proliferation. (A) The MTT assays. HT-29 cells were treated by various concentrations of ABL for 48 h. (B) Cell counting. HT-29 cells were treated by various concentrates of ABL for 24 h. (C) Cell counting of HT-29 cells treated by ABL (150 μmol/l) for 3 to 48 h. $^{*}p<0.05$ or $^{**}p<0.01$, compared with the cells without ABL treatment (n=5).

ABL inhibits the inflammation, proliferation and neointimal formation in VSMCs and induces cell apoptosis in breast and ovarian cancers (5,7). In the present study, we initially tested antiproliferative effect of on human HT-29 colon carcinoma cells using MTT and cell counting assays. HT-29 cells were plated in a 96-well plate, and then treated by different concentrations (0-200 μM) of ABL for 24 h. The results showed that ABL (0, 50, 100, 150, 200 μM) treatment dose-dependently reduced the activity of cell proliferation (Fig. 2A and B). Higher concentrations of ABL (100-200 μM) almost completely inhibited the cell proliferation ($p<0.05$) (Fig. 2A). The results from cell counting were in agreement with MTT assay. Under the same conditions, the cell number decreased by 40, 52, 64 and 76%, compared with the control group, respectively (Fig. 2B). The IC_{50} value (concentration needed for 50% growth inhibition) of ABL was ~95 μM under the present experimental conditions. When HT-29 cells were treated with ABL (150 μM) for different times, the time-dependent reduction was observed in the cell viability as compared with the untreated cells (Fig. 2C). All of these data suggest that ABL can exert its growth inhibitory effect in HT-29 cells.

ABL arrests the cell cycle progression in HT-29 cells. To determine the effect of ABL on cell cycle progression, HT-29 cells were treated with 50-200 μM ABL for 24 h, and then subjected to flow cytometric analysis. Data showed that ABL dose-dependently reduced the number of S phase cells and increased the G0/G1 phase cells ($p<0.05$) (Fig. 3A). The results suggested that the cell cycle of HT-29 cells was arrested in the G0/G1 phase by ABL. Cell cycle progression is controlled by cyclins and CDKs (23). To determine the expression of cyclins and CDKs in ABL-treated HT-29 cells, the lysates of the cells were analyzed by Western blotting. Treatment with ABL (50-200 μM) reduced expression of cyclin E and CDK4, in a concentration-dependent manner with complete inhibition at 100 μM (Fig. 3B and C). Conversely, CDK inhibitor p21 level in HT-29 cells was upregulated under the same conditions (Fig. 3D). However, the levels of cyclin A, cyclin D, CDK2 and CDK6 proteins were not affected by ABL treatment. These results suggest that ABL arrests cell cycle progression in G0/G1 phase through accumulating p21 protein and inhibiting cycin E and CDK4 expression.

ABL induces KLF4 expression in HT-29 cells. Recent evidence has implicated KLF4 as a central regulator of proliferation in various normal and malignant cells. Several proteins regulated by KLF4 such as p21, cyclin E are also targeted by ABL (4,24). Thus, we hypothesized that ABL may reduce proliferation by promoting the KLF4 activity. For this, we first measured the expression of KLF4 protein in ABL-treated cells. After an overnight serum starvation, the cells were treated with ABL at different concentration for 24 h. As shown in Fig. 4A, KLF4 level in HT-29 cells increased in a dose-dependent manner with ABL treatment. Similar results were observed by immnofluorescent. The increased KLF4 protein was mainly located in the nucleus of HT-29 cells and associated with growth inhibition (Fig. 4B). Therefore, we speculated that KLF4 may be involved in ABL-induced growth inhibition in HT-29 cells.

KLF4 is involved in ABL-induced growth inhibition of HT-29 cells. To distinguish the above-mentioned possibilities, we evaluated for possible alterations of the cell proliferation in response to overexpression of KLF4. HT-29 cells were first infected with either pAd–KLF4 or Ad-null for 48 h. The MTT assay showed that the proliferation of HT-29 cells was inhibited by the overexpression of KLF4 in a concentration-dependent manner (Fig. 5A), similar to ABL effect. However, overexpression of KLF5 did not inhibit the cell growth (data not shown). Furthermore, we investigated the effects of KLF4 overexpression on the expression of cell cycle-related proteins using Western blot analysis. The results showed that overexpression of KLF4 resulted in the decrease in cyclin E and CDK4 protein levels, and an increase in the expression of p21 protein (Fig. 5B). To confirm whether KLF4 is necessary for ABL-induced growth arrest of HT-29 cells, we examined the effects of KLF4 knockdown by specific siRNA. The results showed that inhibiting of KLF4 expression impaired

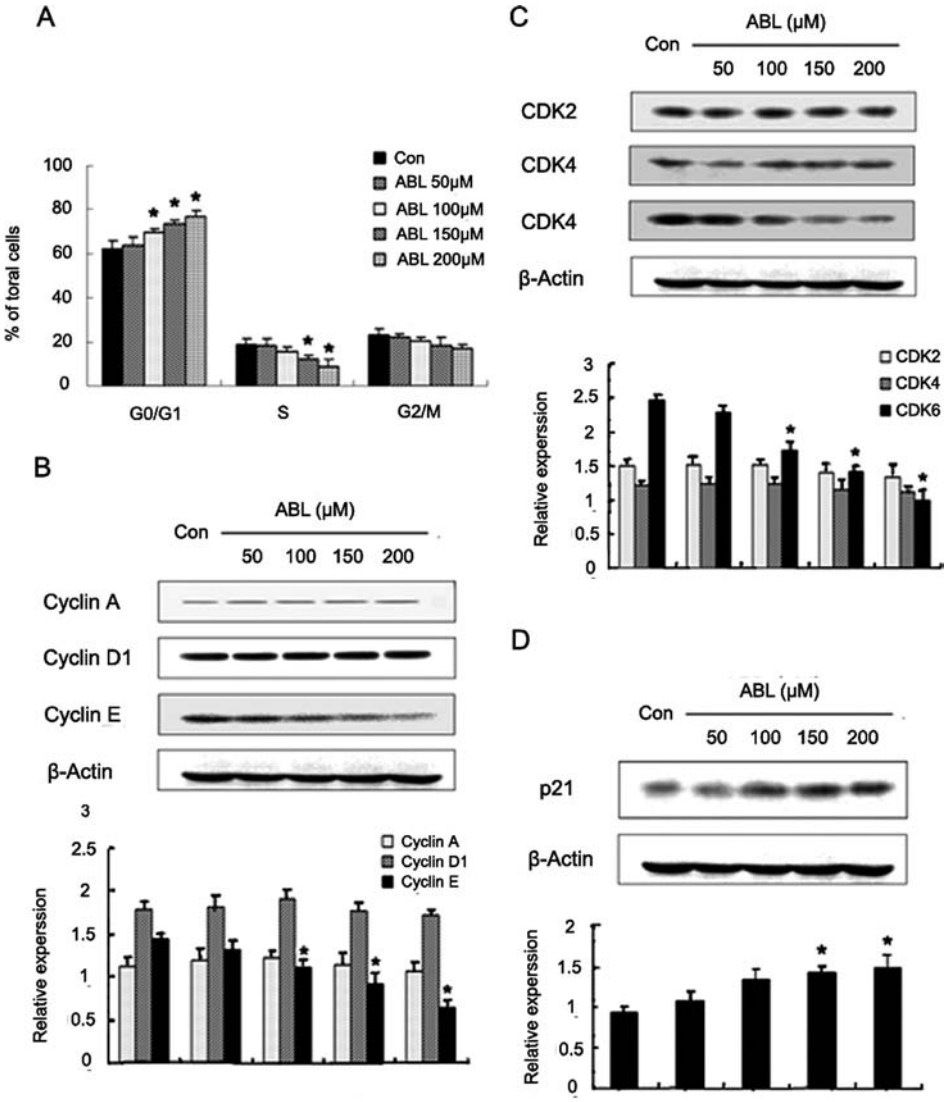

Figure 3. ABL inhibits expression of cyclin E and CDK4, and increases the expression of p21. (A) Flow cytometric analysis. HT-29 cells were treated by ABL at indicated concentration for 24 h (n=3). (B-D) Western blotting for cyclins, CDKs and p21 proteins. HT-29 cells were treated by ABL at the indicated concentration for 24 h. β-actin was used as an internal control. The graphs represent the relative level of these proteins for four independent experiments. *p<0.05, compared with the control.

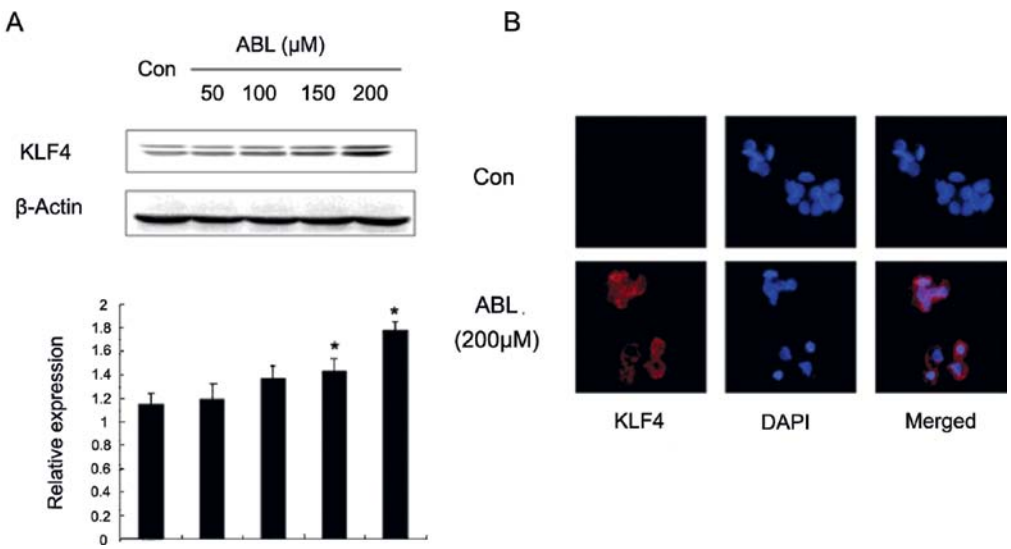

Figure 4. ABL induces KLF4 expression in HT-29 cells. (A) Western blotting. HT-29 cells were treated by ABL at indicated concentration for 24 h. β-actin was used as an internal control. The graphs represent the relative level of KLF4 protein for four independent experiments. *p<0.05, compared with the control. (B) Immunofluorescent staining (x200 magnification).

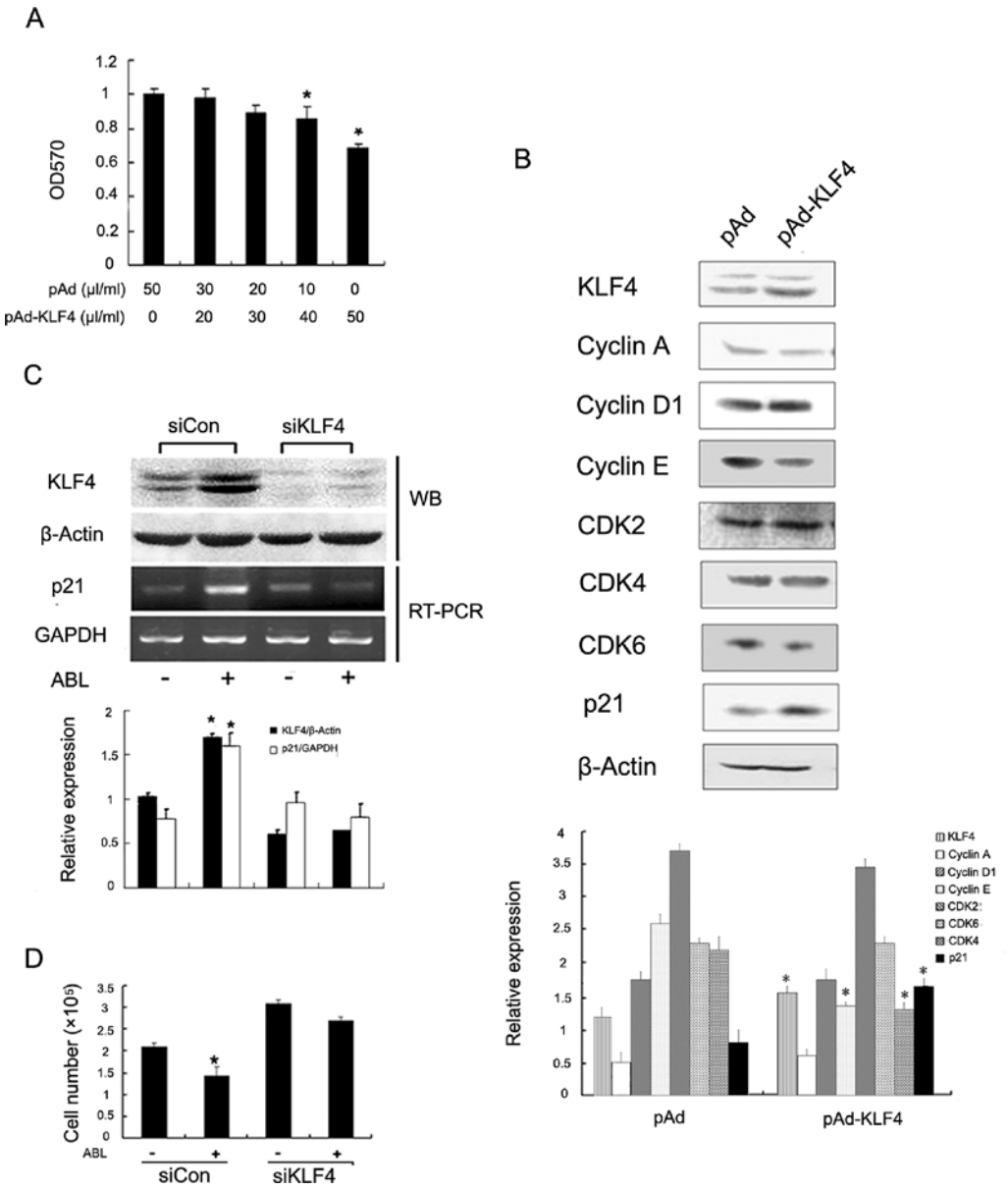

Figure 5. KLF4 is involved in ABL-induced growth inhibition of HT-29 cells. (A) The MTT assay. Cells were infected with the indicated titers of pAd-KLF4 for 48 h. Values are mean ± SD from three different experiments. *p<0.05, compared with pAd (n=3). (B) Western blotting. Cells were infected with pAd-KLF4 for 48 h. The graphs represent the relative level of these proteins for four independent experiments. *p<0.05, compared with pAd. (C) Western blotting (WB) of KLF4 expression (upper panel), and semi-quantitative RT-PCR of p21 mRNA (lower panel). Cells were infected with siKLF4 for 48 h and then treated with ABL (150 μmol/l) for another 24 h. The graphs represent the relative level of KLF4 protein and p21 mRNA for four independent experiments. (D) Cell counting. Cells were infected with siKLF4 for 48 h and then treated with ABL (150 μmol/l) for another 24 h. Values are mean ± SD from three different experiments (n=3). *p<0.05, compared with HT-29 cells treated without ABL.

the growth suppression induced by ABL with reduction of p21 protein level (Fig. 5C). Taken together, these results indicate that KLF4 mediates the growth arrests in HT-29 cells induced by ABL.

Discussion

KLF4 has been recognized as a key therapeutic target for the treatment of several types of cancer since it is a central hub for regulation of cellular processes that are critical for growth and metastasis of human cancers (25,26). Herein, we show that ABL reduced proliferation of HT-29 CRC cells in dose- and time-dependent manners. This prompted us to further study the effect of ABL on KLF4 expression since the anticancer mechanism of ABL remains to be fully elucidated. To this end, we found that ABL induced expression of KLF4 and p21 proteins, as well as downregulation of cyclin E and CDK4 level. Therefore, the antiproliferative effects of ABL in HT-29 cells may be mediated by induction of the KLF4 activity and downstream KLF4-mediated p21 mRNA transcription (21,27).

Previous studies indicate that KLF4 inhibits cell proliferation by activating crucial checkpoints in the cell cycle on overexpression of exogenous KLF4 or following DNA damage (28,29). A critical transcriptional target of KLF4 in these conditions is the cyclin-dependent inhibitor, p21 (28-30). These findings led to the suggestion that KLF4 may function as a tumor suppressor in colorectal cancer.

In cell culture, KLF4 expression can be increased by serum deprivation, contact inhibition, and DNA damage (31,32). Conversely, reduced expression of KLF4 has been reported in various tumors (13,14), and restoration of KLF4 expression can induce growth arrest in colon cancer cells (18). Inhibition of KLF4 gene expression plays a critical role in homeostasis of the normal intestine and in tumorigenesis of colorectal cancers (33,34), suggesting that KLF4 is important in tumor development and progression. Biochemical studies indicate that KLF4 inhibits cell proliferation by blocking progression of the cell cycle at the G1-S and G2-M transitions (31). Studies also show that expression of KLF4 is reduced in colorectal neoplasia including carcinoma and adenoma relative to normal mucosa (20,35). KLF4 has been shown to activate p21 expression (36). In the present study, we showed that increase in KLF4 levels in ABL-treated HT-29 cells was accompanied by an increase in the levels of p21, a downstream mediator of the cell cycle effect of KLF4 (28). Several lines of evidence from our and other studies indicate that p21 is a downstream target of KLF4 (25). In HT-29 cells, overexpression of KLF4 induces p21 expression, which is accompanied by a decrease in the rate of proliferation, and reduction of cyclin E and CDK4 protein levels. Conversely, inhibition of KLF4 by siRNA against KLF4 results in a decrease in p21 expression and an increase in cellular proliferation. Importantly, the increase in KLF4 is correlated with the antitumor activity of ABL in HT-29 cells. In view of the established inhibitory effect of KLF4 on cell proliferation, it is likely that KLF4 is responsible at least in part for the antiproliferation activity of ABL in HT-29 cells.

In summary, we show that the antiproliferative effect of ABL may be mediated by induction of KLF4 expression. ABL produces a dose-dependent increase of KLF4 protein level. Surprisingly, ABL results in a dose-dependent decrease of cyclin E and CDK4 level; this effect may be attributed to a dose-dependent increase in p21 mRNA and protein levels induced by ABL and/or KLF4, suggesting the KLF4 as an important cellular target of ABL in HT-29 cells. The fact that ABL is able to target the human colon tumor KLF4 and inhibits human colon tumor cell growth provides a strong impetus for using ABL as a chemopreventative and/or chemotherapeutic agent for human colon cancer.

Acknowledgements

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