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Original Article

Mistletoe (*Viscum album*) extract targets Axl to suppress cell proliferation and overcome cisplatin- and erlotinib-resistance in non-small cell lung cancer cells

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ABSTRACT

Background: Mistletoe extract of *Visucm album* extract (VAE) contains many biologically active components and has been reported to be not only a complementary and alternative medicine, but also a potent therapeutic agent for many types of cancer.

Purpose: In this study, we examined the effect of VAE on expression and activation of Axl and scrutinized the involvement of Axl in the anti-cancer activity of VAE in parental and chemo-resistant non-small cell lung cancer (NSCLC) cells.

Methods: The levels of Axl protein and mRNA were determined by Western blot analysis and RT-PCR, respectively. Phosphorylation of Axl upon Gas6 stimulation was observed by Western blot analysis. For ectopic expression or gene silencing of Axl, the recombinant plasmid, pcDNA3-Axl, or specific siRNA targeting Axl were transfected into A549 and H460 cells using Lipofectamine 2000, respectively. The anti-cancer activity of mistletoe extract was examined against the parental cells and each of their cisplatin- or erlotinib-resistant cells using trypan blue exclusion assays and colony formation assay.

Results: The levels of Axl mRNA were also reduced by VAE treatment, implying the transcriptional down-regulation of Axl expression by VAE. In addition, the phosphorylation of Axl protein upon its ligand, Gas6, stimulation was found to be abrogated by VAE. We next found cytotoxic effect of VAE on both the parental NSCLC cells and their variants which are resistant to cisplatin (A549/CisR and H460/CisR) or erlotinib (H460/ER and H1975/ER). Treatment of these cells with VAE caused a dose-dependent decrease of cell viability and clonogenicity. This anti-proliferative effect of VAE was attenuated in Axl-overexpressing cells, while it was augmented in cells transfected Axl specific siRNA. Next, we also found that in cisplatin-resistant cells and erlotinib-resistant cells, VAE treatment decreased Axl protein level, colonogenicity. The levels of several cell cycle regulator, p21 and apoptosis related protein, X-linked inhibitor of apoptosis, was found to be induced and reduced by VAE treatment, respectively.

Conclusion: Taken together, our data provide that VAE targets Axl to suppress cell proliferation and to circumvent cisplatin- and erlotinib-resistance in NSCLC cells.

Introduction

Lung cancer is the most common cancer worldwide. About 85% of lung cancer is non-small cell lung cancer (NSCLC), which is less aggressive and relatively insensitive to anti-cancer agents, while small cell lung cancer (SCLC) accounts for 10% of lung cancer and has higher growth rate and is more easily metastasized than NSCLC (Ettinger et al., 2013). In case of NSCLC, platinum- or taxol-based chemotherapy is the standard first line treatment (Chen et al., 2014). Since intrinsic or the acquired resistance to these agents limits their therapeutic effects, targeted therapy or combined regimens has been applied as an alternative approach to overcome these obstacles (Forde and Ettinger, 2013).

Abbreviations: ALK, anaplastic lymphoma kinase; EGFR, epidermal growth factor receptor; GAS6, growth arrest-specific 6; NSCLC, non-small cell lung cancer; RTK, receptor tyrosine kinase; SCLC, small cell lung cancer; siRNA, small interfering RNA; TKI, tyrosine kinase inhibitors; VAE, *Viscum album* extract; XIAP, x-linked inhibitor of apoptosis

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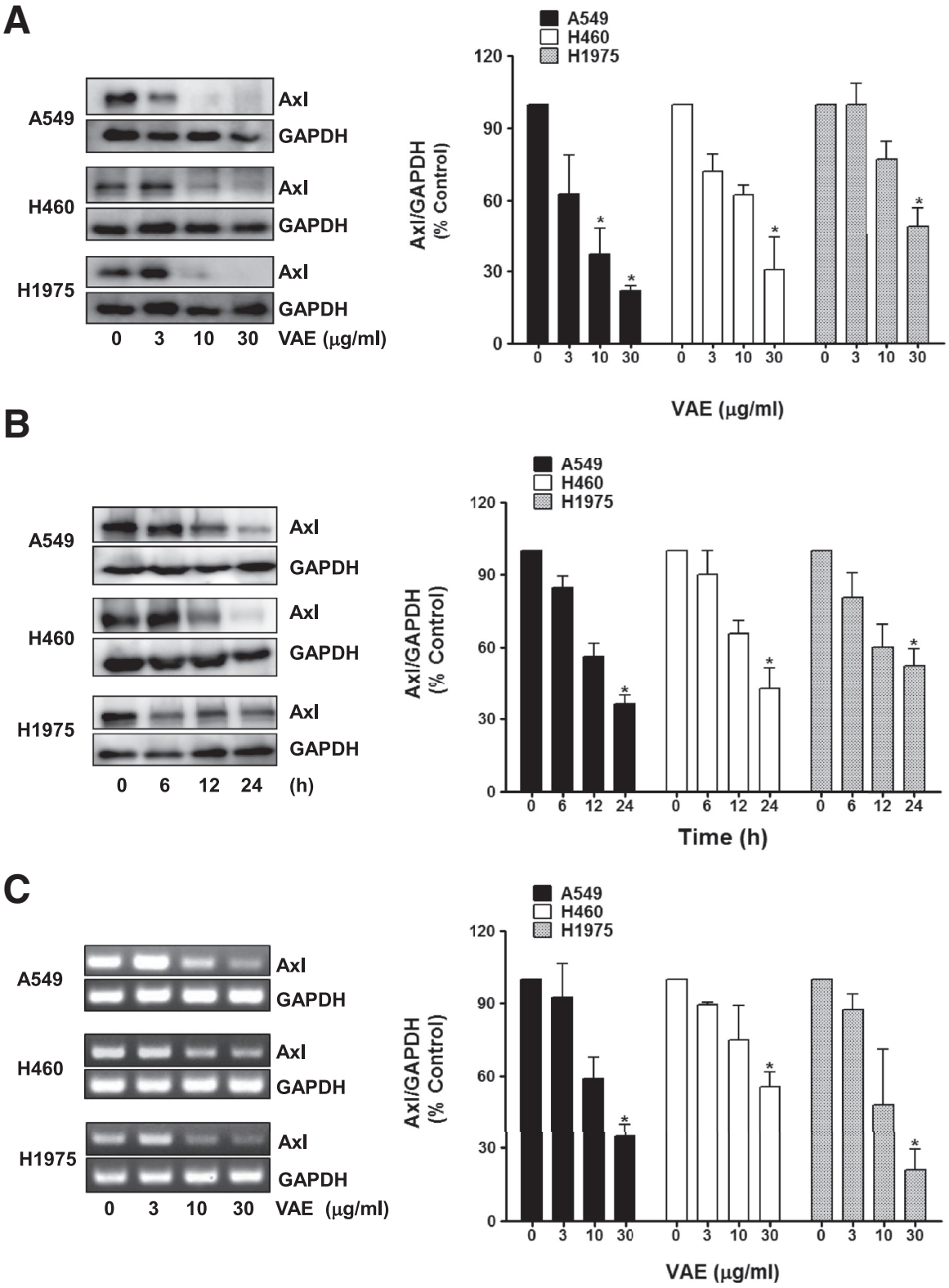

Fig. 1. VAE decreases Axl expression at transcriptional level.
Cells (3×10^5 cells/dish) were seeded onto 60 mm dishes, grown overnight and exposed to VAE. (A) A549, H460 and H1975 cells were treated with 3, 10, 30 μg/ml of VAE for 24 h and then harvested to prepare cell lysates for Western blot analysis. (B) Cells were treated with 20 μg/ml of VAE for 6, 12, 24 h and then harvested. Axl protein levels were determined by Western blot analysis. GAPDH was used as an internal control. Result shown is a representative of three independent experiments. The intensity of protein band was measured and Axl/GAPDH ratio was presented in lower graph. The asterisks indicate the significant difference compared to the control value (*$P < .05$ vs untreated group). (C) For RT-PCR, total RNAs from the cells treated with the indicated concentrations of VAE for 12 h were isolated and used to determine Axl mR NA levels. As an internal control, GAPDH mRNA was also amplified by RT-PCR. The data shown are a representative of three independent experiments. The intensity of amplicon was measured and Axl/GAPDH ratio was presented in lower graph. The asterisks indicate the significant difference compared to the control value (*$P < .05$ vs untreated group).

However, the overall survival rate of NSCLC patients still remains fairly low.

Among 58 receptor tyrosine kinases (RTKs) in the human genome, which classified into 20 subfamilies, TAM family is a small subfamily with only three members which are Tyro3 (also referred to Brt, Dtk, Rse, Sky or Tif), Axl (also referred to Ark, Tyro7, or Ufo) and Mer (also referred to Eyk, Nyk, or Tyro12) (Graham et al., 1994; Lai et al., 1994; O'Bryan et al., 1991). The structural features of these TAM RTKs are so similar, which are the extracellular domain with two immunoglobin-like and two fibronectin type III domains, single transmembrane domain and cytosolic kinase domain (Heiring et al., 2004; Sasaki et al., 2006). In addition, they share several ligands such as growth arrest-specific 6 (Gas 6), protein S, tubby, and tulip to transduce extracellular signals for cell survival, proliferation, invasion, migration (Goruppi et al., 1996; Shiozawa et al., 2010). Gas6 has been reported to be able to engage and activate all three TAM RTKs (Nagata et al., 1996).

Axl is first identified as a novel RTK in 1988 and cloned from chronic myelogenous leukemia patients in 1991 (O'Bryan et al., 1991). Upregulation of Axl expression and its activation has been observed in diverse types of cancers including acute leukemia (Rochlitz et al., 1999), breast (Berclaz et al., 2001), colorectal (Martinelli et al., 2015), ovarian (Rankin et al., 2010) and prostate (Shiozawa et al., 2010) to result in cell survival, proliferation and inhibition of apoptosis. Interestingly, in NSCLC, head and neck cancer, chronic myelogenous leukemia and gastrointestinal stromal tumors, the overexpression and/or activation of Axl found to be a mechanism to get the acquired resistance against epidermal growth factor receptor (EGFR) inhibitors, gefitinib (Bae et al., 2015) or erlotinib (Giles et al., 2013), and tyrosine kinase inhibitor, imatinib (Dufies et al., 2011), respectively. MicroRNAs or monoclonal antibodies which specifically target Axl have been reported to inhibit the proliferation of NSCLC cells in vitro as well as in vivo (using tumor xenografts) (Gazdar, 2010; Wang et al., 2014). Therefore, Axl seems be potent enough as a therapeutic target to treat cancer and overcome chemoresistance.

Mistletoe is universal name for various species of parasitic plant which grows attached to and within the branches of host trees such as apple, elm, oak and pine. Mistletoe extract prepared from *Viscum album*, a species of mistletoe in the family Santalaceae, is generally known as European mistletoe and frequently prescribed to improve numerous health problems including hypertension, diabetes mellitus, inflammation, arthritis, and viral infection (Bock et al., 2014; Glickman-Simon and Pettit, 2015; Gorter et al., 1999; Kim et al., 2014; Tusenius et al., 2005). Especially, it has been steadily considered as a potent complementary and alternative medicine to treat various human solid tumors (Mansky et al., 2013; Shakeel et al., 2014; Weissenstein et al., 2016). Accumulating evidence from in vitro and in vivo studies has proposed various possible mechanisms involved in the anti-tumor activity of mistletoe extract or its biologically active components such as lectins and viscotoxins (Bussing et al., 1996; Schaller et al., 1998), which are inhibition of cell cycle (dela Cruz et al., 2015), induction of apoptosis (Kovacs, 2010), degradation of cytoskeletal proteins (Lavastre et al., 2007), and alteration of expression and/or activity of intracellular molecules which transduce signals for cell growth, survival and proliferation (Lavastre et al., 2002; Lyu and Park, 2007; Ucar et al., 2012). Although the therapeutic values of mistletoe extracts keep growing, the underlying mechanisms to explain its anti-tumor activity is not fully studied.

In this study, we aimed to explore a novel target of *Visucm album* extract (VAE) associated with its anti-tumor effect. Our data demonstrated the inhibitory effects of VAE on Axl expression, its activation upon a ligand binding, cell viability and colony formation in NSCLC cells. To further understand the underlying mechanisms involved in anti-proliferative effect of VAE, we also examined if VAE affects some intracellular molecules related to cell cycle regulation and apoptosis.

Results

VAE downregulates Axl expression at transcriptional level

We first examined the effect of *Visucm album* extract (VAE) on Axl receptor tyrosine kinase (RTK) expression in non-small lung cancer cells (NSCLC) with wild-type EGFR, A549 and H460 cells, as well as with EGFR mutation, H1975 cells. These cells were treated with 3, 10, and 30 μg/ml of VAE for 24 h and Axl protein level was determined by Western blot analysis. As shown in Fig. 1A, VAE treatment of these cells resulted in dose-dependent reduction of Axl protein level. Additionally, this inhibitory effect of VAE on Axl protein level was also found to be time-dependent, since Axl protein levels were gradually decreased, when cells were exposed to 20 μM VAE for 6, 12, and 24 h (Fig. 1B).

Next, RT-PCR was conducted to further examine if VAE affects Axl mRNA level. Consistent with Western blot results, treatment of A549, H460 and H1975 cells with the indicated concentrations of VAE were found to cause significant and dose-dependent decline of Axl mRNA level (Fig. 1C). Taken together, Western blot and RT-PCR results showed the marked reduction of both protein and mRNA levels of Axl by VAE, indicating that VAE downregulates Axl expression at transcriptional level.

VAE suppresses GAS6-dependent activation of Axl

Growth arrest-specific gene 6 (GAS6) is one of the ligands which binds to Axl and causes its homo- or hetero-dimerization and the subsequent phosphorylation of tyrosine residues in cytoplasmic kinase domain (Sasaki et al., 2006). Previously, we reported that Axl was phosphorylated by GAS6 treatment in A549 and H460 cells (Kim et al., 2015). In H1975 cells, Axl phosphorylation in response to GAS6 was also examined. Serum-starved H1975 cells were treated with 200 μg/ml of Gas6 for 10, 15, 30, 60 min and the levels of phosphorylated Axl protein were determined by Western blot analysis. The phosphorylation of Axl after Gas6 treatment was found to be rapid (Fig. 2A), since the phospho-Axl protein level was fairly increased within 10 min and returned back to basal level by 30 min.

We next observed the effect of VAE on Axl phosphorylation upon GAS6 stimulation. As shown in Fig. 2B, pre-incubation of H460 and H1975 cells with 20 μg/ml of VAE substantially reduced Gas6-induced Axl phosphorylation, implying that VAE impedes Axl activation upon its ligand binding.

VAE targets Axl to inhibit cell proliferation and to overcome chemoresistance

Since intracellular signals via Axl play critical roles to induce cell growth, survival, proliferation and to block apoptosis (Goruppi et al., 1996; Li et al., 2009; Linger et al., 2013; Sainaghi et al., 2005; Shiozawa et al., 2010), we examined the effect of VAE on cell proliferation and also inspected if the downregulation of Axl expression by VAE correlates with its cytotoxicity. Treatment of cells with 3, 10, and 30 μg/ml of VAE for 24 h was found to result in dose-dependent reduction of cell viability (Fig. 3A). Of note, exposure of A549, H460 and H1975 cells with 30 μg/ml of VAE diminished the viabilities of each cells to 41%, 37% and 43%, respectively.

Clonogenic assay was also conducted to further confirm the inhibitory effect of VAE on cell proliferation. A549, H460 and H1975 cells were incubated with the indicated concentrations of VAE for 24 h and then let them grow for the next 7–10 days to form colonies by the viable cells. The number of colonies was dose-dependently retarded by VAE treatment and all three cells failed to form visible colonies at higher than 10 μg/ml of VAE (Fig. 3B). It is perceptible that sensitivity of these cells to VAE seems to be regardless of epidermal growth factor receptor (EGFR) status, since A549 and H460 cells express wild-type EGFR, while H1975 cell has EGFR mutation.

To evaluate the significance of Axl targeting of VAE, we prepared

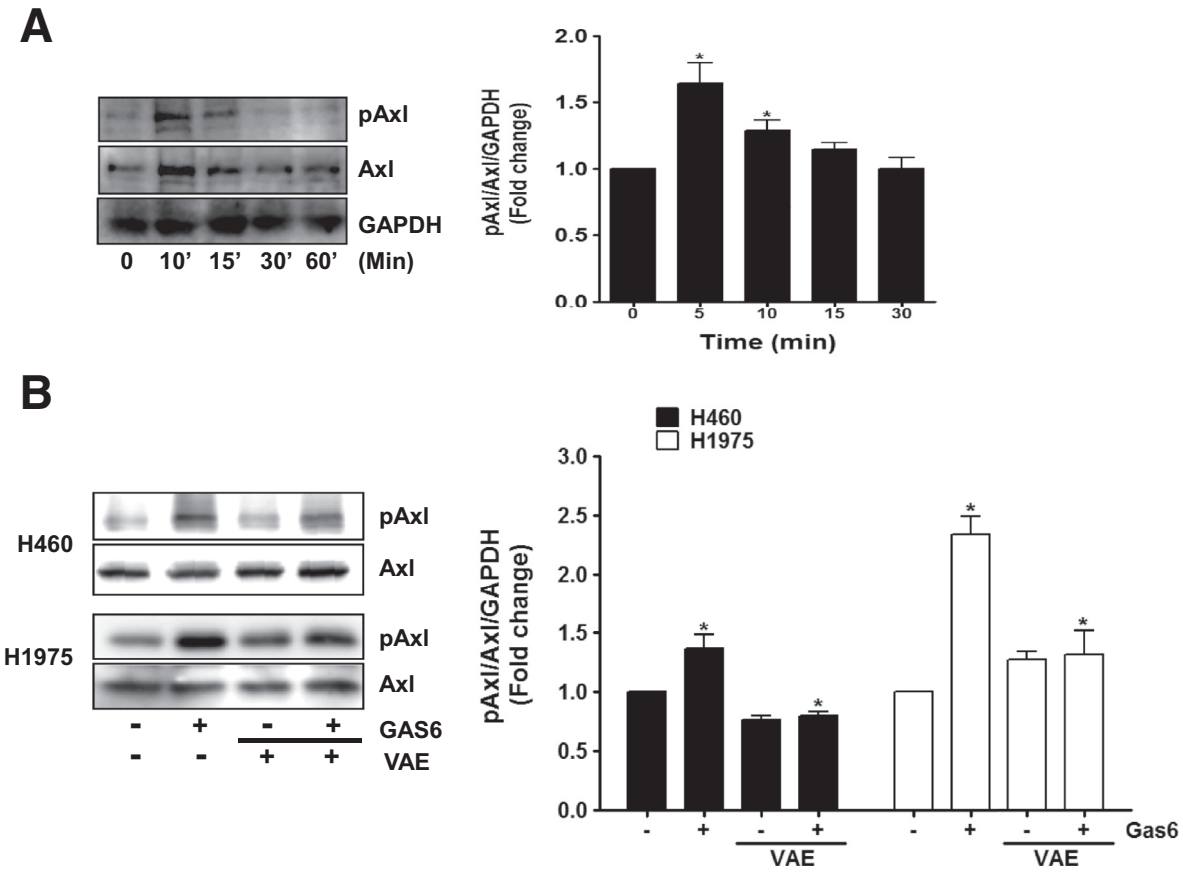

Fig. 2. VAE inhibits Gas6-dependent Axl activation. (A) H1975 cells (3×10^5 cells/dish) were seeded onto 60 mm dishes, serum-starved overnight, and treated with 200 ng/ml Gas6 for 15, 30, and 60 min and then harvested. (B) H460 and H1975 cells were pre-incubated with 20 μg/ml of VAE for 60 min, followed with Gas6 treatment and then harvested. Phosphorylated Axl protein levels were determined by Western blot analysis. Total Axl and GAPDH were used as a loading control. Result shown is a representative of three independent experiments. The intensity of protein band was measured and pAxl/Axl/GAPDH ratio was presented in lower graph. The asterisks indicate the significant difference compared to the control value (*$P < .05$ vs untreated group).

cells which are transfected with a recombinant plasmid containing *Axl* cDNA, pcDNA3-Axl, to overexpress Axl protein or with specific siRNA to knockdown Axl expression. We then examined the effect of VAE on these cells' proliferation. Western blot result showed that the Axl protein level was increased in Axl-overexpressing A549 cells, A549/pcDNA3-Axl, compared to the control cells transfected with pcDNA3 vector, A549/pcDNA3 (Fig. 3C). Moreover, Axl protein levels in A549/pcDNA3-Axl cells were higher than those in control cells even after VAE treatment, indicating a quiet compensation of the inhibitory effect of VAE on Axl protein level by ectopic expression of Axl.

Colony formation assay also showed that Axl-overexpressing cells are relatively less sensitive to VAE compared to the control cells. As shown in Fig. 3D, A549/pcDNA3-Axl cells were found to form colonies at 10 μg/ml of VAE, while A549/pcDNA3 cells were not able to form colonies at the same concentration of VAE.

Next, we observed the augmentation of anti-proliferative effect of VAE in cells transfected with specific siRNA, siAxl, which down-modulates Axl expression. H460 cells were transfected with siAxl, or control siRNA, siCtrl, and then treated with the indicated concentrations of VAE for 24 h. Western blot results showed that Axl protein level was decreased in siAxl-transfected cells and additionally diminished by VAE treatment (Fig. 3E). In accordance with Western blot results, we found that knockdown of Axl expression resulted in the synergistic inhibition of cell proliferation by VAE (Fig. 3F).

Taken together, these results demonstrated that Axl is a novel target of VAE to inhibit cell proliferation, confirming again that Axl protein level is closely correlated with cell proliferation.

VAE inhibits cell proliferation of cisplatin- and erlotinib-resistant cells and results in the elevation of p21 as well as reduction of XIAP expression

Next, we examined the cytotoxic effect of VAE in cisplatin- and erlotinib-resistant cells. To establish each variant cells, the parental cells were exposed to escalating concentrations of cisplatin (A549/CisR and H460/CisR cells) or erlotinib (H460/ER and H1975/ER cells), respectively. Axl protein levels in these chemo-resistant cells were decreased by VAE treatment. Of note, H1975/ER cells seemed to be most sensitive to VAE among these chemo-resistant cells, since in H1975/ER cells, Axl protein level was considerably decreased by as low as 3 μg/ml of VAE at which there was almost no effect on Axl protein level in A549/CisR and H460/ER cells (Fig. 4A and C).

Colony formation assay also showed cytotoxicity of VAE on these chemo-resistant cells. As shown in Fig. 4B and D, treatment of these cells with VAE caused substantial reduction of the number of colonies and the size of each colony. Compared to the effect of VAE in A549/CisR and H460/ER cells, that in H460/CisR and H1975/ER cells were found to be more profound, which are mostly consistent with Western blot result.

To further understand underlying mechanism which might be associated with the inhibitory effect of VAE on cell proliferation, we assessed the levels of several cell cycle regulators including p21 and apoptosis related protein, X-linked inhibitor of apoptosis protein (XIAP). A549, A549/CisR, H1975, and H1975/ER cells were treated with 20 μg/ml of VAE for 24 h and Western bolt analysis showed that VAE induced the expression of cyclin-dependent kinase inhibitor, p21, which causes cell cycle arrest, but reduced that of XIAP, which inhibits

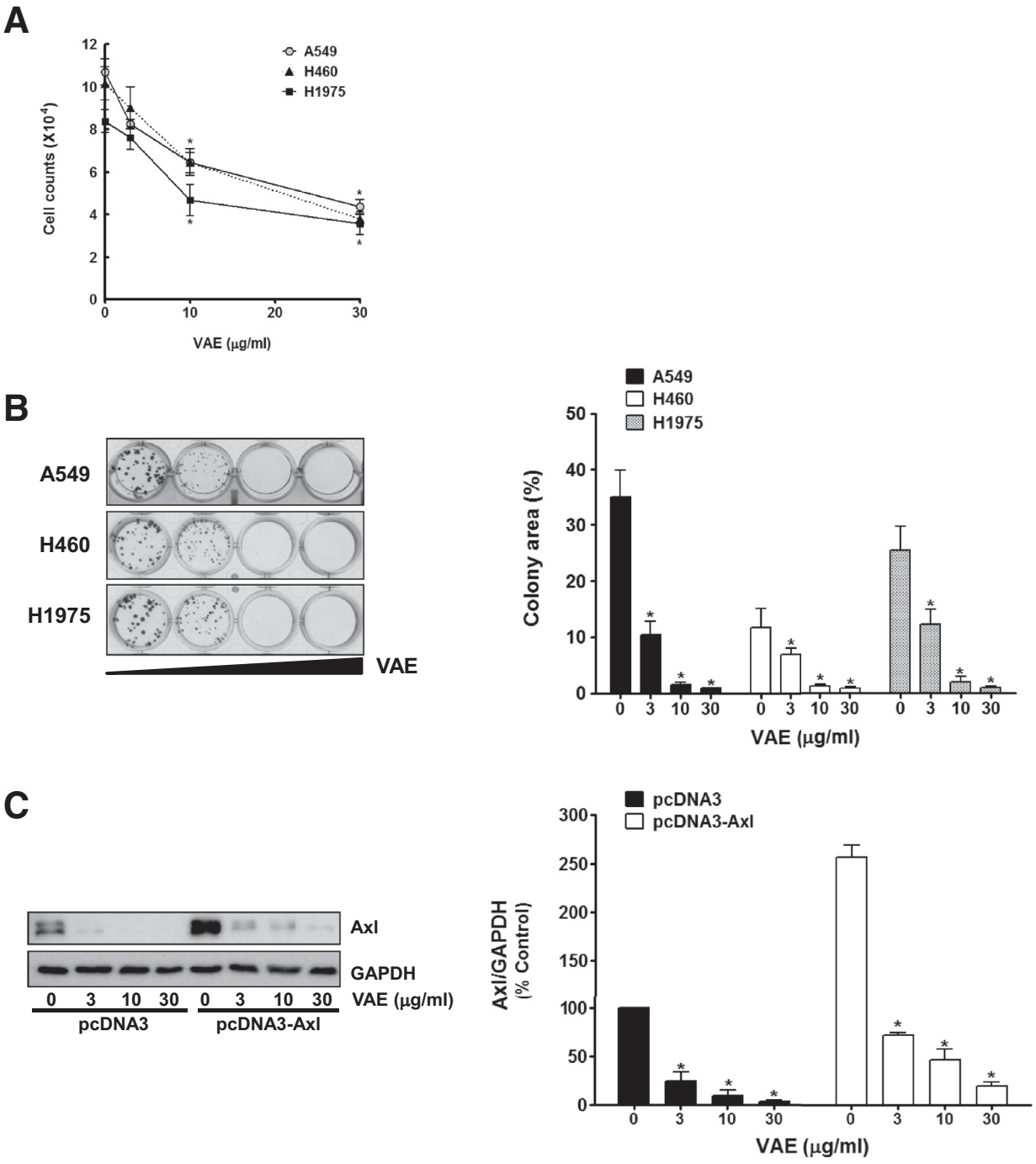

Fig. 3. VAE suppresses cell proliferation, which is attenuated or augmented by elevation or knockdown of Axl expression, respectively. A549, H460 and H1975 cells (3×10^5 cells/dish) were seeded onto 60 mm dishes, grown overnight. (A) Cells were exposed to 3, 10, 30 μg/ml of VAE for 24 h and then used for trypan blue exclusion assay to count the number of viable cells. Data are represented as mean ± SD of at least three independent experiments. The asterisks indicate the significant difference compared to the control value (*$P < .05$ vs untreated group). (B) Cells (2×10^3 cells/well) were seeded onto 24-well plates, treated with the indicated dose of VAE for 24 h and then allowed to grow for next 7–10 days. Colonies were visualized by crystal violet staining and total colony area was quantified and presented in lower graph. The data shown are representative of three independent experiments. The asterisks indicate the significant difference compared to the control value (*$P < .05$ vs untreated group) (C) A549/pc-DNA3 and A549/pc-DNA3-Axl cells which are transfected with pcDNA3 vector or recombinant pcDNA3-Axl plasmid, respectively, were treated with the indicated doses of VAE for 24 h and then harvested. The total cell lysates were prepared and Axl protein level was determined by Western blot analysis. GAPDH was used a loading control. Result shown is a representative of three independent experiments. The intensity of protein band was measured and Axl/GAPDH ratio was presented in lower graph. The asterisks indicate the significant difference compared to the control value (*$P < .05$ vs untreated group). (D) Colonogenic assay was also conducted with A549/pc-DNA3 and A549/pc-DNA3-Axl cells exposed to the indicated concentrations of VAE for 24 h. Colonies formed during 7–10 days of culture were visualized by crystal violet staining and total colony area was quantified and presented in lower graph. The data shown are representative of three independent experiments. The asterisks indicate the significant difference compared to the control value (*$P < .05$ vs untreated group). (E) H460 cells (3×10^5 cells) were transfected with Axl specific siRNA, siAxl, or control siRNA, respectively, and then harvested 24 h later. Cells were divided into two groups and treated with 10 and 20 μg/ml of VAE for 24 h. Axl protein levels were determined by Western blot analysis and GAPDH was used as a loading control. The intensity of protein band was measured and Axl/GAPDH ratio was presented in lower graph. The asterisks indicate the significant difference compared to the control value (*$P < .05$ vs untreated group). (F) Both cells transfected with siAxl or control siRNA were treated with 10 μg/ml of VAE for 24 h, then harvested and stained with tryphan blue to count viable cells. Data are represented as mean ± SD of at least three independent experiments. The asterisks indicate the significant difference compared to the control value (*$P < .05$ vs untreated group).

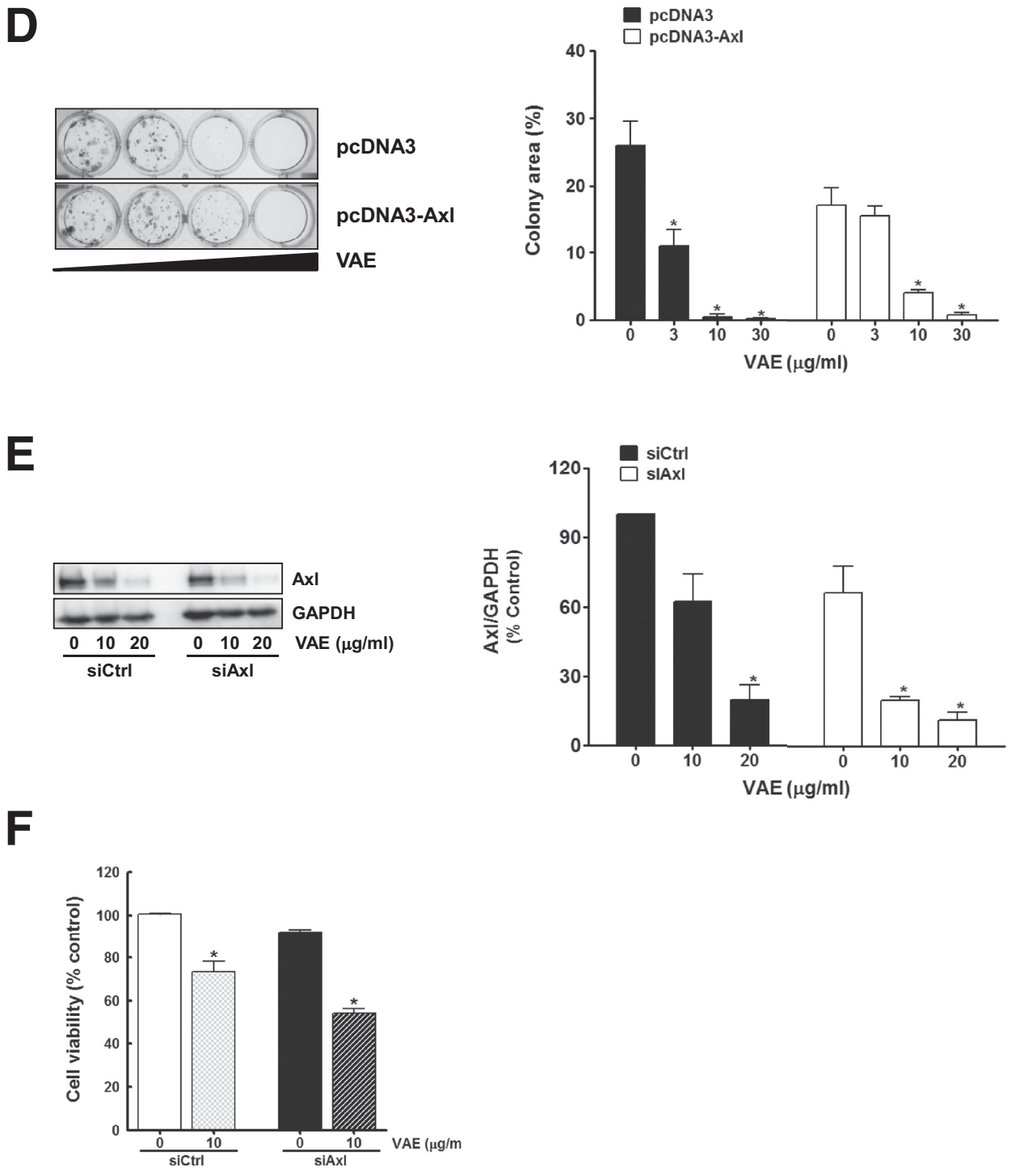

Fig. 3. (*continued*)

apoptosis (Fig. 4E). Taken together these results indicate that VAE downregulates Axl expression, subsequently increases p21 protein level, and decreases XIAP protein level, which results in the inhibition of cell proliferation.

Discussion

Platinum-based regimens and the combination with second-line agents such as docetaxel, pemetrexed, or vinorelbine have been the standard chemotherapy for NSCLC (Al-Farsi and Ellis, 2014). Recent approaches to develop personalized medicine discovered the specific inhibitors targeting epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK) and other tyrosine kinases (Gazdar, 2010; Kwak et al., 2010; Natale et al., 2009).

In case of tumors with driver mutations in *EGFR* and *ALK* genes, EGFR-tyrosine kinase inhibitors (TKIs) such as erlotinib, gefitinib, and afatinib as well as ALK-inhibitor, crizotinib, are considered as first-line anti-cancer agents (Bagcchi, 2015; Kohler and Schuler, 2013). Compared to the standard chemotherapy, the targeted therapy using EGFR-TKIs or ALK inhibitors have been shown to improve progression-free survival (Bruckl et al., 2017; Chuang and Neal, 2015). However, most of patients acquire the tolerance against cisplatin and EGFR-TKIs,

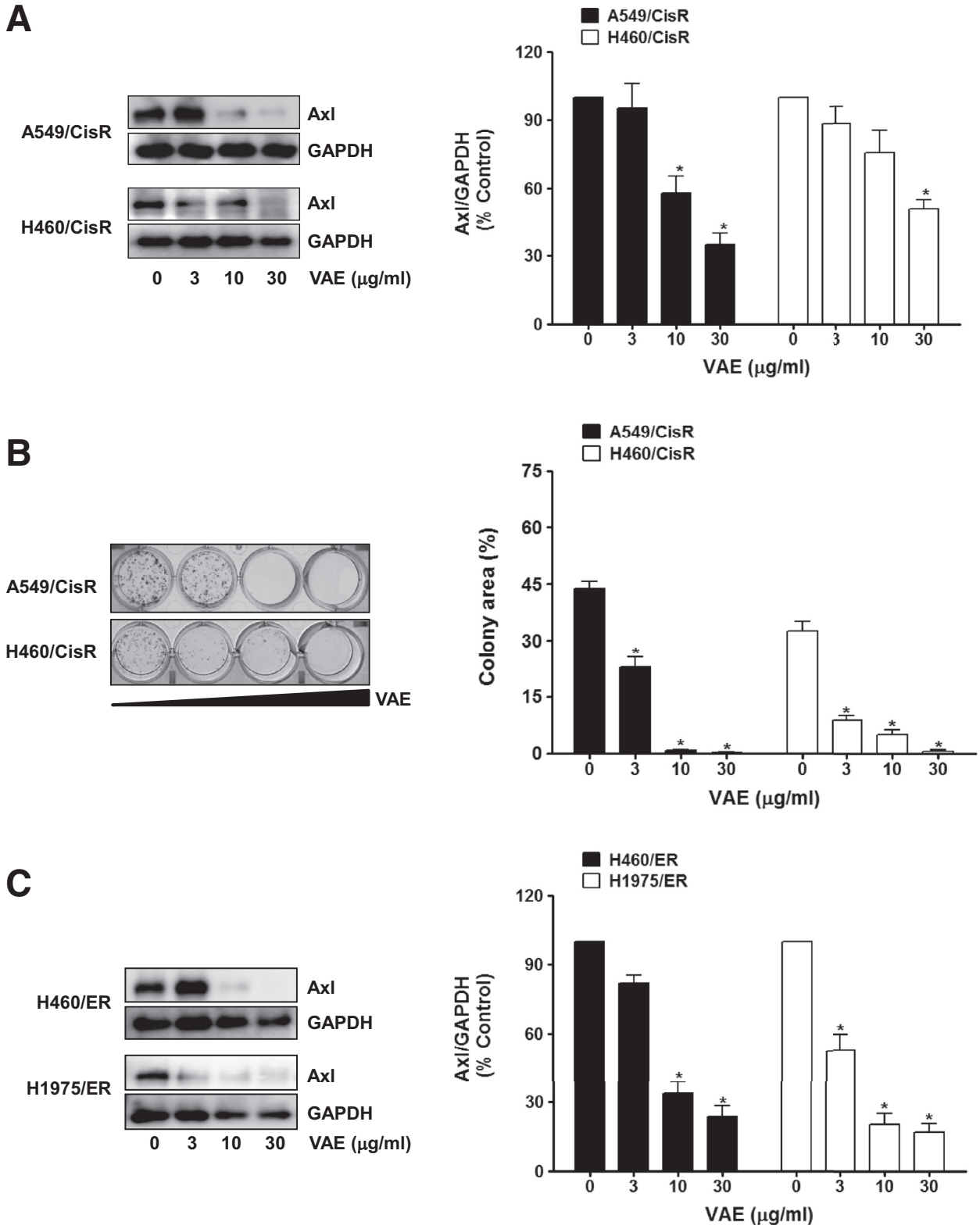

FIG. 4. VAE inhibits proliferation of cisplatin- and erlotinib-resistant cells and causes induction of p21 as well as reduction of XIAP. Each of cisplatin- or erlotinib-resistant cells (3×10^5 cells/dish), which are A549/CisR, H460/CisR, H460/ER and H1975/ER, respectively, were seeded onto 60 mm dishes and grown overnight. (A, C) Cells were incubated with 3, 10, 30 μg/ml of VAE for 24 h and then harvested. Axl protein levels were examined by Western blot analysis. GAPDH was used as a loading control. Result shown is a representative of three independent experiments. The intensity of protein band was measured and Axl/GAPDH ratio was presented in lower graph. The asterisks indicate the significant difference compared to the control value (*$P < .05$ vs untreated group). (B, D) Cells (2×10^3 cells/dish) were seeded onto 24-well plates, treated with the indicated concentrations of VAE for 24 h and let to grow for the next 7–10 days to form colonies. Next, colonies were visualized by crystal violet staining and total colony area was quantified and presented in lower graph. The data shown is representative of three independent experiments. The asterisks indicate the significant difference compared to the control value (*$P < .05$ vs untreated group). (E) Cells (3×10^5 cells/dish) were treated with 20 μg/ml of VAE for 24 h and then harvested. Total cell lysates were prepared and used to determine p21 and XIAP protein levels by Western blot analysis. GAPDH was used as a loading control. Result shown is a representative of three independent experiments. The intensity of protein band was measured and the relative levels of p21 or XIAP were presented in lower graph. The asterisks indicate the significant difference compared to the control value (*$P < .05$ vs untreated group).

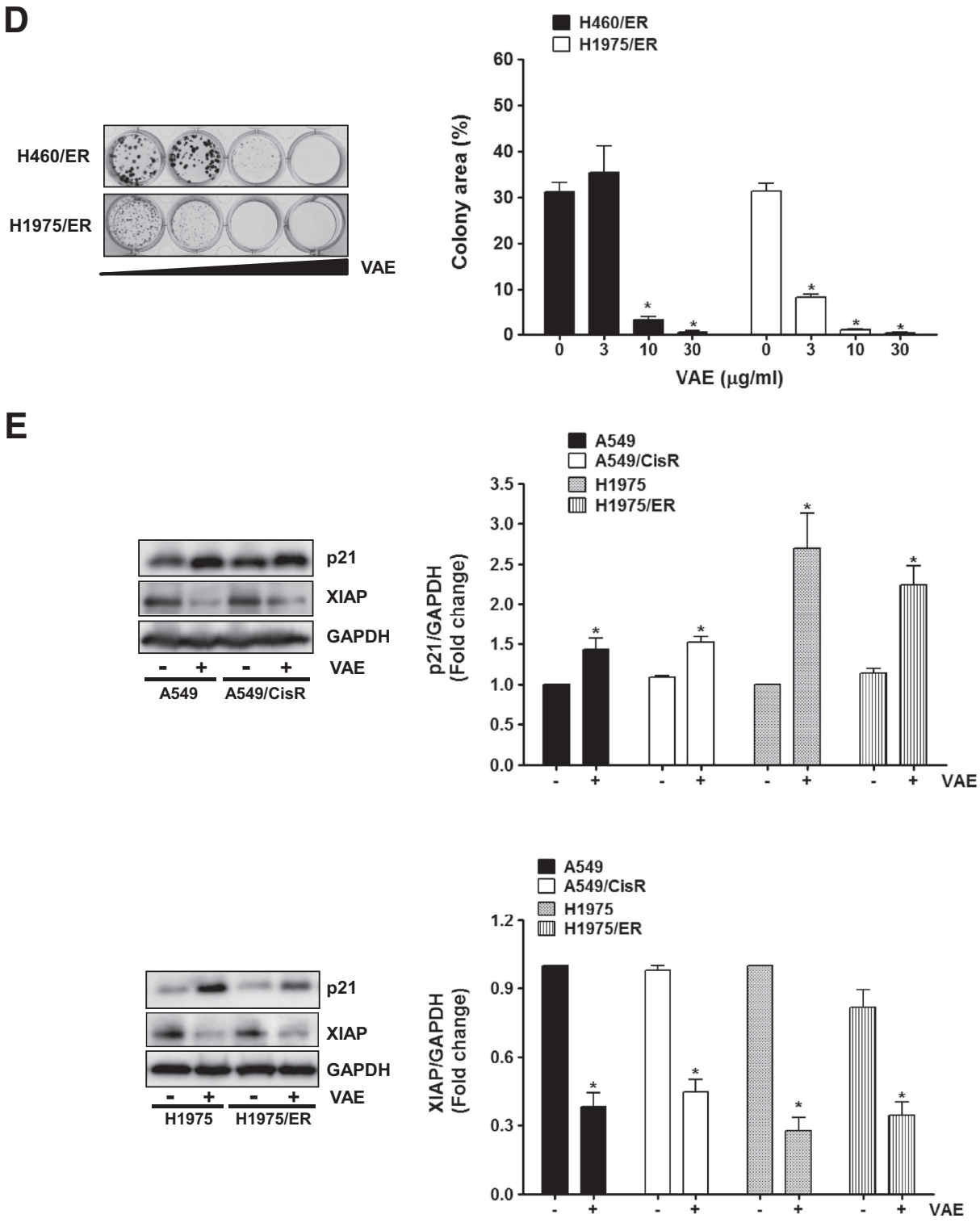

FIG. 4. (*continued*)

which is one of the vicious problems to make the successful treatment of NSCLC difficult.

Axl, a member of TAM RTK family, has been reported to be involved in the acquisition of resistance against anti-cancer drugs including EGFR-TKIs. In this study, we observed the inhibitory effect of mistletoe (*Visucm album*) extract (VAE), an aqueous and injectable preparation on Axl expression and its activation upon ligand stimulation in NSCLC cells (Figs. 1 and 2). Interestingly, we also found that the cytotoxic effect of VAE was inversely proportional to Axl protein level, since it was attenuated or augmented by overexpression or knockdown of Axl, respectively (Fig. 3D and F). These results strongly indicate that VAE is a potent candidate as a complementary and alternative medicine or an adjuvant for treatment of cancer patients. However, the clinical evaluations of the benefit of VAE are manifold and still conflicting, since some studies reported the significant improvement of survival, tumor regression, reduction of cytotoxicity and quality of life, while others demonstrated no positive outcomes for survival, relapse, remission, and quality of life (Kienle et al., 2016).

Previously, several genes such as cytokine (Lyu and Park, 2007), cell motility-associated genes (Schotterl et al., 2017), transforming growth factor-β (TGF-β), TGF-β receptor II which is a cell surface receptor with serine/threonine kinase activity (Jiang et al., 2014; Schotterl et al., 2017), cell cycle dependent kinase (CDK) 2 and CDK4 (Chai and Zhao, 2017; Chen et al., 2016; dela Cruz et al., 2015) have been proved to be modulated by VAE and/or its components, indicating that these genes are the targets of VAE and/or its components to explain the diverse biological activities. In our study, VAE was found to downregulate the expression and activation of Axl, which result in the inhibition of cell proliferation.

Mistletoe has been known to contain various types of biologically active compounds including glycoprotein (lectin), polypeptide (viscotoxin), oligosaccharides, flavonoid, and triterpene acid (Chou et al., 1999; Delebinski et al., 2012; Hostanska et al., 1995; Mueller and Anderer, 1990; Schaller et al., 1998) and many studies have focused on lectins and viscotoxin and demonstrated that these two ingredients of mistletoe are the major constituents to result in the antitcancer and immunomodulatory activities (Bussing et al., 1996; Hostanska et al., 1995; Huber et al., 2002; Lavastre et al., 2002; Mannel et al., 1991; Simon et al., 2013). So, lectins and viscotoxin seem to be the most likely compounds to target Axl, although it must be proven by additional experiments.

Since the evaluation of clinical relevance of in vitro studies is very important and invaluable, we referred to some previous reports. Like the interpretation by Weissenstein et al. (2014), the serum level of VAE might not be reached as high as concentrations active in our study (≥10 μg/ml) by subcutaneous injection. However, in case of intratumoral mistletoe application in pancreatic cancer patients (Schad et al., 2014), the applied mistletoe concentrations (20–160 mg) seemed to be much higher than those used in our study (3–30 μg/ml). So, we assume that the effect of VAE on concentrations used in this study might be clinically feasible.

Next, we also observed that VAE inhibited the cell proliferation of A549, H460, and H1975 cells harboring wild-type or mutant *EGFR* gene (Fig. 3A). Colony formation assay further showed that VAE suppressed clonogenic activity of both parental and the cisplatin/erlotinib-resistant cells, A549/CisR, H460/CisR, H460/ER and H1975/ER (Figs. 3B, 4B and D). These results indicated that VAE is effective enough to abrogate proliferation of NSCLC cells which have *EGFR* mutations and resistances to standard anti-cancer agents or *EGFR*-targeted therapy.

Conclusion

Our data showed that VAE has the inhibitory effects on Axl expression and its activation upon Gas6 stimulation, which are associated with its anti-proliferative activity in parental and cisplatin/paclitaxel-resistant NSCLC cells. Thus, VAE targets Axl RTK to result in the inhibition of cell proliferation and circumvention of chemo-resistance in NSCLC cells.

Materials and methods

Reagents and antibodies

Mistletoe extract which is commercially available and a standardized preparation from the host tree Fraxinus, AbnobaviscumF®, was gifted by Abnova-Korea (Seoul, South Korea). Cisplatin and erlotinib were obtained from Sigma-Aldrich Korea (Seoul, South Korea). A549 and H460 cells, harboring epidermal growth factor receptor (EGFR) wild-type cell lines, and EGFR mutant cell line, H1975 cell, were purchased from the American Type Culture Collection (Manassas, VA, USA). Primers for Axl and GAPDH were synthesized by the domestic company, Bioneer Corp. (Daejeon, Korea). TRI reagent was obtained from Solgent Co., Ltd. (Daejeon, Korea). AmpliTaq DNA polymerase and Lipofectamine 2000 were obtained from Roche Diagnostics Corp. (Indianapolis, IN, USA) and Invitrogen (Carlsbad, CA, USA), respectively. G418 was from Gibco BRL (Gaithersburg, MD, USA. For Western blot analysis, specific antibodies against phospho-Axl and Axl were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibodies against p21, XIAP and GAPDH as well as secondary antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX, USA).

Cell culture and establishment of erlotinib and cisplatin-resistant cells

A549, H460 and H1975 cells were grown in RPMI-1640 (Gibco BRL) containing 10% FBS, 2 mM L-glutamine, 10 U/ml penicillin and 10 mg/ml streptomycin at 37° °C in 5% CO_2 in a water-saturated atmosphere. The variants of H1975 and H460 cells which are resistant to erlotinib (H460/ER and H1975/ER cells) or cisplatin (A549/CisR and H460/CisR) were established by stepwise exposure of those parental cells to escalating concentrations of erlotinib (ranging from 1 μM to 10 μM) or cisplatin (ranging from 0.5 μM to 2 μM), respectively.

RT-PCR

Cells (2×10^5) were seeded in a 60-mm culture dish and grown overnight. They were then treated with 3, 10, and 30 μg/ml of VAE for 24 h. Total RNA was extracted using TRI reagent and subjected to cDNA synthesis and PCR. The specific primers were as follows: Axl sense, 5′-AACCTTCAACTCC TGCCTTCTCG-3′ and antisense, 5′-CAGCTTCTCCTTCAGC TCTTCAC-3′; GAPDH sense, 5′-GGAGCCAAAAGGGTCAT CAT-3′ and antisense, 5′-GTGATGGCATGGACTGTGGT-3′. The mRNA level of Axl was normalized to that of GAPDH. Density of amplicon was analyzed using LAS-3000 Image Reader (GE Healthcare Life science) and MultiGauge 3.0 software.

Western blot analysis

Total cell lysates were prepared from the parental or chemo-resistant cells treated with the indicated concentrations of VAE using lysis buffer [1% Triton X-100, 50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM PMSF, 1 mM Na_3VO_4, and protease inhibitor cocktail]. Untreated cells were used as controls. Protein concentrations were determined using Bio-Rad protein assays. Proteins from the cell lysates (40–60 μg) were separated by 12% SDS-PAGE, and electrotransferred onto nitrocellulose membranes. The membranes were blocked for 30 min at room temperature in Tris-buffered saline with 0.05% Tween-20 (TTBS) containing 5% non-fat dry milk, and then incubated with TTBS containing a primary antibody for 4 h at room temperature. After 3 × 10 min washes in TTBS, the membranes were incubated with peroxidase-conjugated secondary antibody for 1 h. Following 3 additional 10-min washes with TTBS, the protein bands of interest were visualized using an enhanced chemiluminescence detection system (Amersham™ ECL™ Prime Western Blotting Detection Reagent; GE Healthcare, Piscataway, NJ, USA). Density of protein band was analyzed using LAS-3000 Image Reader (GE Healthcare Life science) and MultiGauge 3.0 software.

Cell viability measurement

To assess cell viability, the number of viable cells was counted using Trypan blue. Briefly, 3×10^4 cells were seeded into 60-mm culture dish, grown overnight and then treated with the indicated concentrations of VAE for 24 h. After VAE treatment, cells were harvested and stained with 0.4% Trypan blue solution. Dye-excluding viable cells were counted under the microscope. Values are the mean ± SD of triplicates and normalized to that of control group to determine the % of viability.

Clonogenic assay

Cells were seeded into 24 well plates (5×10^2 cells/well) and treated with the indicated concentrations of VAE. VAE-treated cells were then cultured for the next 7 to 10 days. Colonies of >50 cells were stained with Crystal violet (in 60% methanol; Junsei Chemical Co., Ltd., Tokyo, Japan) and images were acquired using the RAS-3000 Image Analysis System (FujiFilm, Tokyo, Japan). The area percentage occupied by the colonies was calculated using ImageJ software with a plugin "ColonyArea" (Guzman et al., 2014).

Ectopic expression of Axl

To ectopically express Axl, the recombinant plasmid, pcDNA3-Axl, was constructed by cloning the Axl cDNA into the *Eco*RI and *Bam*HI sites of the pcDNA3 vector and 2 μg of purified plasmids were transfected into the H460 cells (3×10^5 cells in a 60-mm dish) using Lipofectamine 2000 (Invitrogen). To establish stable cell lines, which constitutively express Axl, the transfected cells were cultured in the presence of 400 μg/ml of G418. The RPMI 1640 medium containing G418 was refreshed every 3 days. After 3–4 weeks, the Axl-expressing cells were enriched and the Axl expression in these cells was analyzed by western blot analysis.

siRNA transfection

RNA interference-mediated gene silencing was performed to reduce Axl protein level. Cells (5×10^5) were seeded in 60-mm culture dishes, grown overnight and then transfected with 50 nM siRNA targeting Axl (sense, 5′-AAGAUUUGGAGAdACACACUGA-3′ and antisense, 5′-UCAGUGUGUUCUCCAAAUCUU-3′), as previously described (30), or control siRNA. The cells were harvested for 24 and 48 h after transfection and used to evaluate protein expression and cell proliferation, respectively.

Statistical analysis

Data were expressed as the means ± SD of triplicate samples or at least three independent experiments. To determine statistical significance, the Student's *t*-test was used with a *P*-value threshold of $<.05$.

Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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