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Original Research Article

Induction of Cyclin D1 Proteasomal Degradation by Branch Extracts from *Abeliophyllum distichum* Nakai in Human Colorectal Cancer Cells

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Abstract - *Abeliophyllum distichum* Nakai (*A. distichum*) has been reported to exert the inhibitory effect on angiotensin converting enzyme and aldose reductase. Recently, our group found that branch extracts of *A. distichum* (EAFAD-B) induce apoptosis through ATF3 activation in human colon cancer cells. However, anti-cancer reagents exert their activity through the regulation of various molecular targets. Therefore, the elucidation of potential mechanisms of EAFAD-B for anti-cancer activity may be necessary. To elucidate the potential mechanism of EAFAD-B for anti-cancer activity, we evaluated the regulation of cyclin D1 in human colon cancer cells. EAFAD-B decreased cellular accumulation of cyclin D1 protein. However, cyclin D1 mRNA was not changed by EAFAD-B. Inhibition of proteasomal degradation by MG132 attenuated EAFAD-B-mediated cyclin D1 downregulation and the half-life of cyclin D1 was decreased in the cells treated with EAFAD-B. In addition, EAFAD-B induced cyclin D1 phosphorylation at threonine-286 and the point mutation of threonine-286 to alanine attenuated EAFAD-B-mediated cyclin D1 proteasomal degradation. Inhibitions of both ERK1/2 by PD98059 and NF-κB by a selective inhibitor, BAY 11-7082 suppressed cyclin D1 downregulation by EAFAD-B. From these results, we suggest that EAFAD-B-mediated cyclin D1 downregulation may result from proteasomal degradation through its threonine-286 phosphorylation via ERK1/2-dependent NF-κB activation. The current study provides new mechanistic link between EAFAD-B and anti-cancer activity in human colon cancer cells.

Key words - *Abeliophyllum distichum* Nakai, Cyclin D1, Proteasomal degradation, Cancer prevention, Colon cancer

Introduction

Cyclin D1 as proto-oncogene regulates G1 to S phase transition in various cell types and its overexpression is associated with the development and progression of cancer (Alao, 2007). Cyclin D1 forms active complexes with cyclin dependent kinases (CDK) 4 and 6 and subsequently phosphorylates retinoblastoma protein (pRB), resulting in promoting cell cycle progression (Kato *et al*., 1993; Weinberg, 1995). There are growing evidences that many therapeutic agents down-regulate cyclin D1 level (Alao *et al*., 2004; Alao *et al*., 2006; Langenfeld *et al*., 1997; Spinella *et al*., 1999), which indicates the relationship between the down-regulation of cyclin D1 and cancer prevention.

Among cancer types, overexpression of cyclin D1 is detected in 68.3% of human colon cancer cases (Bahnassy *et al*., 2004) and deregulation of cyclin D1 contributes colon tumorigenesis and improves the outcome in colon cancer patients (Holland *et al*., 2001). Therefore, it is accepted that control of cyclin D1 level may be a promising colon cancer preventive and therapeutic way.

Abeliophyllum distichum Nakai (*A. distichum*) has been known to be a deciduous shrub and native to the south and central areas of Korea (Oh *et al*., 2003). Recently, *A. distichum* has been reported to exert the inhibitory effect on angiotensin converting enzyme (Oh *et al*., 2003). In addition, our group, for the first time, has reported that the branch shows the highest anti-cancer properties among the plant parts including branch, leaf and flower from *A. distichum*. In addition, the branch extracts exerts apoptosis through the overexpression of activating transcription factor 3 (ATF3) in human colon cancer cells (Park *et al*., 2014a)

In light of the preventive potential of *A. distichum* in colon

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cancer, this study was performed to elucidate the biological mechanism by which EAFAD-B induces the inhibition of cell growth in human colon cancer cells. Here, for the first time, we report that the branch extracts from *A. distichum* leads to ERK1/2-NF-κB-mediated proteasomal degradation of cyclin D1 through threonine-286 phosphorylation.

Materials and Methods

Materials

Cell culture media, Dulbecco's Modified Eagle medium (DMEM)/F-12 1:1 Modified medium (DMEM/F-12) was purchased from Lonza (Walkersville, MD, USA). BAY 11-7082 were purchased from Sigma Aldrich (St. Louis, MO, USA). PD98059 were purchased from Calbiochem (San Diego, CA, USA). Antibodies against cyclin D1, phospho-cyclin D1 (Thr286), HA-tag, IκKα, p-IκKα, ERK1/2, p-ERK1/2 and β-actin were purchased from Cell Signaling (Bervely, MA, USA). Wild type HA-tagged cyclin D1 and point mutation of T286A of HA-tagged cyclin D1 were provided from Addgene (Cambridge, MA, USA). All chemicals were purchased from Fisher Scientific, unless otherwise specified.

Sample preparation

The branch of *A. distichum* (voucher number: Park1001 (ANH)) was formally identified by Jae Ho Park as the professor of Jungwon University, Korea. The branch was cultivated and collected at Goesan-gun, Chungbuk, Korea on August in 2013. Five hundred gram of fresh branch was extracted with 1,000 ㎖ of 80% methanol with shaking for 24 h. The methanol-soluble fraction was filtered and concentrated to approximately 200 ㎖ volume using a vacuum evaporator. Then, the concentrated fraction was fractionated using 200 ㎖ of petroleum ether three times and subsequently re-fractionated using 200 ㎖ of ethyl acetate three times. The ethyl acetate fractions from the branch of *A. distichum* (EAFAD-B) was separated from the mixture, evaporated by a vacuum evaporator and prepared aseptically and kept in a refrigerator until use.

Cell culture and treatment

Human colon cancer cell lines, HCT116 and SW480 cells were purchased Korean Cell Line Bank (Seoul, Korea) and grown in DMEM/F-12 supplemented with 10% fetal bovine serum (FBS), 100 U/㎖ penicillin and 100 ㎍/㎖ streptomycin. The cells were maintained at 37℃ under a humidified atmosphere of 5% CO_2. EAFAD-B was dissolved in dimethyl sulfoxide (DMSO) and treated to cells. DMSO was used as a vehicle and the final DMSO concentration was not exceeded 0.1% (v/v).

Expression vectors

Transient transfection of the vectors (Wild type HA-tagged cyclin D1 and point mutation of T286A of HA-tagged cyclin D1) was performed using the PolyJet DNA transfection reagent (SignaGen Laboratories, Ijamsville, MD, USA) according to the manufacturers' instruction. After transfection, the cells were treated with DMSO or 100 ㎍/㎖ of EAFAD-B for 24 h.

SDS-PAGE and Western blot analysis

After EAFAD-B treatment, cells were washed with 1×phosphate-buffered saline (PBS), and lysed in radioimmunoprecipitation assay (RIPA) buffer (Boston Bio Products, Ashland, MA, USA) supplemented with protease inhibitor cocktail (Sigma Aldrich) and phosphatase inhibitor cocktail (Sigma Aldrich), and centrifuged at 15,000 × g for 10 min at 4℃. Protein concentration was determined by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). The proteins were separated on SDS-PAGE and transferred to PVDF membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were blocked for non-specific binding with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) for 1h at room temperature and then incubated with specific primary antibodies in 5% nonfat dry milk at 4℃ overnight. After three washes with TBS-T, the blots were incubated with horse radish peroxidase (HRP)-conjugated immunoglobulin G (IgG) for 1 h at room temperature and chemiluminescence was detected with ECL Western blotting substrate (Amersham Biosciences) and visualized in Polaroid film.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was prepared from EAFAD-B-treated cells using a RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and total RNA (1 ㎍) was reverse-transcribed using a Verso

cDNA Kit (Thermo Scientific, Pittsburgh, PA, USA) according to the manufacturer's protocol for cDNA synthesis. PCR was carried out using PCR Master Mix Kit (Promega, Madison, WI, USA) with primers for human cyclin D1 and human GAPDH as follows: human cyclin D1: forward 5'-aactacctggaccgcttcct-3' and reverse 5'-ccacttgagcttgttcacca-3'; human GAPDH: forward 5'-acccagaagactgtggatgg-3' and reverse 5'-ttctagacggcaggtcaggt-3'.

Transient transfection and luciferase activity

Transient transfection was performed using the PolyJet DNA transfection reagent (SignaGen Laboratories) according to the manufacturers' instruction. HCT116 cells were plated in 12-well plates at a concentration of 2×10^5 cells/well. After growth overnight, plasmid mixtures containing 0.5 ㎍ of NF-κB-Luc-plasmid and 0.05 ㎍ of pRL-null vector were transfected for 24 h. The transfected cells were cultured in the absence or presence of EAFAD-B and PD98059. The cells were then harvested in 1 × luciferase lysis buffer, and luciferase activity was normalized to the pRL-null luciferase activity using a dual-luciferase assay kit (Promega).

Statistical analysis

A statistical analysis was performed with the Student's unpaired t-test and one-way ANOVA followed by Dunnett's test. Differences with $*P < 0.05$ were considered statistically significant.

Results

EAFAD-B down-regulates cyclin D1 protein but not mRNA

Our group has reported that EAFAD-B suppresses the proliferation of human colon cancer cells (Park *et al*., 2014a). To investigate if EAFAD-B affects the level of cyclin D1 protein in human colon cancer cells, HCT116 and SW480 cells were treated with 0, 50, 100 and 200 ㎍/㎖ of EAFAD-B for 24 h. As shown in Fig. 1, EAFAD-B decreased cyclin D1 protein level at dose-dependent manner. In time-course experiment (Fig. 1B), cyclin D1 protein started to decrease at 1 h after EAFAD-B treatment in both HCT116 and SW480 cells. To elucidate that EAFAD-B-mediated down-regulation of cyclin D1 protein results from transcriptional effect, mRNA level of cyclin D1 was evaluated by RT-PCR and we found that EAFAD-B did not affect mRNA level of cyclin D1 (Fig. 1C). There results indicate that EAFAD-B may decrease protein stability of cyclin D1.

EAFAD-B induces cyclin D1 proteasomal degradation

To confirm that EAFAD-B affects cyclin D1 proteasomal degradation, HCT116 and SW480 cells pretreated with MG132 as a proteasome inhibitor and then co-treated with EAFAD-B. As shown in Fig. 2A, the level of cyclin D1 was decreased by EAFAD-B in the cells treated with DMSO, while pre-treatment of MG132 blocked EAFAD-B-mediated cyclin D1 downregulation in both HCT116 and SW480 cells. To verify these results, the cells were pre-treated with DMSO or EAFAD-B and then exposed to cycloheximide (CHX) for indicated times. As shown in Fig. 2B, EAFAD-B treatment decreased half-life of cyclin D1 protein in both HCT116 and SW480 cells.

Cyclin D1 proteasomal degradation by EAFAD-B is mediated from threonine-286 phosphorylation

It has been reported that cyclin D1 phosphorylation at threonine-286 is associated with its proteasomal degradation via the ubiquitin-proteasome pathway (Diehl *et al*., 1997). Thus, we tested if EAFAD-B phosphorylates cyclin D1 at threonine-286. As a result (Fig. 3A), cyclin D1 begun to be phosphorylated at threonine-286 at 1 h after EAFAD-B treatment. To verify that threonine-286 phosphorylation of cyclin D1 results in cyclin D1 proteasomal degradation by EAFAD-B, HCT116 cells were transfected with HA-wild type cyclin D1 or HA-T286A cyclin D1. As shown in Fig. 3B, cyclin D1 degradation by EAFAD-B was observed in wild type cyclin D1-transfected cells. However, it was attenuated in T286A cyclin D1-transfected cells. Overall, these data proposed that downregulation of cyclin D1 by EAFAD-B depends on proteolytic proteasomal degradation via threonine-286 phosphorylation.

Cyclin D1 proteasomal degradation by EAFAD-B is dependent on ERK1/2-mdiated NF-κB activation

There are growing evidences that cyclin D1 proteasomal degradation is associated with ERK1/2, p38, GSK3β and NF-

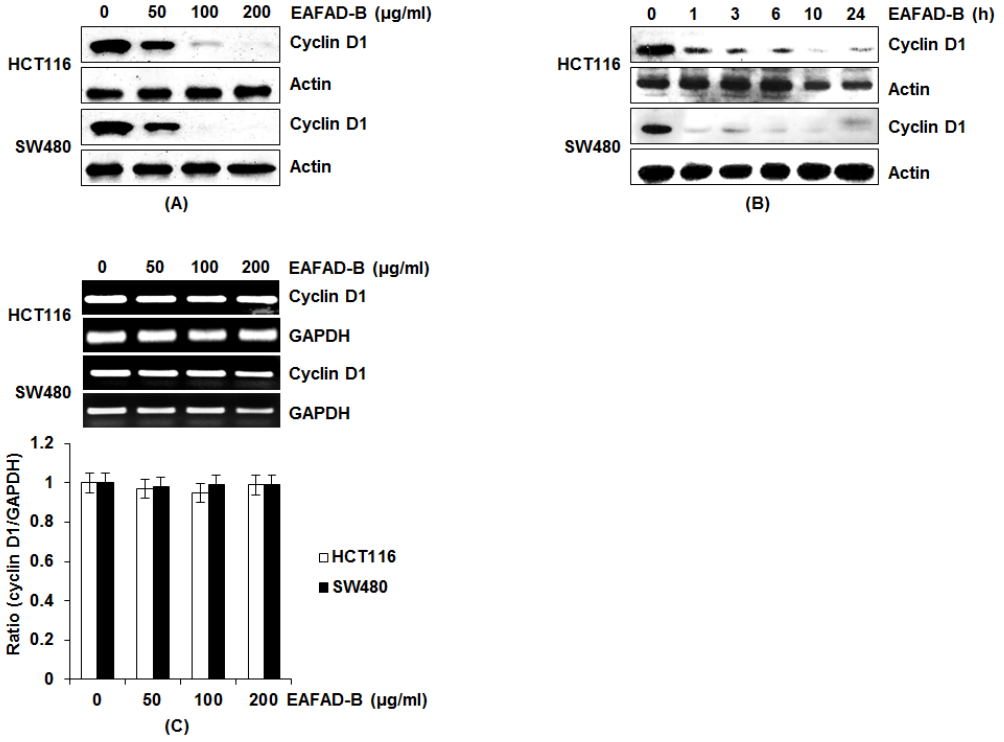

Fig. 1. Downregulation of cyclin D1 by EAFAD-B in HCT116 and SW480 cells. HCT116 and SW480 cells were plated overnight and then exposure to EAFAD-B at the indicated concentrations for 24 h (A). HCT116 and SW480 cells were treated with 100 ㎍/㎖ of EAFAD-B for the indicated times (B). Cell lysates were subjected to SDS-PAGE and Western blot was performed using cyclin D1 antibody. Actin was used as the internal control. HCT116 and SW480 cells were plated overnight and then exposure to EAFAD-B at the indicated concentrations for 24 h. RT-PCR analysis of cyclin D1 gene expression, total RNA was prepared after EAFAD-B treatment for 24 h. GAPDH were used as the internal control (C).

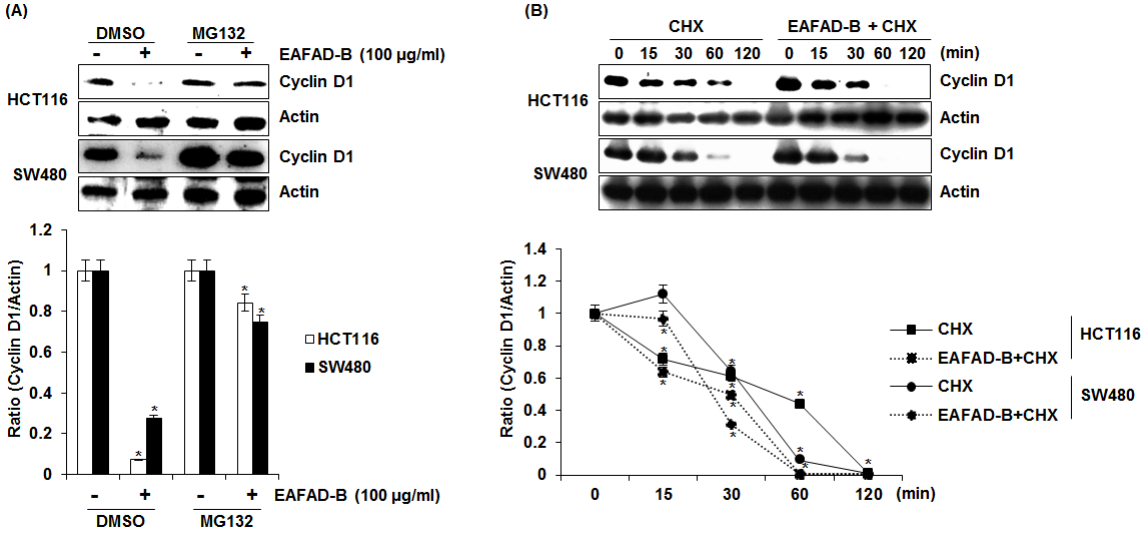

Fig. 2. Proteasomal degradation of cyclin D1 by EAFAD-B in HCT116 and SW480 cells. The cells were pretreated with 20 μM of MG132 for 2 h and then co-treated with 100 ㎍/㎖ of EAFAD-B for the additional 1 h (A). The cells were co-treated with 10 ㎍/㎖ of cycloheximide and 100 ㎍/㎖ of EAFAD-B for the indicated times (B). Cell lysates were subjected to SDS-PAGE and Western blot was performed using cyclin D1 and p-cyclin D1 (Thr286) antibody. Actin was used as the internal control.

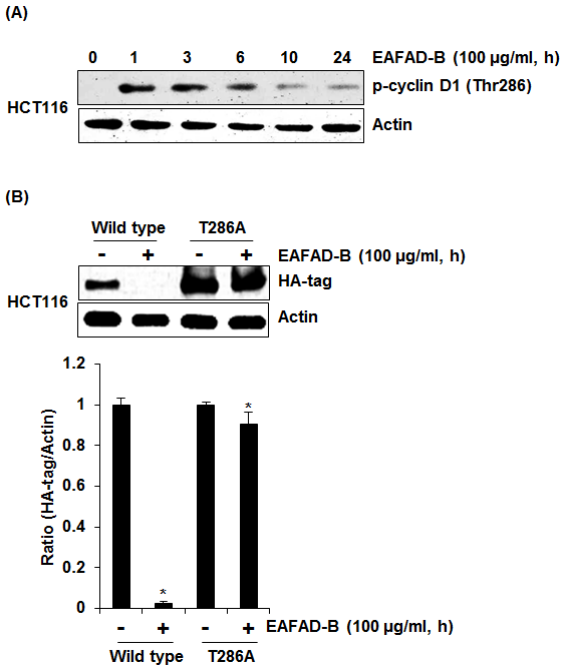

Fig. 3. Effect of cyclin D1 phosphorylation at threonine-286 on EAFAD-B-mediated cyclin D1 proteasomal degradation. HCT116 cells were treated with 100 ㎍/㎖ of EAFAD-B for the indicated times (A). HCT116 cells were transfected with wild type HA-tagged cyclin D1 or HA-tagged T286A cyclin D1 and then treated with 100 ㎍/㎖ of EAFAD-B for 1 h (B). Cell lysates were subjected to SDS-PAGE and the Western blot was performed using antibodies against phospho-cyclin D1 (Thr286) or HA-tag. Actin was used as internal control.

κB (Okabe *et al*., 2006; Thoms *et al*., 2007; Diehl *et al*., 1998; Kwak *et al*., 2005). To determine the upstream kinases involved in EAFAD-B-mediated proteasomal degradation of cyclin D1, HCT116 cells were pretreated with PD98059 as an ERK1/2 inhibitor or BAY 11-7082 as a selective NF-κB inhibitor, and then exposured to EAFAD-B. As a result (Fig. 4A and 4B), inhibition of both ERK1/2 and NF-κB attenuated cyclin D1 proteasomal degradation by EAFAD-B in both HCT116 and SW480 cells, which indicates that activation of ERK1/2 and NF-κB by EAFAD-B can contribute to cyclin D1 proteasomal degradation. Thus, we investigated if EAFAD-B activates ERK1/2 and NF-κB, and found that EAFAD-B increased the phosphorylation levels of ERK1/2 and IκK-α, and NF-κB luciferase activity (Fig. 4C and 4D). In addition, we found that inhibition of both ERK1/2 and NF-κB decreased EAFAD-B-mediated cyclin D1 phosphorylation at threonine-286 (Fig. 4E) and inhibition of ERK1/2 attenuated EAFAD-B-mediated NF-κB activation (Fig. 4F). However, inhibition of p38 and GSK3β did not affect EAFAD-B-mediated cyclin D1 down-regulation (data not shown). These findings indicate that EAFAD-B-mediated cyclin D1 proteasomal degradation may result from ERK1/2-induced NF-κB activation.

Discussion

Abeliophyllum distichum Nakai (*A. distichum*) has been known to be a deciduous shrub and native to the south and central areas of Korea (Oh *et al*., 2003). Although *A. distichum* has been recently used as the functional source in food and cosmetic field, no specific pharmacological effects from *A. distichum* have been described. *A. distichum* has been reported to exert the inhibitory effect on angiotensin converting enzyme and aldose reductase (Oh *et al*., 2003; Li *et al*., 2013). Recently, our group firstly reported that branch extracts from *A. distichum* induces apoptosis through ATF3 activation in human colon cancer cells (Park *et al*., 2014a). However, anti-cancer reagents exert their activity through the regulation of various molecular targets such as cell cycle regulation, apoptosis induction and inhibition of angiogenesis & metastasis. Therefore, the elucidation of potential mechanisms of *A. distichum* for anti-cancer activity may be necessary. Thus, we focused on the cyclin D1 down-regulation because overexpression of cyclin D1 is detected in 68.3% of human colon cancer cases (Bahnassy *et al*., 2004) and has been regarded as the important target for cancer chemoprevention against human colon cancer.

Cyclin D1 can be regulated through transcriptional regulation or the activation of proteasomal degradation. In current study, EAFAD-B decreased the level of cyclin D1 protein, while cyclin D1 mRNA did not be changed by EAFAD-B. In addition, Inhibition of proteasomal degradation by MG132 attenuated EAFAD-B-mediated cyclin D1 downregulation and the half-life of cyclin D1 was decreased in the cells treated with EAFAD-B. These findings indicate that EAFAD-B-mediated downregulation of cyclin D1 may be through cyclin D1 proteasomal degradation.

Threonine-286 phosphorylation of cyclin D1 results in its

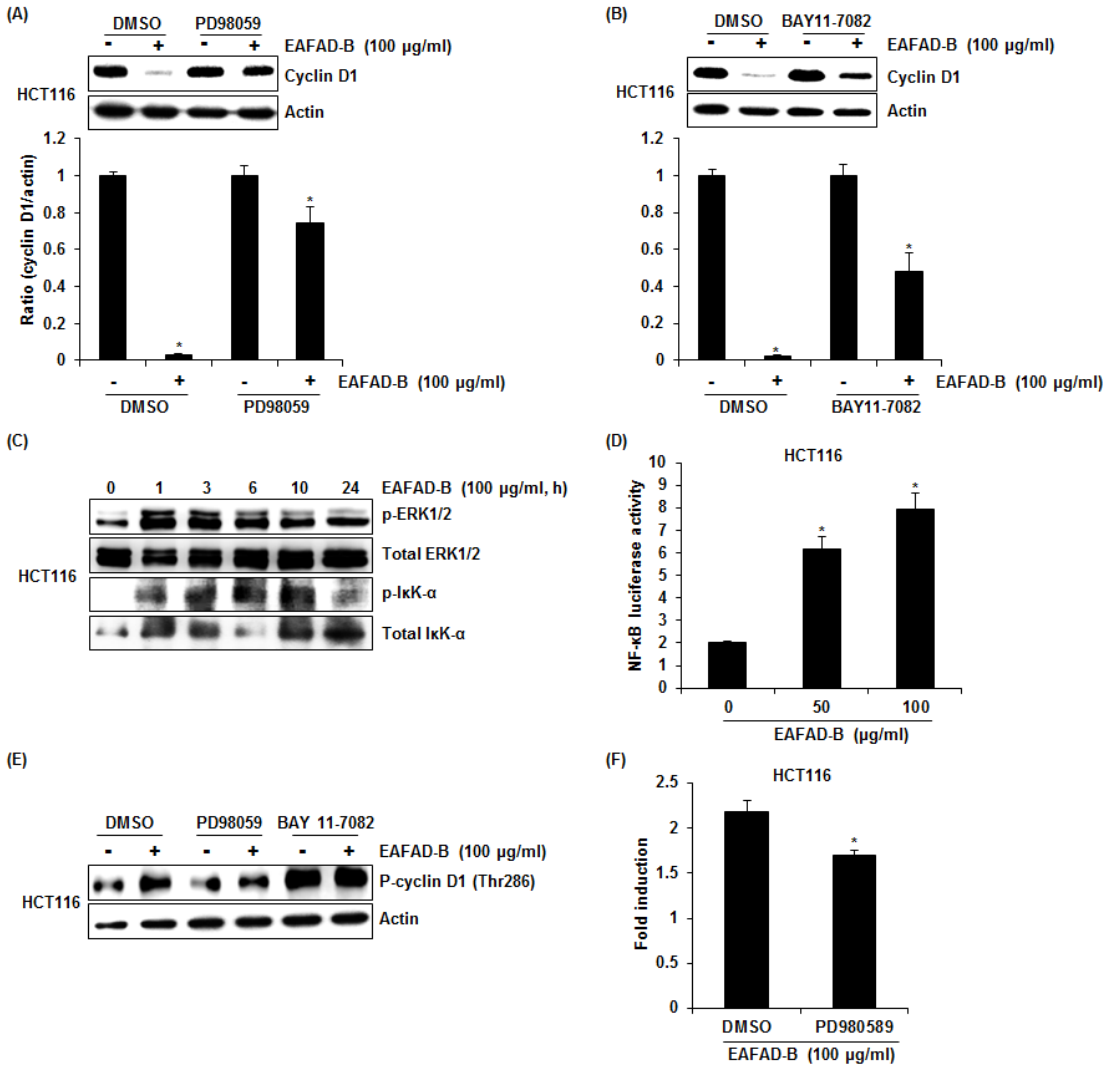

Fig. 4. Attenuation of EAFAD-B-mediated cyclin D1 proteasomal degradation by ERK1/2-dependent NF-κB activation. The cells were pretreated with 50 μM of PD98059 or 1 μM for BAY 11-7082 for 2 h and then co-treated with 100 ㎍/㎖ of EAFAD-B for the additional 1 h. Cell lysates were subjected to SDS-PAGE and Western blot was performed using antibodies against cyclin D1 and p-cyclin D1 (Thr286). Actin was used as the internal control (A, B, E). The cells were treated with 100 ㎍/㎖ of EAFAD-B at the indicated times. Cell lysates were subjected to SDS-PAGE and Western blot was performed using antibodies against p-ERK1/2, total ERK1/2, p-IκK-α and total IκK-α. Actin was used as the internal control (C). The cells transfected with NF-κB-Luc-plasmid were treated with EAFAD-B in presence or absence of PD98029 for 24 h and luciferase activity was measured. *P < 0.05 compared to cells without treatments of EAFAD-B or PD98059, respectively (D, F).

proteasomal degradation (Diehl *et al*., 1997). Human cancer cells are mutated in cyclin D1 that disrupt threonine-286 phosphorylation, which results in preventing cyclin D1 proteasomal degradation (Benzeno *et al*., 2006). We found that EAFAD-B phosphorylated threonine-286 of cyclin D1 in time-course experiments and the point mutation of threonine-286 to alanine (T286A) suppressed EAFAD-B-mediated cyclin D1 proteasomal degradation. Therefore, cyclin D1 proteasomal degradation by EAFAD-B may result from its threonine-286 phosphorylation.

In addition, it has been reported that cyclin D1 proteasomal degradation dependent on threonine-286 phosphorylation is associated with various upstream kinases including ERK1/2, p38, GSK3β and NF-κB (Okabe *et al*., 2006; Thoms *et al*.,

2007; Diehl *et al*., 1998; Kwak *et al*., 2005). EAFAD-B induced ERK1/2-dependent NF-κB activation and inhibition of both ERK1/2 and NF-κB reduced cyclin D1 phosphorylation at threonine-285 and subsequently proteasomal degradation by EAFAD-B. From these findings, it is indicated that ERK1/2-dependent NF-κB activation by EAFAD-B at least in part contributes EAFAD-B-mediated cyclin D1 proteasomal degradation. The roles of NF-κB are complex in cancer progression and anticancer therapeutics. NF-κB activation is associated with the survival of cancer cells, showing that inhibition of NF-κB activity is regarded as the target for anticancer therapy (Verma, 2004; Kim *et al*., 2006). However, some reports suggest that NF-κB activation by aspirin and tolfenamic acid promotes a proapoptotic response in cancer cells (Stark *et al*., 2001; Jeong *et al*., 2013). Although tolfenamic acid induces NF-κB activation in human colorectal cancer cell line, HCT116, it has been reported that tolfenamic acid suppresses inflammatory stimuli-mediated NF-κB signaling pathway in HCT116 cells (Shao *et al*., 2015). Interestingly, we found that the extracts from *A. distichum* inhibit LPS-mediated NF-κB activation in mouse macrophage cells (Park *et al*., 2014b). These two literatures suggest that NF-κB can be differently regulated according to circumstances such as cancer cells or normal cells.

There is a report that four glycosides such as acteoside, isoacteoside, rutin and hisrutrin may be major components of *A. distichum* (Oh *et al*., 2003) and acteoside reduced cyclin D1 protein level in human leukemia cell line, HL-60 (Lee *et al*., 2007). However, it is not clear that the reduction of cyclin D1 protein level by acteoside results from transcriptional regulation or proteasomal degradation. Therefore, the further mechanistic study will be required to investigate if acteoside-mediated decrease of cyclin D1 is resulted from proteasomal degradation.

This present study revealed that EAFAD-B-induced proteasomal degradation of cyclin D1 might inhibit proliferation in human colon cancer cells. Furthermore, the current study provides information on molecular events for the anti-cancer activity of EAFAD-B. In addition, we think that these findings will provide the data for screening the natural plants in developing alternative and complementary medicine for an anti-cancer of human colorectal cancer.

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