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Original Research Article

# Anticancer Activity of the Safflower Seeds (*Carthamus tinctorius* L.) through Inducing Cyclin D1 Proteasomal Degradation in Human Colorectal Cancer Cells

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**Abstract** - The seed of safflower (*Carthamus tinctorius* L) has been reported to suppress human cancer cell proliferation. However, the mechanisms by which safflower seed inhibits cancer cell proliferation have remained nuclear. In this study, the inhibitory effect of the safflower seed (SS) on the proliferation of human colorectal cancer cells and the potential mechanism of action were examined. SS inhibited markedly the proliferation of human colorectal cancer cells (HCT116, SW480, LoVo and HT-29). In addition, SS suppressed the proliferation of human breast cancer cells (MDA-MB-231 and MCF-7). SS treatment decreased cyclin D1 protein level in human colorectal cancer cells and breast cancer cells. But, SS-mediated downregulated mRNA level of cyclin D1 was not observed. Inhibition of proteasomal degradation by MG132 attenuated cyclin D1 downregulation by SS and the half-life of cyclin D1 was decreased in SS-treated cells. In addition, SS increased cyclin D1 phosphorylation at threonine-286 and a point mutation of threonine-286 to alanine attenuated SS-mediated cyclin D1 degradation. Inhibition of ERK1/2 by PD98059 suppressed cyclin D1 phosphorylation and downregulation of cyclin D1 by SS. In conclusion, SS has anti-proliferative activity by inducing cyclin D1 proteasomal degradation through ERK1/2-dependent threonine-286 phosphorylation of cyclin D1. These findings suggest that possibly its extract could be used for treating colorectal cancer.

**Key words** - Safflower seed, Anticancer activity, Cyclin D1, Human colorectal cancer, Cancer chemoprevention

## Introduction

AHerbal plants have been used for the drug discovery and development (Kim *et al*., 1999; Kim *et al*., 2003). Recently, the seed of safflower (*Carthamus tinctorius* L.) has been reported to play possible roles in the improvement of osteoporosis induced-ovariectomized rats and to protect estrogen deficiency-induced bone loss (Alam *et al*., 2006; Hong *et al*., 2002). In addition, safflower seed lowers plasma and hepatic lipids in rats fed high-cholesterol diet (Moon *et al*., 2001), and has multidrug-resistance reversing activity (Cha *et al*., 2004). In anti-cancer activity, safflower seeds have been reported to suppress cancer cell proliferation in MCF-7 human breast cancer cells, HepG-2 human hepatocellular carcinoma cells and Hela human cervicse adenocarcinoma cells (Bae *et al*., 2002). However, the mechanisms by which safflower seed inhibits cancer cell proliferation have remained nuclear.

Cell proliferation has been long considered to play an essential role in cancer development, implying that the control of cancer cell proliferation may be important for cancer prevention (Mori *et al*., 1999; Mori *et al*., 2001). Many proto-oncogenes have been reported to relate to cancer cell proliferation (Mori *et al*., 2001). Although it regulates G1-to-S phase transition in normal cells, amplification of cyclin D1 gene functions as a proto-oncogene. Thus, overexpression of cyclin D1 has been observed in various human cancers and the role of cyclin D1 in tumorigenesis is well established (Sherr, 1996; Diehl, 2002; Landis *et al*., 2006; Lee and Sicinski, 2006; Li *et al*., 2006). Especially, cyclin D1 has been reported to be overexpressed in 68.3% of colorectal cancer cases, which indicates that deregulation of cyclin D1 may be associated with colorectal tumorigenesis (Holland *et al*., 2001; Bahnassy

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*et al*., 2004). Therefore, it has been accepted that the control of cyclin D1 level may provide a promising chemopreventive and therapeutic way for human colorectal cancer.

In this study, we evaluated anti-proliferative effect of safflower seeds on human colorectal cancer cells and elucidated the potential mechanism for the suppression of the cell proliferation by safflower seeds. Here, we propose a novel mechanism involved in the induction of cyclin D1 proteasomal degradation by safflower seeds. Safflower seeds induced cyclin D1 proteasomal degradation through ERK1/2-dependent threonine-286 phosphorylation of cyclin D1.

## 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). PD98059, SB203580, SP600125, LiCl, BAY11-7082, MG132, cycloheximide (CHX), and 3-(4,5-dimethylthizaol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Aldrich (St. Louis, MO, USA). Antibodies against cyclin D1, phospho-cyclin D1 (Thr286), HA-tag, CDK4, CDK6 and β-actin were purchased from Cell Signaling (Bervely, MA, USA). All chemicals were purchased from Fisher Scientific, unless otherwise specified.

### Sample preparation

The seed of safflower (*Carthamus tinctorius* L.) was purchased from Humanherb, Korea and formally identified by Jin Suk Koo as the professor of Andong National University, Korea. One kilogram of the safflower seed was extracted with 2000 ㎖ of 80% methanol with shaking for 24 h. After 24 h, the methanol-soluble fraction was filtered and concentrated to approximately 400 ㎖ volume using a vacuum evaporator and then fractionated with 400 ㎖ of petroleum ether three times, and then 400 ㎖ of ethyl acetate three times in a separating funnel. The ethyl acetate fraction was separated from the mixture, evaporated by a vacuum evaporator, and prepared aseptically and kept in a refrigerator.

### Cell culture and treatment

Four human colorectal cancer cell lines (HCT116, SW480, LoVo and HT-29) and two breast cancer cell lines (MDA-MB-231 and MCF-7) were purchased from Korean Cell Line Bank (Seoul, Korea) and grown in DMEM/F-12 supplemented with 10% fatal bovine serum (FBS), 100 U/㎖ penicillin and 100 ㎍/㎖ streptomycin. The cells were maintained at 37℃ under a humidified atmosphere of 5% $CO_2$. The extracts from the safflower seed (SS) was dissolved in dimethyl sulfoxide (DMSO) and treated to cells. DMSO was used as a vehicle and the final DMSO concentration did not exceed 0.1% (v/v).

### Cell proliferation assay

Cell growth was measured using MTT assay system. Briefly, cells were plated onto 96-well plated and grown overnight. The cells were treated with the varying concentrations of SS for 24 h. Then, the cells were incubated with 50 ㎕ of MTT solution (1 ㎎/㎖) for an additional 2 h. The resulting crystals were dissolved in DMSO. The formation of formazan was measured by reading absorbance at a wavelength of 570 ㎚.

### SDS-PAGE and Western blot

After SS 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% non-fat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) for 1 h at room temperature and then incubated with specific primary antibodies in 5% non-fat 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, Piscataway, NJ, USA) and visualized in Polaroid film.

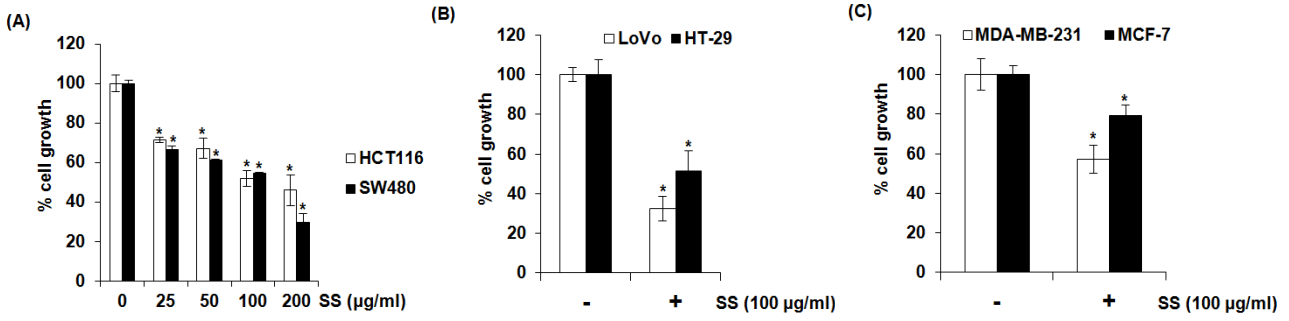


Fig. 1. Anti-proliferative effect of SS in human colorectal cancer cells (HCT116, SW480, LoVo and HT-29) and breast cancer cells (MDA-MB-231 and MCF-7). The cells were plated overnight and then treated with SS. Cell proliferation was measured using MTT assay as described in Materials and methods. *P<0.05 compared to cell without SS treatment.

**Reverse transcriptase-polymerase chain reaction (RT-PCR)**

After SS treatment, total RNA was prepared 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 human primers for cyclin D1 and GAPDH as followed : cyclin D1: forward 5'-aactacctggaccgcttcct-3' and reverse 5'-ccacttgagcttgttcacca-3', GAPDH: forward 5'-acccagaagactgtggatgg-3' and reverse 5'-ttctagacggcaggtcaggt-3'.

**Expression vectors**

Wild type HA-tagged cyclin D1 and T286A HA-tagged cyclin D1 were provided from Addgene (Cambridge, MA, USA). Transient transfection of the vectors was performed using the PolyJet DNA transfection reagent (SignaGen Laboratories, Ijamsville, MD, USA) according to the manufacturers' instruction.

**Statistical analysis**

All the data are shown as mean ± SEM (standard error of mean). Statistical analysis was performed with one-way ANOVA followed by Dunnett's test. Differences with *P <0.05 were considered statistically significant.

## Results

**Effect of SS on the proliferation of human cancer cells**

The effect of SS on cell proliferation was evaluated in human colorectal cancer cells (HCT116, SW480, LoVo and HT-29) and human breast cancer cells (MCF-7 and MDA-MB-231) by MTT assay. As shown in Fig. 1A, cell proliferation of human colorectal cancer cells was significantly suppressed by SS treatment as 29% and 34% at 25 ㎍/㎖, 33% and 39% at 50 ㎍/㎖, 49% and 46% at 100 ㎍/㎖, and 54% and 71% at 200 ㎍/㎖ in HCT116 and SW480 cells, respectively. In addition, SS reduced the proliferation of LoVo and HT-29 cells by 68% and 49% at 100 ㎍/㎖, respectively (Fig. 1B). In the cell proliferation tested in human breast cancer cells lines, the proliferation of MDA-MB-231 and MCF-7 cells was also reduced by SS treatment as 43% and 21% at 100 ㎍/㎖, respectively (Fig. 1C).

**Decreased level of cyclin D1 protein by SS treatment**

Among proto-oncogenes involved in the abnormal proliferation of cancer cells, cyclin has been frequently observed to be overexpressed in cancer cells (Takahashi-Yanaga and Sasaguri, 2008). Thus, we investigated whether SS can regulate the level of cyclin D1 protein. As shown in Fig. 2A, the level of cyclin D1 protein was dose-dependently downregulated by SS treatment in both HCT116 and SW480 cells. In addition, down-regulatory effect of SS on cyclin D1 protein level was observed in SS-treated cells such as HT-29, LoVo, MCF-7 and MDA-MB-231 (Fig. 2B and 2C). In time-course experiment (Fig. 2D), cyclin D1 protein level started to be decreased at 1 h after SS treatment. To determine whether decreased cyclin D1 protein level by SS results from transcriptional regulation, the level of cyclin D1 mRNA was evaluated by RT-PCR. As shown in Fig. 2E, cyclin D1 mRNA level was not changed by

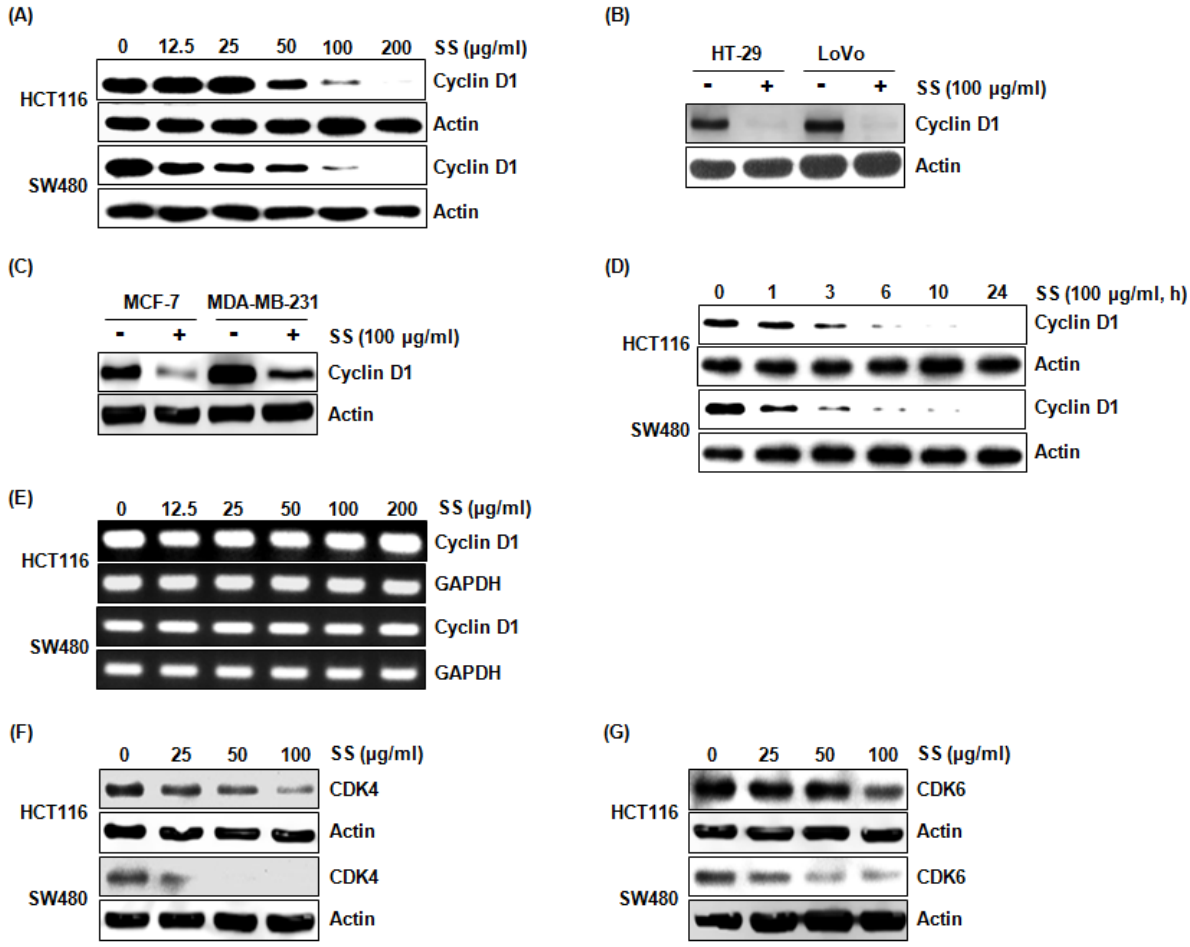


Fig. 2. Downregulation of cyclin D1 by SS. (A-C) The cells were plated overnight and then treated with SS at the indicated concentrations for 24 h. (D) HCT116 and SW480 cells were plated overnight and then treated with 100 ㎍/㎖ of SS for the indicated times. (E) For RT-PCR analysis of cyclin D1 gene expression, total RNA was prepared after SS treatment for 24 h. (F, G) The cells were plated overnight and then treated with SS at the indicated concentrations for 24 h. Cell lysates were subjected to SDS-PAGE and the Western blot was performed using antibody against cyclin D1, CDK4 or CDK6. Actin and GAPDH were used as internal control for Western blot analysis and RP-PCR, respectively.

SS treatment. Next, we investigated the regulatory effect of SS on CDK4 and CDK6 since CDK4 and CDK6 have been reported to be major partners for the cell proliferation. As shown in Fig. 2F and 2G, SS induced the downregulation of CDK4 and CDK6 in HCT116 and SW480 cells.

**Induction of cyclin D1 proteasomal degradation by SS through threonine-286 phosphorylation**

We observed that SS decreases cyclin D1 protein level but not cyclin D1 mRNA, which indicates that SS-mediated decrease of cyclin D1 protein may result from the regulation of cyclin D1 protein stability. To determine the effect of SS on cyclin D1 protein stability, the cells were pretreated with MG132 as a proteasome inhibitor and then co-treated with SS. As shown in Fig. 3A, the inhibition of the proteasome activity by MG132 attenuated SS-mediated decrease of cyclin D1 protein. To verify these results, the cells were pretreated with DMSO or SS, and then exposed to cycloheximide. As shown in Fig. 3B, SS treatment decreased half-life of cyclin D1 protein. These data suggest that downregulation of cyclin D protein level by SS is involved in the proteasomal degradation.

Threonine-286 phosphorylation of cyclin D1 has been known to contribute to proteasomal degradation. Thus, we

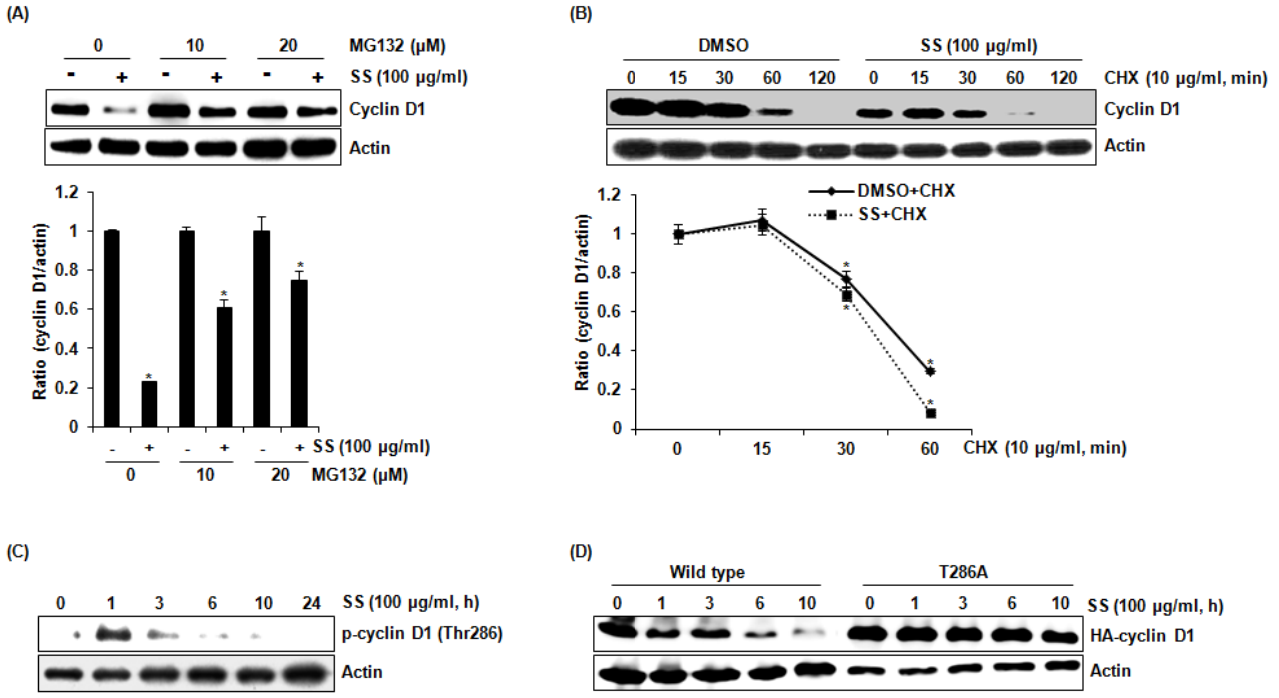


Fig. 3. Proteasomal degradation of cyclin D1 through cyclin D1 phosphorylation at threonine-286 by SS. (A) HCT116 cells were pretreated with MG132 for 2 h and then co-treated with SS (100 ㎍/㎖). (B) HCT116 cells were pretreated with DMSO or MRB (100 ㎍/㎖), and then co-treated with 10 ㎍/㎖ of cycloheximide (CHX) for the indicated times. (C) HCT116 cells were treated with SS (100 ㎍/㎖) for the indicated times. (D) HCT116 cells were transfected with wild type HA-tagged cyclin D1 or HA-tagged T286A cyclin D1 expression vector and then treated with SS (100 ㎍/㎖). Cell lysates were subjected to SDS-PAGE and the Western blot was performed using antibody against cyclin D, p-cyclin D1, HA-tag or actin. Actin was used as internal control.

determine whether SS induces cyclin D1 phosphorylation at threonine-286 and its phosphorylation regulates SS-mediated cyclin D1 proteasomal degradation. As shown in Fig. 3C, threonine-286 phosphorylation of cyclin D1 started to be observed at 1 h after SS treatment. Furthermore, mutation of threonine-286 to alanine (T286A) blocked SS-induced cyclin D1 proteasomal degradation compared to wild type (Fig. 3D). These data indicate that SS may induce cyclin D1 proteasomal degradation through phosphorylating threonine-286 of cyclin D1.

**Dependency of ERK1/2 on SS-mediated cyclin D1 proteasomal degradation and threonine-286 phosphorylation**

Cyclin D1 phosphorylation at threonine-286 and subsequent proteasomal degradation can be regulated by the activation of the upstream kinases such as MAPKs (ERK1/2, p38 and JNK), GSK3β or IκK-$\alpha$. To elucidate the upstream kinases involved in SS-induced phosphorylation and proteasomal degradation of cyclin D1, the cells were pretreated with each inhibitor such as PD98059 (ERK1/2 inhibitor), SB203580 (p38 inhibitor), SP600125 (JNK inhibitor), LiCl (GSK3β inhibitor) or BAY11-7082 (IκK-$\alpha$ inhibitor), and then co-treated with SS. As shown in Fig. 4A-4B, SS activated ERK1/2, and inhibition of ERK1/2 by PD98059 attenuated cyclin D1 proteasomal degradation and threonine-286 phosphorylation by SS treatment. However, SS-mediated cyclin D1 proteasomal degradation was not changed in the inhibition of other kinases such as p38, JNK, GSK3β or IκK-$\alpha$ (Fig. 4C-4F). These data indicate that ERK1/2 may be a major upstream kinase for SS-mediated threonine-286 phosphorylation and subsequent proteasomal degradation of cyclin D1.

## Discussion

Cyclin D1 has been reported to be frequently deregulated in cancer and be a biomarker of cancer phenotype and disease

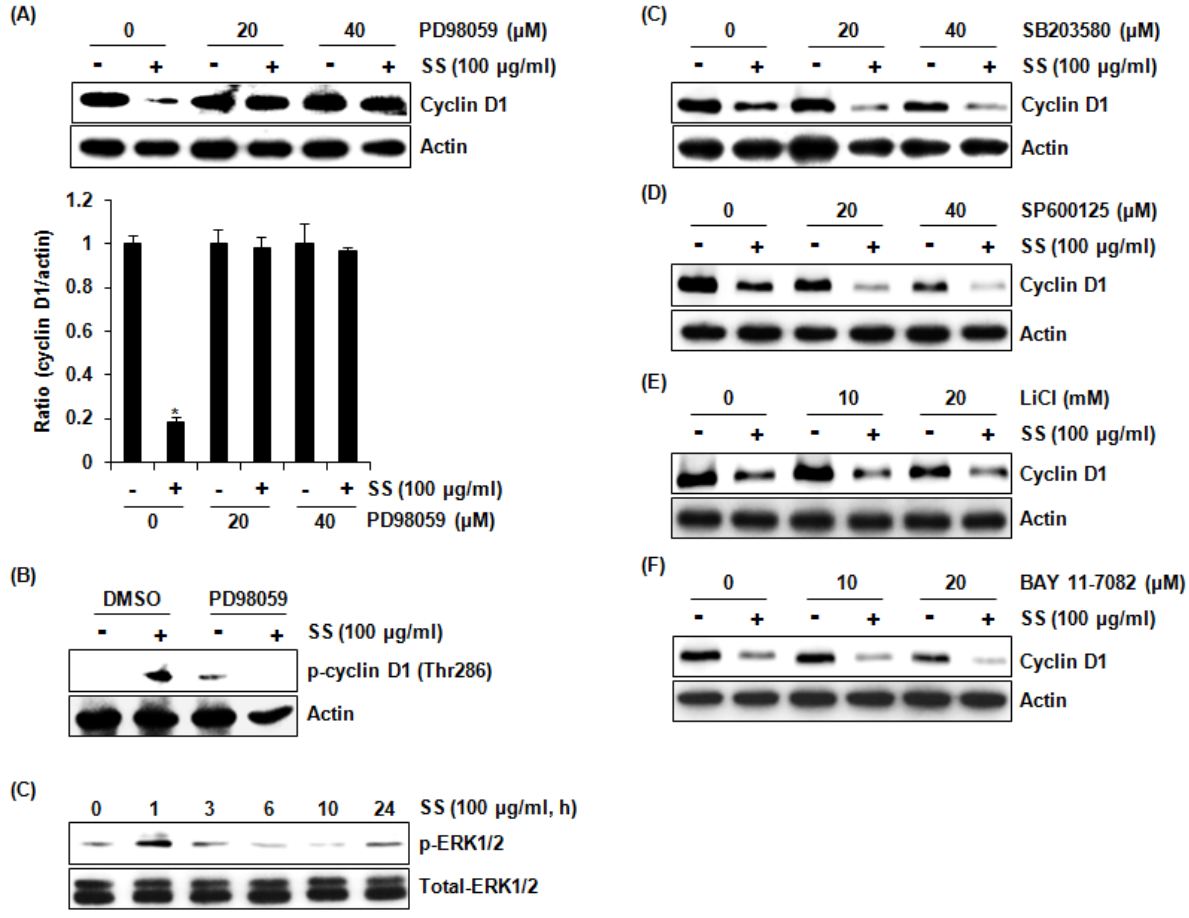


Fig. 4. Dependency of ERK1/2 on SS-mediated phosphorylation at threonine-286 and degradation of cyclin D1. (A, C, D, E, F) HCT116 were pretreated with PD98059, SB203580, SP600125, LiCl or BAY11-7082 and then co-treated with SS (100 ㎍/㎖). (B) HCT116 cells were pretreated with PD98059 and then co-treated with SS (100 ㎍/㎖). (C) HCT116 cells were treated with SS (100 ㎍/㎖) for the indicated times. Cell lysates were subjected to SDS-PAGE and the Western blot was performed using antibody against cyclin D1, phospho-cyclin D1, p-ERK1/2, total-ERK1/2, or actin. Actin was used as internal control.

progression (Musgrove *et al*., 2011). The ability of cyclin D1 to activate G1-to-S phase transition by forming the complex with CDK4 is the most extensively documented mechanism for their oncogenic actions and provides an attractive therapeutic target (Musgrove *et al*., 2011).

Genetic lesions such as amplification of the cyclin D1 gene can account for the cases of tumor-specific cyclin D1 over-expression, but not all. (Shan *et al*., 2009). In this study, we showed that mRNA level of cyclin D1 was not changed by SS treatment, while decreased level of cyclin D1 protein was observed in SS-treated cells. These data indicate that SS may decrease cyclin D1 protein stability. There is growing evidence that inhibition of cyclin D1 degradation can contribute to the increased levels of cyclin D1 in many types of human cancers (Barbash and Diehl, 2008). Thus, the regulation of cyclin D1 degradation can provide the molecular target for preventive and therapeutic purposes. In this study, we showed that cyclin D1 proteasomal degradation may contribute to SS-mediated decrease of cyclin D1 protein using the proteasome inhibitor, MG132 and the protein synthesis inhibitor, cycloheximide.

Although several distinct mechanisms can inhibit cyclin D1 proteolysis, threonine-286 phosphorylation of cyclin D1 is associated with cyclin D1 degradation (Benzeno *et al*., 2006). Human cancer cells harbor mutations in cyclin D1 disrupting the threonine-286 phosphorylation and preventing ubiquitin-mediated proteolysis (Benzeno *et al*., 2006).

Furthermore, cyclin D1 mutant T286A has been reported to be resistant to ubiquitination and subsequently induces a highly stable protein *in vitro* and *in vivo* (Okabe *et al*., 2006). In this study, we found that SS increases the phosphorylation status of cyclin D1 at threonine-286 and mutation of threonine-286 to alanine blocked SS-induced cyclin D1 degradation, which indicates that threonine-286 phosphorylation of cyclin D1 may be a potential molecular target for the downregulation of cyclin D1 by SS.

Cyclin D1 phosphorylation at threonine-286 has been known to be regulated by various kinases such as MAPK (ERK1/2, p38 and JNK), GSK3β and IκK-$\alpha$. GSK3β phosphorylates cyclin D1 at threonine-286, promotes nuclear-to-cytoplasmic redistribution of cyclin D1 and subsequently decreases cyclin D1 protein stability (Alt *et al*., 2000; Diehl *et al*., 1998). Inhibition of GSK3β activity by the upstream oncogenic event such as those targeting Wnt or Ras decreases cyclin D1 turnover (Diehl *et al*., 1998; Rimerman *et al*., 2000). In addition, activation of Ras/Raf/MEK/MAPK signaling cascade can phosphorylate threonine-286 of cyclin D1 and strengthens cyclin D1 degradation (Okabe *et al*., 2006). On the relationship between IκK-$\alpha$ and cyclin D1 phosphorylation at threonine-286, there is growing evidence that cyclin D1 is overexpressed and localized in the nucleus in IκK-$\alpha$-/- cells, and IκK-$\alpha$ regulates proteolysis by phosphorylation of cyclin D1 at threonine-286 (Kwak *et al*., 2005). In this study, we found that inhibition of ERK1/2 attenuated SS-mediated cyclin phosphorylation at threonine-286 and subsequent degradation of cyclin D1, but not the inhibition of other kinases. These findings indicate that ERK1/2 may be a major kinase for SS-induced cyclin D1 degradation.

In conclusion, SS may induce cyclin D1 proteasomal degradation through ERK1/2-dependent threonine-286 phosphorylation of cyclin D1, which may contribute to the inhibition of the proliferation in human colorectal cancer cells. These findings can provide detailed account of preclinical studies conducted to determine the utility of SS as a therapeutic and chemopreventive agent for the treatment of human colorectal cancer.

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