Korean J. Plant Res. 28(6):727-733(2015)
http://dx.doi.org/10.7732/kjpr.2015.28.6.727

Print ISSN 1226-3591
Online ISSN 2287-8203

Original Research Article

Downregulation of Cyclin D1 by Sophorae Flos through Proteasomal Degradation in Human Colorectal Cancer Cells

Jin Wook Lee, Gwang Hun Park, Hyun Ji Eo and Jin Boo Jeong*

Department of Bioresource Sciences, Andong National University, Andong 36729, Korea

Abstract - Although Sophorae Flos (SF) has been reported to exert an anti-cancer activity, molecular targets and mechanisms associated with anti-cancer activity of SF have been unclear. Because cyclin D1 has been regarded as an important regulator in the cell proliferation, we focused cyclin D1 and investigated the effect of SF on the cyclin D1 regulation in light of elucidating the molecular mechanism for SF's anti-cancer activity. The treatment of SF decreased cellular accumulation of cyclin D1 protein. However, SF did not change the level of cyclin D1 mRNA. Inhibition of proteasomal degradation by MG132 attenuated SF-mediated cyclin D1 downregulation and the half-life of cyclin D1 was decreased in the cells treated with SF. In addition, a point mutation of threonine-286 to alanine attenuated SF-mediated cyclin D1 downregulation. Inhibition of ERK1/2 by a selective inhibitor, PD98059 suppressed cyclin D1 downregulation by SF. From these results, we suggest that SF-mediated cyclin D1 downregulation may result from proteasomal degradation through its threonine-286 phosphorylation via ERK1/2. SF-induced proteasomal degradation of cyclin D1 might inhibit proliferation in human colorectal cancer cells. The current study provides information on molecular events for an anti-cancer activity of SF

Key words - Sophorae Flos, Cyclin D1, Anti-cancer, Human colorectal cancer

Introduction

As one of the well-known traditional Chinese medicinal herbs, Sophorae Flos as the flower buds of *Sophora japonica* L. (*S. japonica*) have been used for treating bleeding related disorders such as hematochezia, hemorrhoidal bleeding, dysfunctional uterine bleeding and diarrhea (Lo *et al*., 2009). In addition, Sophorae Flos have been reported to exhibit various pharmacological properties such as anti-diabetes (Jung *et al*., 2006), anti-inflammation (Kim *et al*., 2003a; Kim *et al*., 2003b), anti-oxidant (Tang *et al*., 2002) and anti-cancer (Abe *et al*., 1996; Kang *et al*., 2006). In anti-cancer activity, our group has reported that the flower buds of Sophorae Flos exert anti-cancer activity through upregulating activating transcription factor 3 in human colorectal cancer cells (Lee *et al*., 2015). However, another molecular target and mechanism associated with anti-cancer activity of Sophorae Flos has been unclear. Many medicinal plants exert their anti-cancer activity through the regulation of various molecular targets. Thus, the elucidation of additional potential mechanisms of Sophorae Flos for anti-cancer activity may be necessary.

Cyclin D1 as one of the proto-oncogene is an important regulator of G1 to S progression by forming active complex with cyclin-depending kinase 4 and 6 in many different cell types (Alao, 2007). In addition, cyclin D1 has been regarded to be important for the development and progression of several cancers such as breast, oesophagus, bladder and lung (Hall and Peters, 1996; Vermeulen *et al*., 2003; Knudsen *et al*., 2006). Among cancer types, cyclin D1 overexpression has been observed in 68.3% of human colorectal cancer, which is involved in colorectal tumorigenesis (Bahnassy *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 the present study, we investigated the effect of Sophorae Flos against cyclin D1 expression in human colorectal cancer cell lines, HCT116 and SW480. We found that Sophorae Flos

*Corresponding author. E-mail : jjb0403@anu.ac.kr
Tel. +82-54-820-7757

facilitated the proteasomal degradation of cyclin D1. Furthermore, threonine-286 phosphorylation and ERK1/2 might be involved in cyclin D1 degradation induced by Sophorae Flos.

Materials and Methods

Materials

Dulbecco's Modified Eagle medium (DMEM)/F-12 1:1 Modified medium (DMEM/F-12) was purchased from Lonza (Walkersville, MD, USA). Antibodies against cyclin D1, HA-tag and β-actin were purchased from Cell Signaling (Bervely, MA, USA). MG132, cycloheximide and PD98059 were purchased from Calbiochem (San Diego, CA, USA). All chemicals were purchased from Fisher Scientific, unless otherwise specified.

Sample preparation

Sophorae Flos ((voucher number: Park1003 (ANH)) was kindly provided by the Bonghwa Alpine Medicinal Plant Experiment Station, Korea and was formally identified by Jae Ho Park as the professor of Jungwon University, Korea. One kilogram of Sophorae Flos was extracted with 1000 ㎖ of 80% methanol with shaking for 24 h. After 24 h, the methanol-soluble fraction was filtered and concentrated to approximately 200 ㎖ volume using a vacuum evaporator and then fractionated with petroleum ether and ethyl acetate in a separating funnel. The ethyl acetate fraction was separated from the mixture, evaporated by a vacuum evaporator (EYELA N-N series, Tokyo Rikakikai Co., Japan) prepared aseptically and kept in a refrigerator.

Cell culture and treatment

Human colorectal cancer cell lines, HCT116 and SW480 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 ethyl acetate fraction from Sophorae Flos (SF) were 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).

SDS-PAGE and Western blot

After SF 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, St. Louis, MD. USA) 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 1h 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.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was prepared using a RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and total RNA (1 ㎍) was reverse-ranscribed 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 point mutation of T286A of 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 using Microsoft Excel. Differences with *P <0.05 were considered statistically significant.

Results

Effect of SF on protein and mRNA level in human colorectal cancer cells

To assess whether SF affects cyclin D1 expression, we treated HCT116 and SW480 cells with 12.5, 25 and 50 ㎍/㎖ of SF for 24 h. As shown in Fig. 1A, SF dose-dependently downregulated cyclin D1 expression. In time-course experiment (Fig. 1B), cyclin D1 level begins to rapidly decrease at 3 h in HCT116 cells and 1 h in SW480 cells after SF treatment. To determine if SF-mediated downregulation of cyclin D1 is responsible for transcriptional downregulation, we tested mRNA level of cyclin D1 in both HCT116 and SW480 cells after treatment with different concentrations of SF. As a result (Fig. 1C), mRNA level was not affected by treatment of SF. These results indicate that SF may decrease protein stability of cyclin D1.

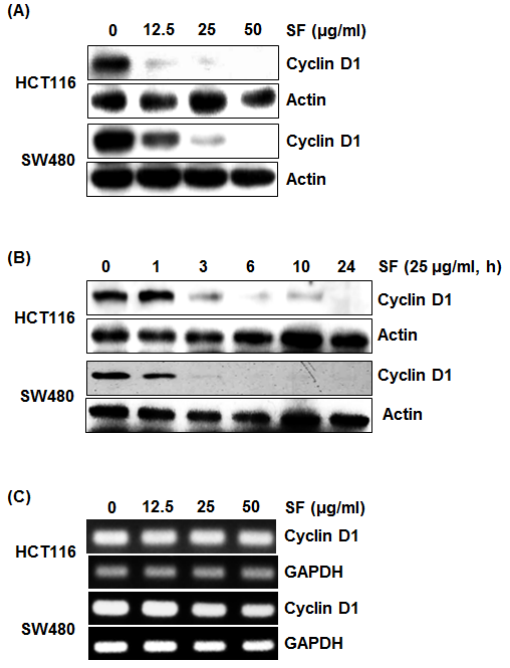

Fig. 1. Downregulation of cyclin D1 by SF in HCT116 and SW480 cells. (A) HCT116 and SW480 cells were plated overnight and then treated with SF at the indicated concentrations for 24 h. Cell lysates were subjected to SDS-PAGE and the Western blot was performed using antibody against cyclin D1. (B) HCT116 and SW480 cells were plated overnight and then treated with 25 ㎍/㎖ of SF for the indicated times. Cell lysates were subjected to SDS-PAGE and Western blot was performed using antibody against cyclin D1. (C) HCT116 and SW480 cells were plated overnight and then treated with SF at the indicated concentrations for 24 h. RT-PCR analysis of cyclin D1 gene expression, total RNA was prepared after SF treatment for 24 h. Actin and GAPDH were used as internal control for Western blot analysis and RP-PCR, respectively.

SF-mediated proteasomal degradation of cyclin D1 in human colorectal cancer cells

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

Involvement of threonine-286 (Thr286) phosphorylation of cyclin D1 in SF-mediated cyclin D1 degradation in human colorectal cancer cells

Cyclin D1 phosphorylation at threonine-286 is associated with its proteasomal degradation via the ubiquitinproteasome pathway (Diehl *et al*., 1997). Thus, we transfected HA-tagged wild type cyclin D1 or HA-tagged T286A cyclin D1 expression vectors in HCT116 and SW480 cells, and then the transfected cells were treated with SF. As a result (Fig. 3), SF decreased HA-tag in both HCT116 and SW480 cells transfected with wild type cyclin D1. However, the transfection of T286A cyclin D1 expression vector blocked SF-mediated downregulation of HA-tag. These results indicate that threonine-286 phosphorylation of cyclin D1 may contribute to SF-mediated cyclin D1 degradation.

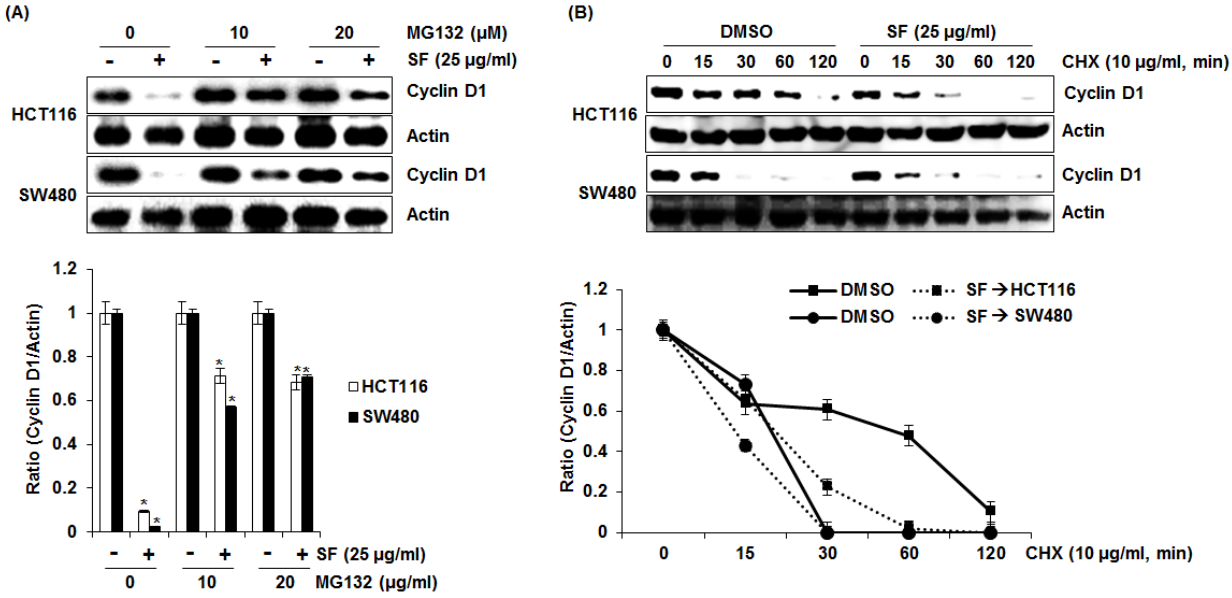

Fig. 2. Proteasomal degradation of cyclin D1 by SF in HCT116 and SW480 cells. (A) HCT116 and SW480 cells were plated overnight. The cells were pretreated with MG132 for 2 h and then co-treated with SF for the addition 3 h. (B) HCT116 and SW480 cells were pretreated with DMSO for ㎍/㎖ of SF for 1 h and then co-treated with 10 ㎍/㎖ of cycloheximide (CHX) for the indicated times. Cell lysates were subjected to SDS-PAGE and the Western blot was performed using antibodies against cyclin D1. Actin was used as internal control.

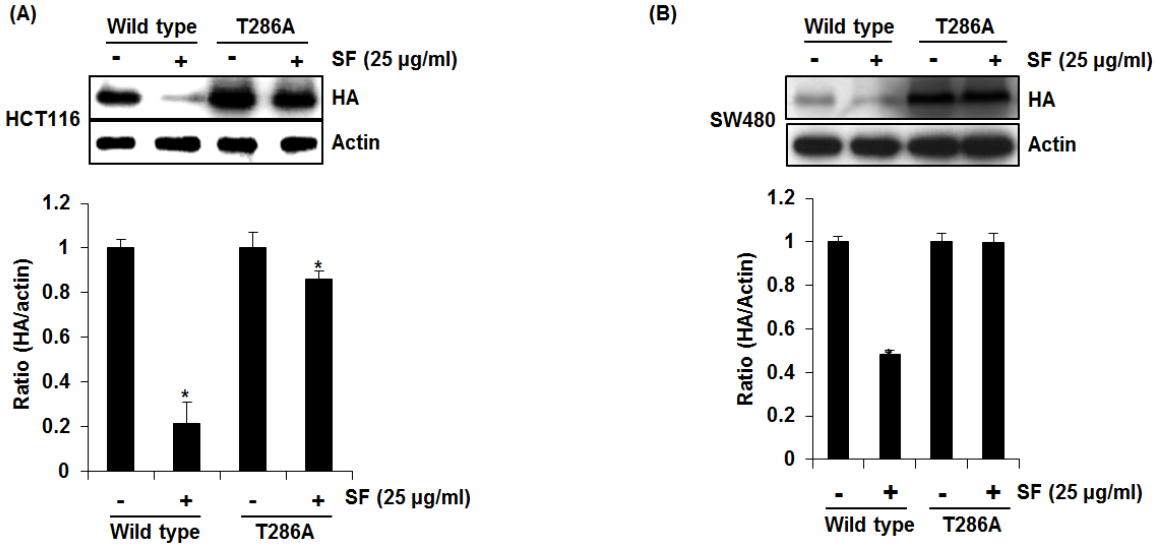

Fig. 3. Involvement of cyclin D1 at threonine-286 in SF-mediated cyclin D1 degradation. (A, B) HCT116 and SW480 cells were transfected with wild type HA-tagged cyclin D1 for 24 h and then treated with 25 ㎍/㎖ of SF. Cell lysates were subjected to SDS-PAGE and the Western blot was performed using antibodies against HA-tag. Actin was used as internal control.

Contribution of ERK1/2 activation by SF to cyclin D1 degradation in human colorectal cancer cells

To investigate the upstream kinases associated with SF-mediated cyclin D1 degradation, HCT116 and SW480 cells were pretreated with specific inhibitors such as PD98059 (ERK1/2 inhibitor), SB203580 (p38 inhibitor), SB216763 (GSK3β inhibitor) and BAY11-7082 (NF-κB inhibitor) and then co-treated with SF. As shown in Fig. 4A and 4B, SF

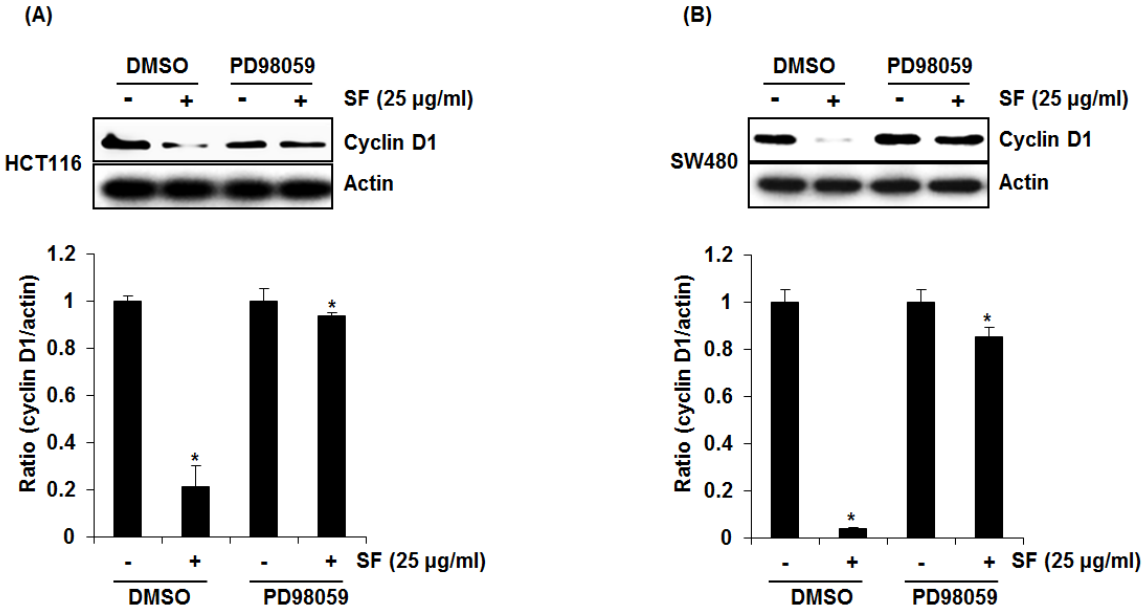

Fig. 4. Effect of ERK1/2 on SF-mediated cyclin D1 degradation. (A, B) HCT116 and SW480 cells were pretreated with 40 μM of PD98059 as an ERK1/2 inhibitor and then co-treated with 25 ㎍/㎖ of SF. Cell lysates were subjected to SDS-PAGE and the Western blot was performed using antibodies against cyclin D1. Actin was used as internal control.

degraded cyclin D1 in both HCT116 and SW480 cells without the inhibitors. However, ERK1/2 inhibition by PD98059 attenuated SF-mediated cyclin D1 degradation. In addition, we found that inhibitions of p38, GSK3β and NF-κB did not affect cyclin D1 degradation induced by SF (data not shown). These results indicate that ERK1/2 may be an important upstream kinase involved in SF-mediated cyclin D1 degradation.

Discussion

Although Sophorae Flos (SF) have an anti-cancer activity (Abe *et al*., 1996; Kang *et al*., 2006), molecular targets and mechanisms associated with anti-cancer activity of SF have been unclear. Currently, we reported that activating transcription factor 3 is overexpressed by SF via its transcriptional upregulation, which contributes to SF-mediated apoptosis and cell growth inhibition in human colorectal cancer lines, HCT116 and SW480 (Lee *et al*., 2015).

Because cyclin D1 has been regarded as an important regulator in the cell proliferation, we focused cyclin D1 and investigated the effect of SF on the cyclin D1 regulation in light of elucidating the molecular mechanism for SF's anti-cancer activity.

In this study, we found that SF inhibited cyclin D1 expression in human colorectal cancer cell lines, HCT116 and SW480 cells. It has been reported that cyclin D1 is frequently overexpression in human cancers and its overexpression can be attributed to transcriptional activation, translation and increased protein stability (Kim *et al*., 2009). Our data shows that SF downregulated cyclin D1 protein level, but not mRNA level, which indicates that SF-mediated downregulation of cyclin D1 protein may result from proteasomal degradation.

Alterations in cyclin D1 turnover lead to abnormal accumulation of cyclin D1 independent of change in cyclin D1 transcription and translation (Kim *et al*., 2009). In addition, there is growing evidence that cyclin D1 accumulation induced by genetic alterations is observed by 10%, while upregulation of cyclin D1 level by the alteration of proteasomal degradation pathway accounts for 50% in breast and esophagus cancer (Barnes *et al*., 1998; Bartkova *et al*., 1994; Shinozaki *et al*., 1996). Among the alteration of proteasomal degradation pathways, cyclin D1 turnover can be dependent on threonine-286 phosphorylation and regulated by ubiquitin-dependent proteasomal degradation (Diehl *et al*., 1997). Interestingly, mutation of threonine-286 into alanine (T286A) abolished SF-mediated cyclin D1 degradation, which indicates that SF-mediated

cyclin D1 degradation may be dependent on threonine-286 phosphorylation. Indeed, many anti-cancer agents including All-trans retinoic acid, aspirin and rapamycin have been shown to induce threonine-286 dependent cyclin D1 degradation (Alao, 2007). Especially, Lang *et al*. (1997) has reported that All-trans retinoic acid requires threonine-286 phosphorylation for inducing cyclin D1 degradation since the T286A mutation of cyclin D1 abolished its proteasomal degradation (Langenfeld *et al*., 1997).

Cyclin D1 proteasomal degradation by threonine-286 phosphorylation has been shown to occur dependently of ERK1/2, p38, GSK3β and NF-κB (Okabe *et al*., 2006; Thoms *et al*., 2007; Diehl *et al*., 1998; Kwak *et al*., 2005). From our data that SF-mediated cyclin D1 degradation by selective inhibition of ERK1/2 by PD98059, ERK1/2 activation by SF at least in part contributes to SF-mediated cyclin D1 proteasomal degradation.

Together, SF-induced proteasomal degradation of cyclin D1 might inhibit proliferation in human colorectal cancer cells. The current study provides information on molecular events for an anti-cancer activity of SF

Acknowledgement

This work was supported by a grant from 2015 Research Funds of Andong National University.

References

Abe, M., O. Suzuki, K. Tasaki, K. Tominaga and H. Wakasa. 1996. Analysis of lectin binding properties on human Burkitt's lymphoma cell lines that show high spontaneous metastasis to distant organs in SCID mice: the binding sites for soybean agglutinin lectin masked by sialylation are closely associated with metastatic lymphoma cells. Pathol. Int. 46:977-983.

Alao, J.P. 2007. The regulation of cyclin D1 degradation: roles in cancer development and the potential for therapeutic invention. Mol. Cancer 6:24.

Bahnassy, A.A., A.R. Zekri, S. El-Houssini, A.M. El-Shehaby, M.R. Mahmoud, S. Abdallah and M. El-Serafi. 2004. Cyclin A and cyclin D1 as significant prognostic markers in colorectal cancer patients. BMC Gastroenterol. 23:22-24.

Barnes, D.M. and C.E. Gillett. 1998. Cyclin D1 in breast cancer. Breast Cancer Res. Treat. 52:1-5.

Bartkova, J., J. Lukas, H. Muller, D. Lutzhoft, M. Strauss and J. Bartek. 1994. Cyclin D1 protein expression and function in human breast cancer. Int. J. Cancer. 57:353-361.

Diehl, J.A., M. Cheng, M.F. Roussel and C.J. Sherr. 1998. Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. Genes Dev. 12:3499-3511.

Diehl, J.A., F. Zindy and C.J. Sherr. 1997. Inhibition of cyclin D1 phosphorylation on threonine-286 prevents its rapid degradation via the ubiquitin-proteasome pathway. Genes Dev. 11:957-972.

Hall, M. and G. Peters. 1996. Genetic alterations of cyclins, cyclin-dependent kinases, and Cdk inhibitors in human cancer. Adv. Cancer Res. 68:67-108.

Jung, C.H., S. Zhou, G.X. Ding, J.H. Kim, M.H. Hong, Y.C. Shin, G.J. Kim and S.G. Ko. 2006. Antihyperglycemic activity of herb extracts on streptozotocin-induced diabetic rats. Biosci. Biotechnol. Biochem. 70:2556-2559.

Kang, S.C., C.M. Lee, H. Choi, J.H. Lee, J.S. Oh, J.H. Kwak and O.P. Zee. 2006. Evaluation of oriental medicinal herbs for estrogenic and antiproliferative activities. Phytother. Res. 20:1017-1019.

Kim, B.H., E.Y. Chung, B.K. Min, S.H. Lee, M.K. Kim, K.R. Min and Y. Kim Y. 2003a. Anti-inflammatory action of legume isoflavonoid sophoricoside through inhibition on cyclooxygenase-2 activity. Planta Med. 69:474-476.

Kim, B.H., E.Y. Chung, J.C. Ryu, S.H. Jung, K.R. Min and Y. Kim. 2003b. Anti-inflammatory mode of isoflavone glycoside sophoricoside by inhibition of interleukin-6 and cyclooxygenase-2 in inflammatory response. Arch. Pharm. Res. 26:306-311.

Kim, J.K. and J.A. Diehl. 2009. Nuclear cyclin D1: An oncogenic driver in human cancer. J. Cell. Physiol. 220:292-296.

Knudsen, K.E., J.A. Diehl, C.A. Haiman and E.S. Knudsen. 2006. Cyclin D1: polymorphism, aberrant splicing and cancer risk. Oncogene 25:1620-1628.

Kwak, Y.T., R. Li, C.R. Becerra, D. Tripathy, E.P. Frenkel and U.N. Verma. 2005. IkappaB kinase alpha regulates subcellular distribution and turnover of cyclin D1 by phosphorylation. J. Biol. Chem. 280:33945-33952.

Langenfeld, J., H. Kiyokawa, D. Sekula, J. Boyle and E. Dmitrovsky. 1997. Post-translational regulation of cyclin D1 by retinoic acid: a chemoprevention mechanism. Proc. Natl. Acad. Sci. USA. 94:12070-12074.

Lee, J.W., G.H. Park, H.J. Eo, H.M. Song, M.K. Kim, M.J.

Kwon, J.S. Koo, J.R. Lee, M.H. Lee and J.B. Jeong. 2015. Anti-cancer activity of the flower bud of *Sophora japonica* L. through upregulating activating transcription factor 3 in human colorectal cancer cells. Korean J. Res. Plants 28(3): 297-304.

Lo, Y.H., R.D. Lin, Y.P. Lin, Y.L. Liu and M.H. Lee. 2009. Active constituents from *Sophora japonica* exhibiting cellular tyrosinase inhibition in human epidermal melanocytes. J. Ethnopharmacol. 124:625-629.

Okabe, H., S.H. Lee, J. Phuchareon, D.G. Albertson, F. McCormick and O. Tetsu. 2006. A critical role for FBXW8 and MAPK in cyclin D1 degradation and cancer cell proliferation. PloS One 1:e128.

Shinozaki, H., S. Ozawa, N. Ando, H. Tsuruta, M. Terada, M. Ueda and M. Kitajima. 1996. Cyclin D1 amplification as a new predictive classification for squamous cell carcinoma of the esophagus, adding gene information. Clin. Cancer Res. 2:1155-1161.

Tang, Y.P., Y.F. Li, J. Hu and F.C. Lou. 2002. Isolation and identification of antioxidants from *Sophora japonica*. J. Asian Nat. Prod. Res. 4:123-128.

Thoms, H.C., M.G. Dunlop and L.A. Stark. 2007. p38-mediated inactivation of cyclin D1/cyclin-dependent kinase 4 stimulates nucleolar translocation of RelA and apoptosis in colorectal cancer cells. Cancer Res. 67:1660-1669.

Vermeulen, K., D.R. Van Bockstaele and Z.N. Berneman. 2003. The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. Cell Prolif. 36:131-149.

(Received 27 August 2015 ; Revised 5 November 2015 ; Accepted 10 November 2015)