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Original Research Article

Anticancer Activity of the Branch Extracts from *Vaccinium oldhamii* through Cyclin D1 Proteasomal Degradation in Human Cancer Cells

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Abstract - In this study, we investigated the effect of the extracts from *Vaccinium oldhamii* on cell proliferation and the regulatory mechanisms of cyclin D1 protein level in human cancer cells. The branch extracts from *Vaccinium oldhamii* (VOB) showed higher inhibitor effect against the cell growth than leave extracts (VOL) and fruit extracts (VOF) in human colorectal cancer, breast cancer, prostate cancer, non-small lung cancer, pancreatic cancer and liver cancer cells. In addition, VOB decreased cyclin D1 level at both protein and mRNA level. MG132 treatment attenuated VOB-mediated cyclin D1 downregulation. A point mutation of threonine-286 to alanine attenuated cyclin D1 degradation by VOB. In addition, the inhibition of nuclear export by leptomycin B (LMB) attenuated cyclin D1 degradation by VOB. But, the treatment of PD98059 (ERK1/2 inhibitor), SB203580 (p38 inhibitor), SP600125 (JNK inhibitor), LiCl (GSK3β inhibitor), LY294002 (PI3K inhibitor) or BAY 11-7082 (IκK inhibitor) did not affect VOB-induced cyclin D1 degradation. In conclusion, VOB induced cyclin D1 degradation through redistribution of cyclin D1 from the nucleus to cytoplasm via T286 phosphorylation of cyclin D1, which resulted in the inhibition of cancer cell proliferation.

Key words – Chemoprevention; Chemotherapy; Functional plant resources; Proteasomal degradation

Introduction

Forest resources have been reported to be beneficial to human health (Karjalainen *et al*., 2010). Among various factors provided by the forest for human health, forest has been regarded as a source of bioactive compounds and drugs (Karjalainen *et al*., 2010). The tree and plants in the forest contain various bioactive compounds such as polyphenols, phytoestrogens, stilbenes and carotenoids (Karjalainen *et al*., 2010) which possess biological activities such as anticancer activity, antiatherogenic, and antioxidant potential (Kris-Etherton *et al*., 2002). Thus, tree and plants in the forest has been used for the commercialization of the human health-promoting agents such as xylitol produced from deciduous hardwood tree (Uhari *et al*., 1996) and taxol from Taxus bark (Joo, 2003; Wall and Wani, 1995).

Vaccinium oldhamii Miquel (*V. oldhamii*) is a deciduous shrub belonging to Ericaceae and is native to Korea. In Korea, *V. oldhami* grows in the southern part of Korea and the middle of the mountains of Jeju island. *V. oldhamii* has been used to treat gonorrhea, vomiting, diarrhea, and eruption (Lee *et al*., 2004). *V. oldhamii* has been reported to have various pharmacological activities such as antioxidant and anticancer activity (Hirotoshi *et al*., 2013). In addition, *V. oldhami* inhibits α-amylase and acetylcholinesterase (Lee *et al*., 2004; Oh and Koh, 2009). The fruit of *V. oldhami* called native blueberry has been reported to have a larger amount of polyphenols and greater antioxidant activity than blueberries (Hirotoshi *et al*., 2013). Thus, *V. oldhamii* has been regarded as the useful resource for developing new blueberry cultivars (Baba *et al*., 2016).

Although the pharmacological properties of *V. oldhamii* have been reported, it is still insufficient. In this study, we evaluated anti-proliferative effect of *V. oldhamii* in human cancer cells and elucidated the potential mechanism.

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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, LY294002, BAY 11-7082, MG132, leptomycin B (LMB) 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, HA-tag and Actin were purchased from Cell Signaling (Bervely, MA, USA). All chemicals were purchased from Fisher Scientific, unless otherwise specified.

Sample preparation

Vaccinium oldhamii (VO) were friendly provided from Forest Medicinal Resources Research Center, National Institute of Forest Science, Yongju, Korea. Five gram of the branches leaves or fruits from VO were extracted with 100 ml of 70% ethanol for 72 h under shaking at the room temperature. After 72 h, the ethanol-soluble fraction was filtered and concentrated to approximately 30 ml volume using a vacuum evaporator and then freeze-dried. The ethanol extracts from the branches (VOB), leaves (VOL) or fruits (VOF) of VO were kept in a refrigerator until use.

Cell culture and treatment

Human colorectal cancer cell lines (HCT116 and SW480), human breast cancer cell lines (MDA-MB-231 and MCF-7), human prostate cancer cell lines (PC-3/nKR and PC-3), human non-small lung cancer cells (A549), human pancreatic cancer cells (AsPC-1) and human liver cancer cells (HepG-2) 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. VOB, VOL and VOF 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).

Cell proliferation assay

Cell growth was measured using MTT assay system. Briefly, the cells were plated onto 96-well plate and grown overnight. The cells were treated with VOB, VOL or VOF 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 analysis

After 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 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)

After 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'-ccacttgagctt gttcacca-3', GAPDH: forward 5'-acccagaagactgtggatgg-3' and reverse 5'-ttctagacggcaggtcaggt-3'.

Expression vectors

Wild type HA-tagged cyclin D1 and HA-tagged T286A 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 ± SD (standard deviation). Statistical analysis was performed with one-way ANOVA followed by Dunnett's test. Differences with *P < 0.05 were considered statistically significant.

Results and discussion

Effect of VOB on the cell proliferation and cyclin D1 expression in human cancer cells

Cancer has been regarded a major public health problem worldwide (Siegel *et al*., 2018). In Korea, a total of 204,909 new cancer cases and 82,155 cancer deaths are expected to occur in 2018 (Jung *et al*., 2018). Because synthetic anti-cancer drugs can induce a lot of side effects, the plant-based resources have been focused for development of a novel anti-cancer agent without side effects (Mahadevappa and Kwok, 2017).

Abnormal cell proliferation and cell cycle progression have been associated with cancer development (Chao *et al*., 2008). In the cell proliferation and cell cycle, cyclin D1 as one of the oncogenes induces G1 to S-phase cell cycle transition, which results in promoting cell proliferation (Balcerczak *et al*., 2005; Baldin *et al*., 1993; Hunter and Pines, 1994). Indeed, cyclin D1 has been reported to be overexpressed in various human cancers such as endometrial (Moreno-Bueno *et al*., 2004), thyroid (Seybt *et al*., 2012), urothelial bladder (Mhawech *et al*., 2004), breast (Stendahl *et al*., 2004), brain gliomas (Tan *et al*., 2004), esophageal (Wu *et al*., 2004) and colorectal cancers (Kristt *et al*., 2000). Thus, cyclin D1 has been regarded as a molecular target for the prevention and treatment of cancer.

Firstly, we compared anti-proliferative effect of the extracts from the branches (VOB), leaves (VOL) and fruit (VOF) of *V. oldhamii* in human colorectal cancer cell lines such as HCT116 and SW480 cells. As shown in Fig. 1A, VOB dramatically

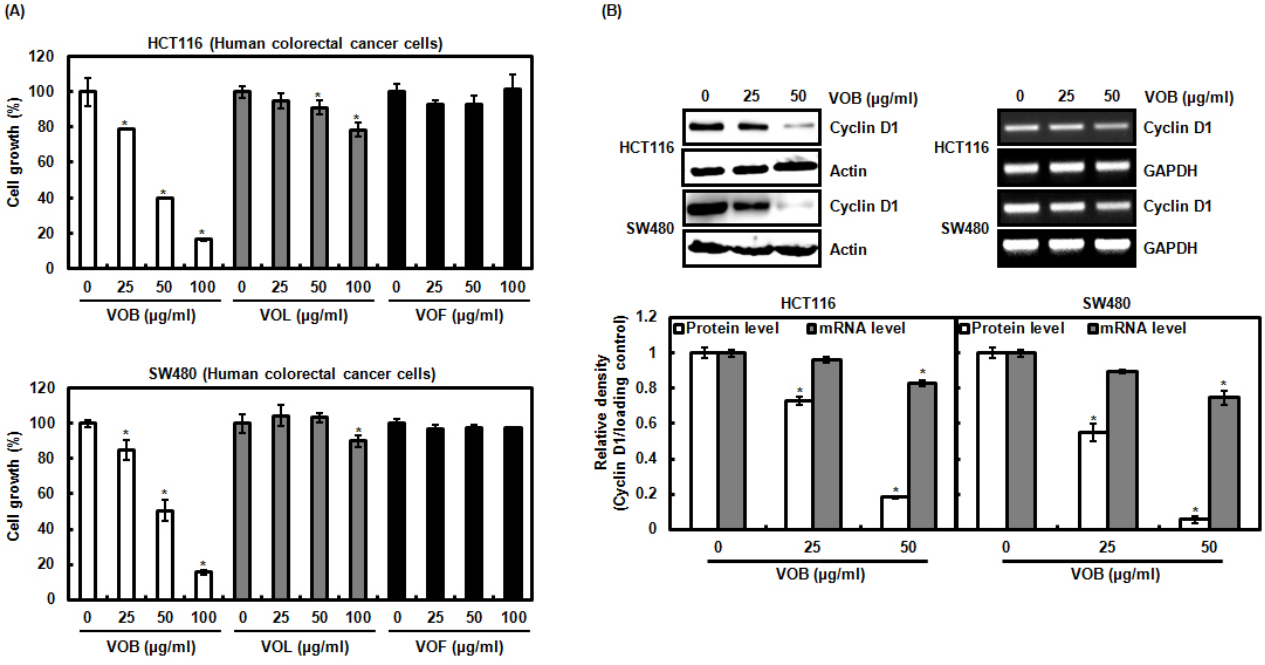

Fig. 1. Effect of VOB on the cell growth and cyclin D1 expression in human colorectal cancer cells. (A) Cells were treated with VOB, VOL or VOF for 24 h. Cell proliferation was measured using MTT assay. *P<0.05 compared to cell without VOB, VOL or VOF. (B) Cells were treated with VOB at the indicated concentrations for 24 h. For Western blot analysis, cell lysates were subjected to SDS-PAGE and the Western blot was performed using antibody against cyclin D1. Actin was used as internal control for Western blot analysis. For RT-PCR analysis of the gene expression of cyclin D1, total RNA was prepared. GAPDH was used as internal control for RP-PCR. *P<0.05 compared to cell without VOB.

inhibited the proliferation of HCT116 and SW480 cells, while the cell growth of HCT116 and SW480 cells was minimally suppressed by VOL. However, VOF did not affect the cell proliferation of these cells. Thus, we chose VOB for the further study and investigated whether VOB affects cyclin D1 expression. As shown in Fig. 1B, VOB treatment downregulated cyclin D1 level in both protein and mRNA level.

In human breast cancer cells such as MDA-MB-231 and MCF-7 cells, anti-proliferative effect against MDA-MB-231 cells was highest in VOB treatment. VOL and VOF minimally suppressed the growth of MDA-MB-231 cells. However, VOB, VOL and VOF did affect the proliferation of MCF-7 cells (Fig. 2A). In addition, we observed that the downregulation of cyclin D1 protein level by VOB treatment was observed in MDA-MB-231 cells but not in MCF-7 cells (Fig. 2B).

In human prostate cancer cells such as PC-3/nKR and PC-3 cells, VOB, VOL and VOF dose-dependently inhibited the cell growth (Fig. 3A). However, the inhibitory effect of VOB on the proliferation of PC-3/nKR and PC-3 cells was more significant that VOL or VOF (Fig. 3A). Furthermore, decreased cyclin D1 protein level by VOB was observed in both PC-3/nKR and PC-3 cells (Fig. 3B).

We also investigated the effect of VOB, VOL and VOF on the cell growth in human non-small lung cancer cells (A549), human pancreatic cancer cells (AsPC-1) and human liver cancer cells (HepG-2). As shown in Fig. 4A, VOB inhibited the cell proliferation at 25, 50 and 100 ㎍/㎖ in A549 cells, while the growth of AsPC-1 and HepG-2 cells was decreased at 50 and 100 ㎍/㎖ of VOB. However, VOL and VOF did not affect the growth of A549, AsPC-1 and HepG-2 cells. Similar to the inhibitory effect of VOB on cell growth, cyclin D1 protein level of A549 was decreased at 25 and 50 ㎍/㎖ of VOB, while VOB attenuated cyclin D1 protein level at 50 ㎍/㎖ in AsPC-1 and HepG-2 cells (Fig. 4B).

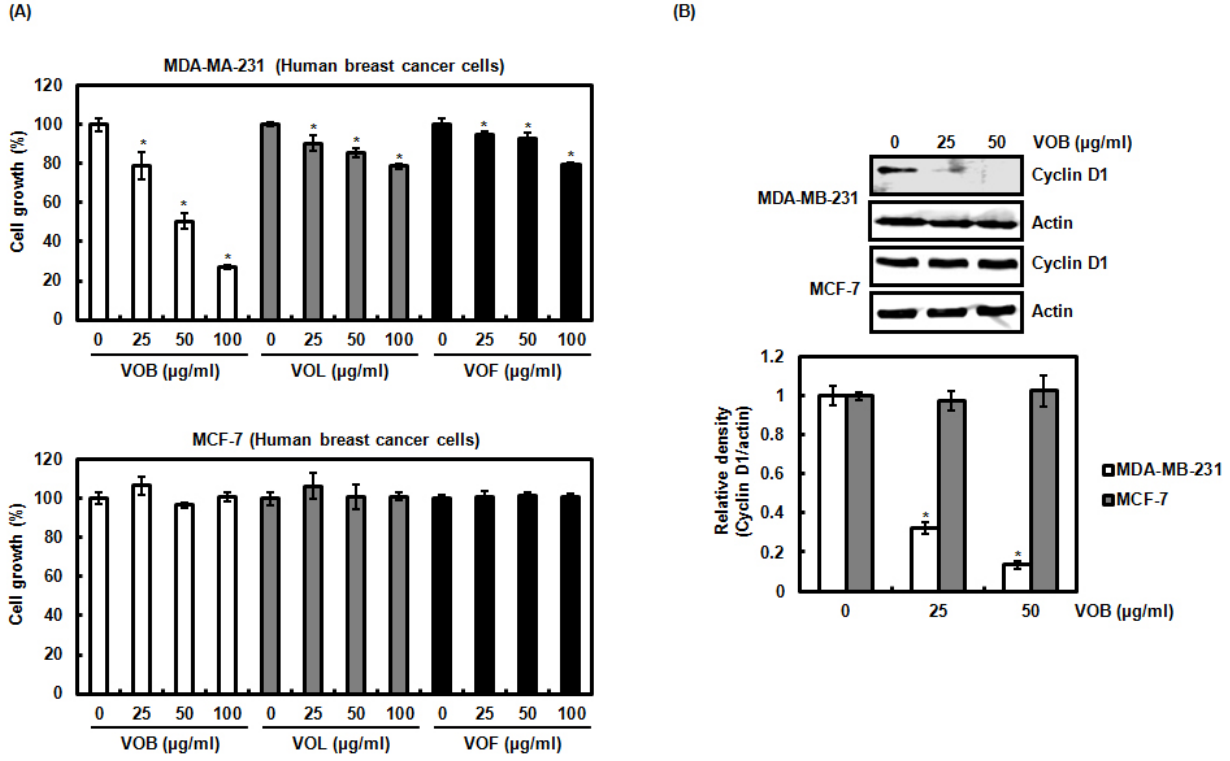

Fig. 2. Effect of VOB on the cell growth and cyclin D1 expression in human breast cancer cells. (A) Cells were treated with VOB, VOL or VOF for 24 h. Cell proliferation was measured using MTT assay. *P<0.05 compared to cell without VOB, VOL or VOF. (B) Cells were treated with VOB at the indicated concentrations for 24 h. For Western blot analysis, cell lysates were subjected to SDS-PAGE and the Western blot was performed using antibody against cyclin D1. Actin was used as internal control for Western blot analysis. *P<0.05 compared to cell without VOB.

From these results indicate that anti-proliferative effect may be highest in VOB and VOB-induced cyclin D1 downregulation may contribute to the inhibition of cell proliferation.

Induction of cyclin D1 proteasomal degradation by VOB in human cancer cells

Interestingly, VOB-mediated downregulation of cyclin D1 protein level was more significant than mRNA level (Fig. 1B), which indicates that VOB may affect cyclin D1 protein stability. Although the elevated cyclin D1 protein level has been observed in various human cancer cells, the increase of cyclin D1 protein is not entirely due to cyclin D1 gene amplification (Park *et al*., 2017). Indeed, the gene amplification and elevated protein of cyclin D1 has been reported to account for 2.5% and 55% in human colorectal cancer cells (Musgrove *et al*., 2011), and defective cyclin D1 degradation pathway can be contribute to the increase of cyclin D1 protein level (Gillett *et al*., 1994; Russell *et al*., 1999). Thus, cyclin D1 degradation has been regarded as a useful treatment for anti-cancer.

To evaluate whether VOB induces cyclin D1 proteasomal degradation, the cells were pretreated with MG132 as a proteasome inhibitor and the co-treated with VOB. As shown in Fig. 5, VOB decreased cyclin D1 protein level in absence of MG132. However, the presence of MG132 attenuated VOB-induced cyclin D1 downregulation, which indicates that VOB-mediated cyclin D1 downregulation may be attributed to cyclin D1 proteasomal degradation.

Contribution of threonine-286 (T286) phosphorylation of cyclin D1 by VOB to cyclin D1 degradation

The degradation of cyclin D1 is known to be induced by phosphorylation of threonine-286 (T286) of cyclin D1 (Alao, 2007; Diehl *et al*., 1997). To evaluate whether T286 phosphorylation of cyclin D1 is associated with VOB-induced cyclin D1

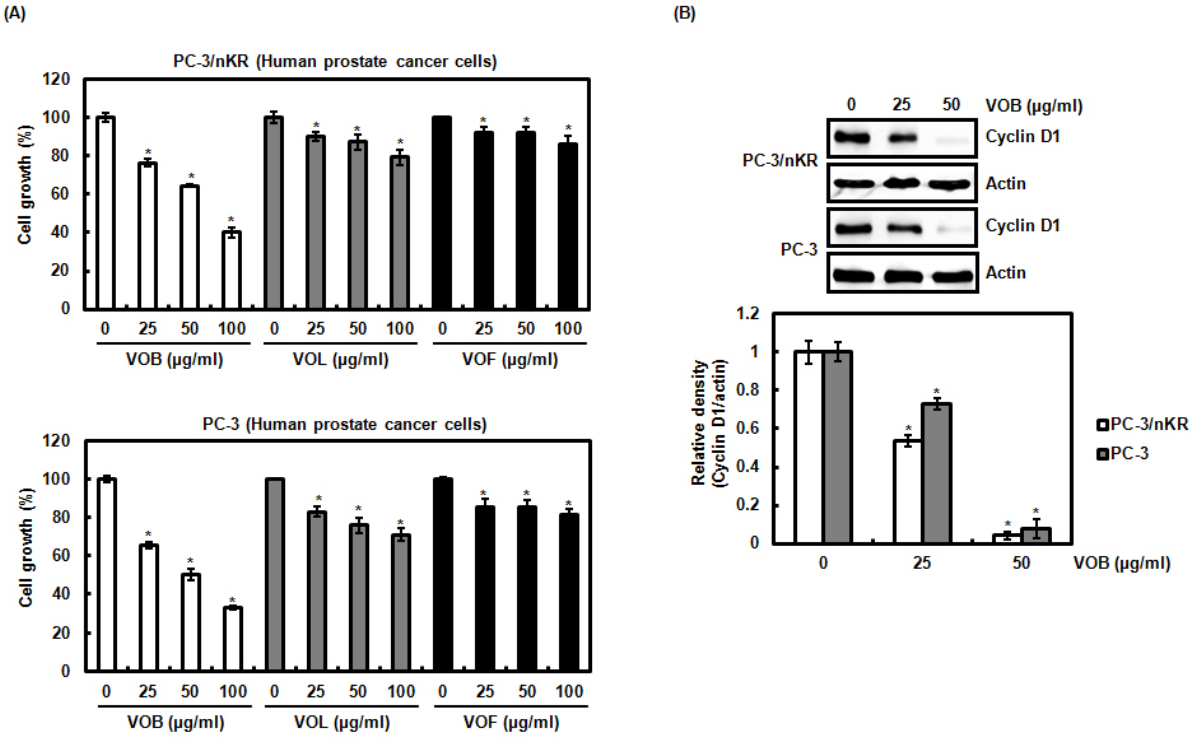

Fig. 3. Effect of VOB on the cell growth and cyclin D1 expression in human prostate cancer cells. (A) Cells were treated with VOB, VOL or VOF for 24 h. Cell proliferation was measured using MTT assay. *P<0.05 compared to cell without VOB, VOL or VOF. (B) Cells were treated with VOB at the indicated concentrations for 24 h. For Western blot analysis, cell lysates were subjected to SDS-PAGE and the Western blot was performed using antibody against cyclin D1. Actin was used as internal control for Western blot analysis. *P<0.05 compared to cell without VOB.

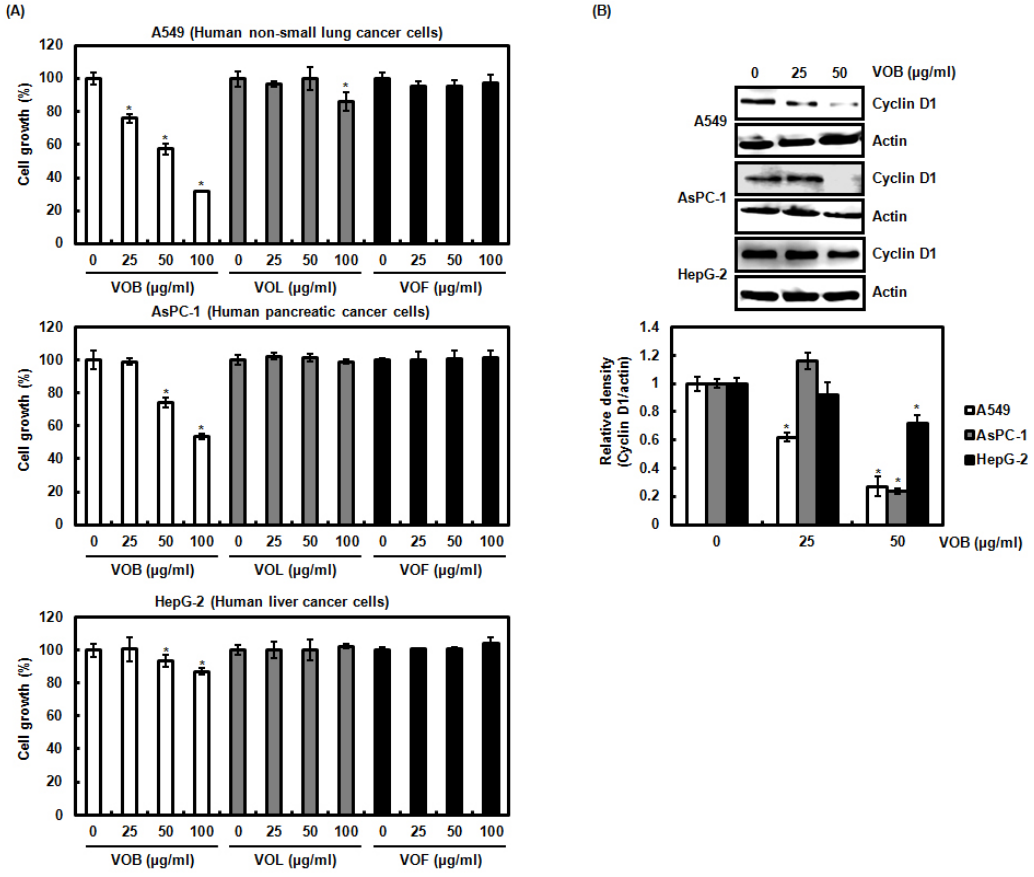

Fig. 4. Effect of VOB on the cell growth and cyclin D1 expression in human non-small lung cancer, pancreatic cancer and liver cancer cells. (A) Cells were treated with VOB, VOL or VOF for 24 h. Cell proliferation was measured using MTT assay. *P<0.05 compared to cell without VOB, VOL or VOF. (B) Cells were treated with VOB at the indicated concentrations for 24 h. For Western blot analysis, cell lysates were subjected to SDS-PAGE and the Western blot was performed using antibody against cyclin D1. Actin was used as internal control for Western blot analysis. *P<0.05 compared to cell without VOB.

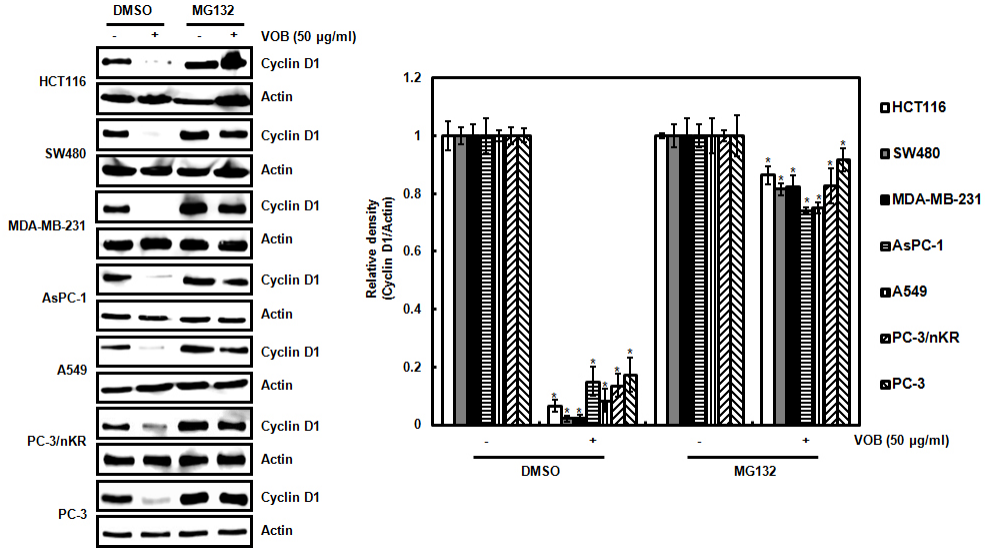

Fig. 5. Effect of VOB on cyclin D1 degradation. The cells were pretreated with MG132 (20 μM) for 2 h and then co-treated with VOB (50 μg/ml). Cell lysates were subjected to SDS-PAGE and the Western blot was performed using antibody against cyclin D1. *P<0.05 compared to cell without VOB.

degradation, HCT116 cells were transfected with HA-tagged wild type cyclin D1 and T286A cyclin D1 constructs and then co-treated with VOB. As a result, VOB decreased HA-cyclin D1 in the cells transfected with wild type-cyclin D1 constructs, but point mutation of T286 to alanine by transfection of T286A-cyclin D1 constructs blocked VOB-induced decrease of HA-cyclin D1 (Fig. 6A). This result indicates that T286 phosphorylation of cyclin D1 may be contribute to VOB-induced cyclin D1 degradation.

The degradation of cyclin D1 by T286 phosphorylation has been reported to be accompanied by redistribution of cyclin D1 from the nucleus to cytoplasm (Diehl *et al*., 1998). To assess whether the redistribution of cyclin D1 from the nucleus to cytoplasm results in VOB-induced cyclin D1 degradation, the cells were treated with LMB as a nuclear export inhibitor and then co-treated with VOB. As shown in Fig. 6B, cyclin D1 degradation is observed in VOB-treated cells in absence of LMB, but the inhibition of cyclin D1 redistribution from the nucleus to cytoplasm by LMB blocked VOB-induced cyclin D1 degradation. These findings suggest that VOB may phosphorylate T286 of cyclin D1 and subsequently redistribute cyclin D1 from the nucleus to cytoplasm, which induce cyclin D1 degradation.

Determination of upstream kinases associated with VOB-induced cyclin D1 degradation

T286 phosphorylation of cyclin D1 has been reported to be regulated by various the upstream kinases such as MAPK (ERK1/2, p38 and JNK), GSK3β, PI3K and IκK, and the activation of these kinases can cause cyclin D1 degradation (Diehl *et al*., 1998; Kwak *et al*., 2005; Okabe *et al*., 2006; Thoms *et al*., 2007). To determine the upstream kinases involved in VOB-mediated cyclin D1 degradation, the cells

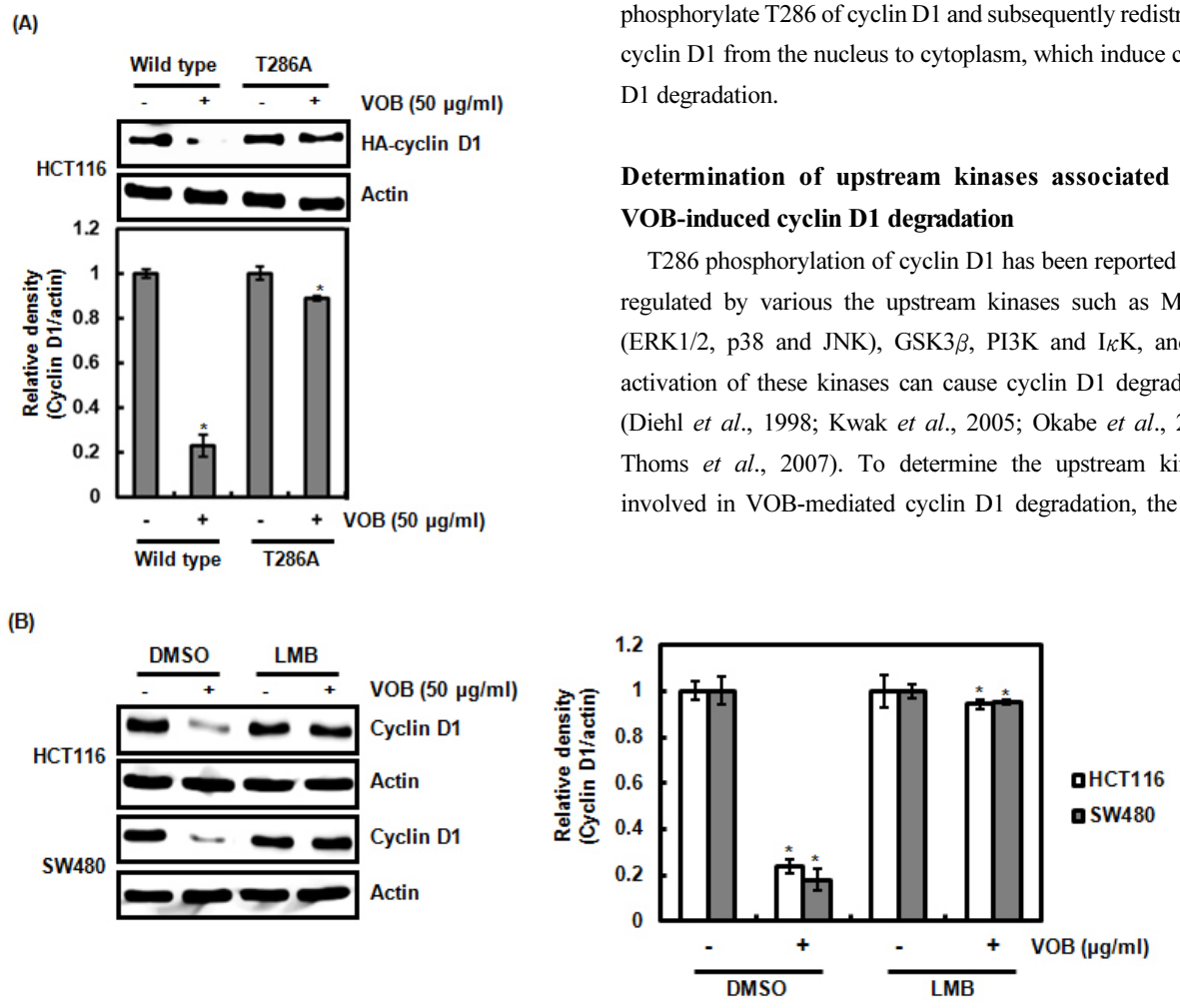

Fig. 6. Cyclin D1 degradation by VOB through redistribute cyclin D1 from the nucleus to cytoplasm via T286 phosphorylation. (A) The cells were transfected with wild type HA-tagged cyclin D1 or HA-tagged T286A cyclin D1 expression vector for 24 h, and then treated with VOB (50 ㎍/㎖). Cell lysates were subjected to SDS-PAGE and the Western blot was performed using antibody against HA-cyclin D1. Actin was used as internal control for Western blot analysis. *P<0.05 compared to cell without VOB. (B) The cells were pretreated with LMB (50 ng/㎖) for 2 h and then co-treated with VOB (50 ㎍/㎖). Cell lysates were subjected to SDS-PAGE and the Western blot was performed using antibody against cyclin D1. Actin was used as internal control for Western blot analysis. *P<0.05 compared to cell without VOB.

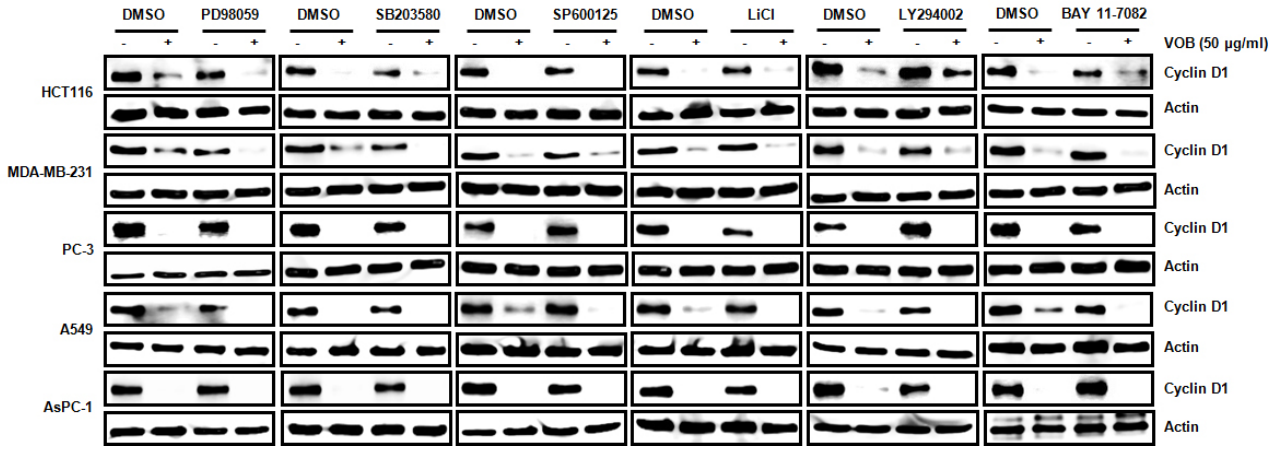

Fig. 7. Determination of the upstream kinases involved in VOB-induced cyclin D1 degradation. The cells were pretreated with PD98059 (20 μM), SB203580 (20 μM), SP600125 (20 μM), LiCl (20 mM), LY294002 (20 μM) or BAY 11-7082 (10 μM) for 2 h and then co-treated with VOB (50 μM). Cell lysates were subjected to SDS-PAGE and the Western blot was performed using antibody against cyclin D1. Actin was used as internal control for Western blot analysis. *P<0.05 compared to cell without VOB.

was pretreated with the specific kinase inhibitors such as PD98059 (ERK1/2 inhibitor), SB203580 (p38 inhibitor), SP600125 (JNK inhibitor), LiCl (GSK3β inhibitor), LY294002 (PI3K inhibitor) or BAY 11-7082 (IκK inhibitor), and then co-treated with VOB. As a result, the treatment of these inhibitors did not inhibit VOB-induced cyclin D1 degradation (Fig. 7). This result indicates that VOB-mediated cyclin D1 degradation may be independent on the activation of MAPK (ERK1/2, p38 and JNK), GSK3β, PI3K and IκK.

In the overall results of this study, we showed that VOB induces cyclin D1 degradation through redistribution of cyclin D1 from the nucleus to cytoplasm via T286 phosphorylation of cyclin D1, which results in the inhibition of cancer cell proliferation. From these findings, VOB may have potential value as a candidate for the development of chemoprevention or therapeutic agents against cancer.

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