

RESEARCH ARTICLE

***Viscum Album Var* Hot Water Extract Mediates Anti-cancer Effects through G1 Phase Cell Cycle Arrest in SK-Hep1 Human Hepatocarcinoma cells**

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Abstract

Viscum album var (VAV) also known as mistletoe, has long been categorized as a traditional herbal medicine in Asia. In addition to its immunomodulating activities, mistletoe has also been used in the treatment of chronic hepatic disorders in China and Korea. There are numerous reports showing that VAV possesses anti-cancer effects, however influence on human hepatocarcinoma has never been elucidated. In the present study, hot water extracts of VAV was evaluated for its potential anti-cancer effect in vitro. SK-Hep1 cells were treated with VAV (50-400ug/ml) for both 24 and 48 hours then cell viability was measured by cell counting kit-8 (CCK-8). Flow cytometry analysis was used to measure the proportion of SK-Hep1 in the different stages of cell cycle. RT-PCR and Western blot analysis were conducted to measure expression of cell cycle arrest related genes and proteins respectively. VAV dose dependently inhibited the proliferation of SK-Hep1 cells without any cytotoxicity with normal Chang liver cell (CCL-13). Flow cytometry analysis showed that VAV extract inhibited the cell cycle of SK-Hep1 cells via G1 phase arrest. RT-PCR and Western blot analysis both revealed that cyclin dependent kinase 2 (Cdk2) and cyclin D1 gene expression were significantly down regulated while p21 was upregulated dose dependently by VAV treatment. Combined down regulation of Cdk2, Cyclin D1 and up regulation of p21 can result in cell death. These results indicate that VAV showed evidence of anti-cancer activity through G1 phase cell cycle arrest in SK-Hep1 cells.

Keywords: *Viscum album var* - anticancer activity - cyclin D1 - G1 phase cell cycle arrest - human hepatocarcinoma cells

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Introduction

Cancer is a leading cause of death in both more and less economically developed countries; the burden is expected to grow worldwide due to the growth and aging of the population, particularly in less developed countries, in which about 82% of the world's population resides (Torre et al., 2015). Liver cancer also known as hepatic adenocarcinoma or hepatocellular carcinoma (HCC) is a malignant tumor in the liver which was reported to rank sixth most frequent cancer globally and second leading cause of cancer death (World Cancer Report, 2014). As stated in the study of Gao et al. (2012) confirmed risk factor includes cirrhosis and hepatitis viral infections which play an important role in hepatocarcinogenesis.

Recent studies have been carried out to find cancer chemo-preventive and/or chemo-therapeutic agents from edible and natural resources. Some studies have reported that natural products have positive effects against cancer compared with chemotherapy. Therefore, many vegetables, fruits and medicinal herbs have been examined to identify

new and effective anticancer compound (Kim et al., 2012). Natural products prevent cancer in different mechanism of action such as DNA synthesis, cell cycle arrest and apoptosis exhibited by the study of Byambaragchaa et al. (2014), anti-metastatic effect (Byambaragchaa et al., 2013) and endoplasmic stress (Jung et al., 2014) and preventing tumorigenic chemicals (Chu et al., 2014).

In addition would be *Viscum album var* (VAV), a species of mistletoe. It belongs to Santalaceae family, commonly known as European mistletoe, common mistletoe or simply as mistletoe (Old English mistle). It is native to Europe and western and southern Asia. It is a hemi-parasitic shrub which grows on the stems of other trees. Mistletoe leaves and young twigs are used by herbalists especially in Germany and Europe for treating circulatory and respiratory system problems (Ernst et al., 2003). VAV preparations have been used for centuries to treat epilepsy, infertility, hypertension and arthritis (Ernst, 2006). Mistletoe extract has been shown to boost the immune system by increasing the number of white blood cells (Grossarth-Maticek, 2001). Aside from the

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immunomodulatory effect, recent study of Marvibaigi et al. (2014) exhibited the potential of mistletoe against breast cancer. Many studies on the anti-cancer effect of VAV have been established but nothing specific for human hepatic adenocarcinoma. Therefore, the aim of this study was to evaluate the anti-cancer properties of *Viscum album var* aqueous extract to SK-Hep1 cells and specifically, to determine the mechanism of action of the extract in the cancer cell death.

Materials and Methods

Chemicals and reagents

Fetal bovine serum (FBS), phosphate buffered saline (PBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from GIBCO BRL (grand Island, NY, USA). Penicillin/streptomycin mix was purchased from Lonza (Walkersville, MD, USA). CCK-8 reagent was purchased from Dojindo (Kumamoto, Japan). Trizol was purchased from Invitrogen (MA, USA) and M-MuLV reverse transcriptase was purchased from Fermentas (USA). Protein extraction solution was purchased from Intron Biotechnology and Bio-Rad protein assay was purchased from Bio-Rad Laboratories (Richmond, CA).

Plant material collection, extraction and preparation

The hot water extract of *Viscum album var* was obtained by subjecting to the following process. The air dried VAV was freeze dried and pulverized to powder form. An exact amount of 100 grams of the dried VAV powder was soaked into a flask with 1 liter boiling water (90°C) for 4 hours and mixed every 30 minutes. After which, the flask was cooled at room temperature and the suspension was filtered. The filtered aqueous extract was placed in a clean container for freeze drying and stored at -70°C. A stock solution was then prepared by dissolving the extract powder in distilled water and experimental concentrations were diluted in the basal medium. Extracts were sterilized by filtration using sterile 0.22 µm pore filter.

Cell culture

Human hepatocarcinoma cells (SK-Hep1) were purchased from Korean Cell Line Bank (KCLB), South Korea. Chang liver normal cells were obtained from Konkuk University (South Korea). All cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100U/ml), streptomycin (100mg/ml), and 3.7mg/ml of NaHCO₃. Cells were cultured in a humidified atmosphere and incubated at 37°C in 5% CO₂.

Cell viability assay

Cell counting kit-8 (CCK-8, Dojindo, Japan) was used to determine cell viability, according to the manufacturer's instructions. Briefly, SK-Hep1 cells were seeded in a 96-well plate with a concentration of 1 x 10⁵ cells/ml and then incubated for 24 hours. The cell suspension was treated with 50, 100, 200 and 400 µg/ml of VAV plant extract for both 24 and 48 hours. After treatment, medium containing VAV was removed and replaced with fresh

media containing 10 µL of CCK-8 solution and incubated at 37°C for two hours. Absorbance at 450nm was measured with an ELISA plate reader (Tecan, Switzerland). For the treated cells, viability is expressed as the percentage of control cells.

Cell cycle distribution analysis

The proportion of SK-Hep1 cancer cell in different stages of cell cycle was measured and analyzed using flow cytometry. After treatment of the seeded cells with VAV (0-400 µg/ml), the cells were harvested and washed twice with phosphate buffered saline (PBS). Cells were stained using 50 µg/ml propidium iodide (PI) and 100 µg/ml RNase A and incubated in the dark for 30 minutes at room temperature. Cell cycle was analyzed using the BD FACSCalibur™ Flow Cytometer. The data was analyzed using the BD CellQuest™ Pro software (Becton, Dickinson and Company, USA).

RNA isolation and polymerase chain reaction

Total RNA was isolated from VAV treated cells using Trizol (Invitrogen, USA) according to the manufacturer's protocol. RNA samples were reverse-transcribed with M-MuLV reverse transcriptase (Fermentas, Lithuania) and specific primers were used to amplify Cdk2 with the primer sequence of Forward: 5'-GCA TCC CAT GTC AAA AAC TTG G-3' and Reverse: 5'-GGA TGA TTC AGT GCC ATT TTG C-3', cyclin D1 with the primer sequence of Forward: 5'-AGA CCT GCG CGC CCT CGG TG-3' Reverse: 5'-GTAG TAG GGA CAG GAA GTT GTT C-3' and p21 with the primer sequence of Forward: 5'-ACC CTT GTG CCT CGC TCA G-3' and Reverse: 5'-GGT CTG CCG CCG TTT TC-3'. The optimum number of cycles for each primer was determined experimentally. The "housekeeping gene" GAPDH was used to verify that equal amounts of RNA were added in the PCR reaction. All gene expression values were normalized against the GAPDH expression.

Western blot analysis

The cells treated with 0 - 400 µg/ml VAV for 24 hours was lysed using a protein extraction solution (Intron Biotechnology). Total protein was determined by the Bio-Rad protein assay. Next, 30 µg of protein was diluted and heated at 95°C for 10 minutes prior to SDS-PAGE gel analysis. The proteins were then transferred to nitrocellulose membranes and blocked overnight with 5% skim milk in TBST (20mM Tris-HCl, pH 7.6, 140mM NaCl, 0.1% Tween 20). The membranes were then rinsed four times with TBST and incubated for 2 hours with 2% skim milk containing 1:1000 dilution of primary antibody: Cdk2, cyclin D1 and p21. After four washes with TBST buffer, the membranes were incubated for 2 hours in horse radish peroxidase-conjugated secondary antibody diluted at 1:2000. The membranes were washed again and developed using enhanced chemiluminescence (ECL Western Blot Analysis System Kit, Amersham Biosciences). The "housekeeping gene" β-actin was used to verify that equal amounts of protein were added. All protein expression values were normalized against the β-actin expression.

Statistical analysis

All experiments were performed in triplicate and the results were expressed as means + standard deviation. Differences between means were evaluated using ANOVA test (one-way) followed by Duncan Multiple Range Test. $p < 0.05$ was taken as statistically significant using SAS/STAT® software.

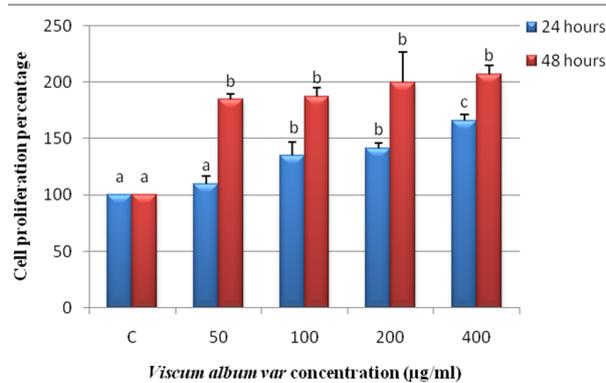


Figure 1. Effect of Viscum album var (VAV) on the Proliferation of Normal Liver Cell (Chang cell) Incubated for 24 and 48 hours. Data are means \pm SD (n=3). means with different superscript are significantly different at $p > 0.05$

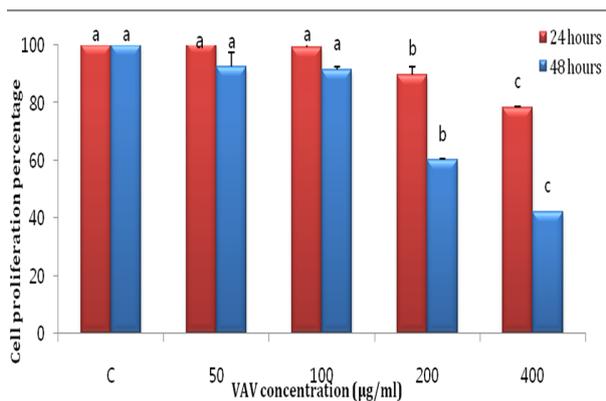


Figure 2. Cytotoxic Effect of VAV Aqueous Extract on Cell Proliferation of SK-Hep1 Cells Incubated at 24 and 48 hours. Data are means \pm SD (n=3). Means with Different Superscript are significantly Different at $p < 0.05$

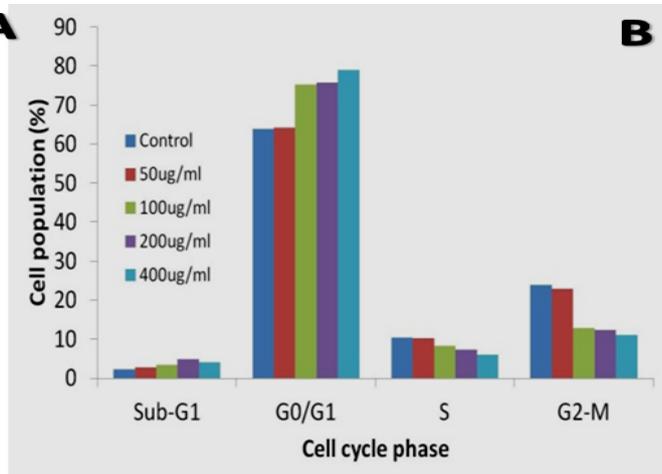
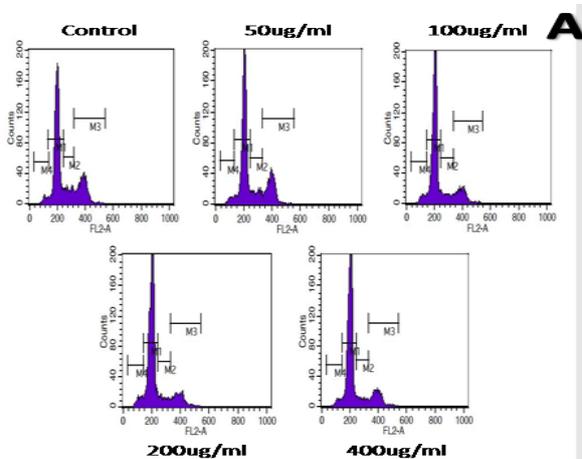


Figure 3. The Effect of Viscum Album Var Hot Water Extract on SK-Hep1 Cell Cycle. (A) Cells treated with different concentrations of VAV for 48 hrs and analyzed by flow cytometry after staining with PI; (B) Histogram showing the number of cells in each phase of the cell cycle

Results

VAV extracts inhibits SK-Hep1 proliferation in vitro

The cytotoxicity of VAV hot water extract on normal cells was evaluated using normal Chang liver cell line before its evaluation for anti-cancer activity. VAV stimulated the proliferation of normal liver cells without any cytotoxicity observed in concentration of up to 400 µg/ml (Figure 1). SK-Hep1 cell line was used to examine the anti-proliferative effects of VAV on cancer cells. Figure 2 showed the dose dependent cytotoxic potential of VAV hot water extract on SK-Hep1 cells for both 24 and 48 hours.

VAV hot water extract induces G1 phase cell cycle arrest

Flow cytometry analysis was done to evaluate if VAV extract induces cell cycle arrest in SK-Hep1 as shown in Figure 3. Histogram analysis (Figure 3B) revealed that cells accumulated in G0/G1 phase of the cell cycle when treated with VAV hot water extract dose dependently. It is supported by a significant increase from 65% of the control to 80% of SK-Hep1 treated with 400 µg/ml VAV. Other phases of the cell cycle showed decrease in SK-Hep1 treated cell population. S phase from 10% to 5% and G2/M phase from 25% to 10%. The increasing cell population in the G0/G1 phase followed by abrupt decrease in the other cell cycle phase means that cell cycle cannot follow after the G1 phase. Figure 3 demonstrated that VAV dose dependently inhibited cellular proliferation of SK-Hep1 cells through G0/G1 phase cell cycle arrest.

Gene expression changes stimulated by VAV hot water extract

To further understand the action mechanism of VAV in the G0/G1 cell cycle arrest, related genes were examined under RT-PCR (Figure 4). The gene expression of Cdk2, which regulates entry to the S phase, and cyclin D1, regulator of Cdks were either decreased or down regulated dose dependently compared to the control. Cyclin dependent kinase inhibitor, p21, had an increased expression after 48 hour treatment of VAV hot water extract.

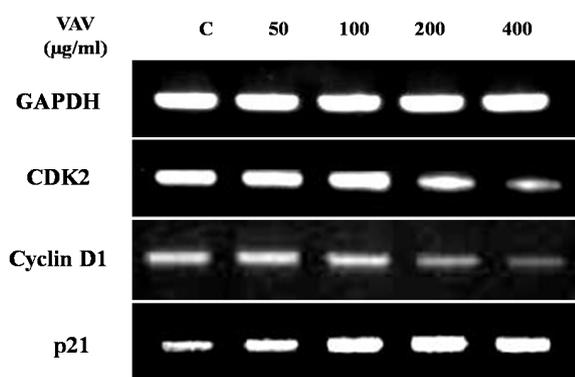


Figure 4. Effect of VAV Hot Water Extract on mRNA Expression of G1 Phase Cell Cycle Related Genes in SK-Hep1 Cell Line

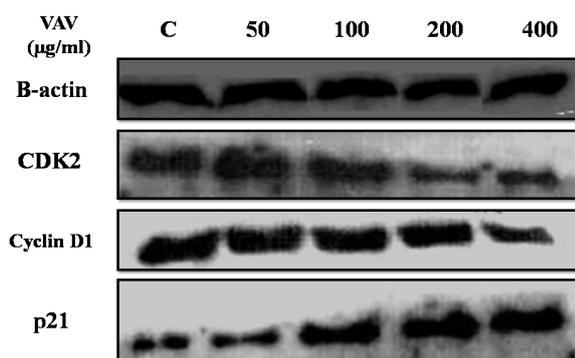


Figure 5. Expression of Cell Cycle Arrest Related Proteins in SK-Hep1 Cell Treated with Different Concentrations of VAV Hot Water Extract

Effects of VAV on the protein expression of related cell cycle arrest genes

To determine the possible molecular action mechanism of VAV on SK-Hep1 cells, the protein expression of G0/G1 cell cycle arrest proteins were evaluated using Western blot analysis. SK-Hep1 cells treated with VAV hot water extract (0-400µg/ml), showed the up regulation of p21 protein, a Cdk inhibitor, and also the down regulation of Cdk2 and cyclin D1 proteins which prevents cell proliferation and arrest growth presented in Figure 4.

Discussion

The present study investigated the anti-cancer effect of *Viscum album var* (VAV) and determined its possible mode of action on SK-Hep1 cell line. The cell viability assay demonstrated the dose dependent inhibition of SK-Hep1 cells proliferation without affecting normal liver cells after both 24 and 48 hour treatment with VAV. This property gives VAV an advantage over the conventional methods of cancer therapy such as radiation and chemotherapy since a successful anticancer treatment should kill or incapacitate cancer cells without causing excessive damage to normal cells. One characteristic of a good anti-cancer agent is the ability to selectively induce cell death in cancer cells with very minimal cytotoxicity to normal cells (Cruz et al., 2014).

Cells respond to DNA damage by halting their cell

cycle progression at G1, S and/or G2 phase due to the activation of cell cycle checkpoints. It follows a signal transduction pathway that generates indicators to inhibit key cell cycle regulators especially the cyclin dependent kinases complexes that govern cell cycle progression (Elledge, 1996; Bartek and Lukas, 2001a).

Activation of different checkpoints involves inhibition of activities of different Cdk complexes. In relation to the G1 phase cell cycle arrest obtained from this study, complexes involving cyclin D1 and Cdk2 will be the main focus. DNA damage induced Cdk inhibition can be caused by numerous processes as established by previous studies which includes the downregulation of cyclins (Muschel et al., 1991; Datta et al., 1992; Poon et al., 1996; Agami and Berbaris, 2000; Miyakawa et al., 2001), inhibition of complex formation between a Cdk and its cyclin partner (Zhan et al., 1999), induction of inhibitory tyrosine phosphorylation of Cdks (Terada et al., 1995; Blasina et al., 1997; Rhind et al., 1997; Poon et al., 2006) inhibition of activating phosphorylation of Cdks (Smits et al., 2000) or induction of the Cdk inhibitor p21 (Dulis et al., 1994; el-Deiry et al., 1994). To assess which of the following processes were involved in the mechanism of action by which VAV hot water extract promote G1 phase cell cycle arrest, related genes and protein expression such as p21, Cdk2 and cyclin D1 were investigated through RT-PCR and Western blot analysis. It is shown that there was an up regulation of p21 and down regulation of both Cdk2 and cyclin D1 in RNA and protein analysis. G1 phase cell cycle checkpoint progress because of the inhibition of complexes formed along with cyclin D or Cdk2. In addition to this would be the activation of the cyclin dependent kinase inhibitor, p21. p21 prevents cell proliferation and responsible for growth arrest and cellular senescence. Particularly, inhibiting the activity of cyclin-Cdk complexes.

In conclusion, the findings acquired in this study elucidated the potential of *Viscum album var* as therapeutic agent against human hepatocarcinoma through G1 phase cell cycle arrest. Further investigations should be done to widen its specific mode of action against liver cancer.

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