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Urushiol V Suppresses Cell Proliferation and Enhances Antitumor Activity of 5-FU in Human Colon Cancer Cells by Downregulating FoxM1

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Abstract

Colorectal cancer (CRC) is one of the most common malignant tumor. 5-FU is commonly used for the treatment of CRC. However, the development of drug resistance in tumor chemotherapy can seriously reduce therapeutic efficacy of 5-FU. Recent data show that FoxM1 is associated with 5-FU resistance in CRC. FoxM1 plays a critical role in the carcinogenesis and drug resistance of several malignancies. It has been reported that urushiol V isolated from the cortex of *Rhus verniciflua* Stokes is cytotoxic to several types of cancer cells. However, the underlying molecular mechanisms for its antitumor activity and its potential to attenuate the chemotherapeutic resistance in CRC cells remain unknown. Here, we found that urushiol V could inhibit the cell proliferation and induced S-phase arrest of SW480 colon cancer cells. It inhibited protein expression level of FoxM1 through activation of AMPK. We also investigated the combined effect of urushiol V and 5-FU. The combination treatment reduced FoxM1 expression and consequently reduced cell growth and colony formation in 5-FU resistant colon cancer cells (SW480/5-FUR). Taken together, these result suggest that urushiol V from *Rhus verniciflua* Stokes can suppress cell proliferation by inhibiting FoxM1 and enhance the antitumor capacity of 5-FU. Therefore, urushiol V may be a potential bioactive compound for CRC therapy.

Key Words: Urushiol V, FoxM1, Colorectal cancer, 5-FU, Drug resistance, Antitumor

INTRODUCTION

Colorectal cancer (CRC) is the third most commonly diagnosed cancer worldwide. It was estimated that there were over 1.9 million new CRC cases and 935,000 deaths in 2020 according to Global Cancer Statistics (Sung *et al*., 2021). Currently, treatments for CRC patients mainly include surgery and chemotherapy. 5-fluorouracil (5-FU), a synthetic fluorinated pyrimidine analog, is a commonly used chemotherapeutic drug for CRC patients. It exerts an antitumor activity by suppressing nucleotide synthetic enzyme thymidylate synthase (TYMS or TS) activity and reducing synthesis of DNA and RNA (Longley *et al*., 2003). However, the antitumor activity of 5-FU can be decreased due to development of drug resistance (Zhang *et al*., 2008). Many genes including TS and FoxM1 have been reported to be associated with 5-FU resistance in CRC (Popat *et al*., 2004; Varghese *et al*., 2019).

FoxM1 is an oncogenic transcription factor that plays a significant role in the initiation, progression, metastasis, and drug resistance of a variety of human tumors, including CRC (Chu *et al*., 2012). It is a critical cell cycle regulator. It is highly expressed in a wide range of human cancers (Kalin *et al*., 2006; Kim *et al*., 2006; Chan *et al*., 2008). Previous studies have shown that aberrant expression of FoxM1 is associated with the development of drug resistance (Koo *et al*., 2012). Thus, inhibiting FoxM1 might decrease cell proliferation and attenuate drug resistance in various types of tumors.

Rhus verniciflua Stokes (lacquer tree, Anacardiaceae) has been traditionally used as a food supplement or a herbal medicine in Korea and China (Kitts and Lim, 2001). Extracts of *Rhus verniciflua* Stokes have been reported to possess anti-oxidant (Lim *et al*., 2001), anti-tumorigenic (Kitts and Lim, 2001), and anti-inflammatory activities (Choi *et al*., 2014). Urushiol V (Fig. 1A), one of urushiol analogues from cortex of *Rhus verniciflua* Stokes, is a compound of 3-substituted catechols with three double bonds in the C15 side chain (ElSohly *et al*., 1982). It has been reported that urushiol V exhibits cytotoxic effects on 29 human cancer cell lines originating from nine organs (Hong

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et al., 1999). However, the underlying mechanisms of such cytotoxic effects of urushiol V are not fully understood yet.

Thus, the objective of this study was to investigate the anticancer activity of urushiol V and explore its underlying mechanisms using SW480 colon cancer cells. In addition, the efficacy of urushiol V on restoring the antitumor activity of 5-FU in a 5-FU resistant SW480 colon cancer (SW480/5-FUR) cells was determined.

MATERIALS AND METHODS

Extraction and Isolation

Dried cortex of *Rhus verniciflua* Stokes (600 g) was extracted twice with 70% EtOH under reflux for 2 h. The extracted solution was filtered through Whatman's filter paper No. 1 and evaporated to obtain the crude extract (119.8 g), which was suspended in distilled water and partitioned with EtOAc. The EtOAc soluble fraction (50 g) was subjected to silica gel column chromatography using n-hexane: acetone solvent system (50:1 → 1:1), to yield 14 fractions. A part (850 mg) of the fraction 5 was separated by medium pressure liquid chromatography (MPLC; RediSep® Rf C18 column, flow rate 75 mL/min, ELSD, UV 214 nm, and 365 nm) and eluted with a gradient of increasing acetonitrile (50% → 100%) in water to give seven fractions. Fraction 5-2 (157.8 mg) was further separated by high performance liquid chromatography (HPLC; acetonitrile-water, 90:10, flow rate 2 mL/min) to afford urushiol V (105.4 mg). The structure of urushiol V was determined based on the analysis of its NMR data along with comparisons with those in the literature (ElSohly *et al*., 1982).

Cell cultures

Human colon cancer SW480 cells were purchased from Korean Cell Line Bank (KCLB; Seoul, Korea). 5-FU-resistant SW480/5-FUR cells were established by repeatedly culturing SW480 cells with constant treatment by 1 μM 5-FU for three months. SW480 and SW480/5-FUR cells were cultured with RPMI1640 (Corning Inc., Corning, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; TCB, Tulare, CA, USA), penicillin (100 U/mL), and streptomycin (10 μg/mL). All cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C.

MTT assay

SW480 and SW480/5-FUR cells were seeded into 96-well plates at density of 2×10^3 cells/well and allowed to adhere overnight. They were incubated with various concentrations of urushiol V or 5-FU. After treatment, MTT solution (0.5 mg/mL) was added and incubated for 2 h at 37°C. Then, 100 μL dimethyl sulfoxide (DMSO) was added to each well and the plate was shaken for 5 min. Absorbance was measured at 540 nm with a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Colony formation assay

Cells were seeded into 6-well plates at 200 cells/well and treated with indicated concentrations of urushiol V. After 72 h of treatment, cells were maintained with drug free medium for 12-14 days with media changes every 3 days. Colonies were fixed with 4% formaldehyde and stained with crystal violet (0.05%). The plate was then photographed and the number of colonies was counted using Image J software (NIH, Bethesda, MD, USA).

Cell cycle analysis

Treated cells were dispersed and washed with cold phosphate buffered saline (PBS) before adding pre-cooled 70% ethanol. Cells were then washed with PBS and incubated with 500 μL propidium iodide (PI)/RNase solution for 30 min in the dark. Prepared cellular samples were immediately analyzed using a FACS Calibur flow cytometer (BD Biosciences, San

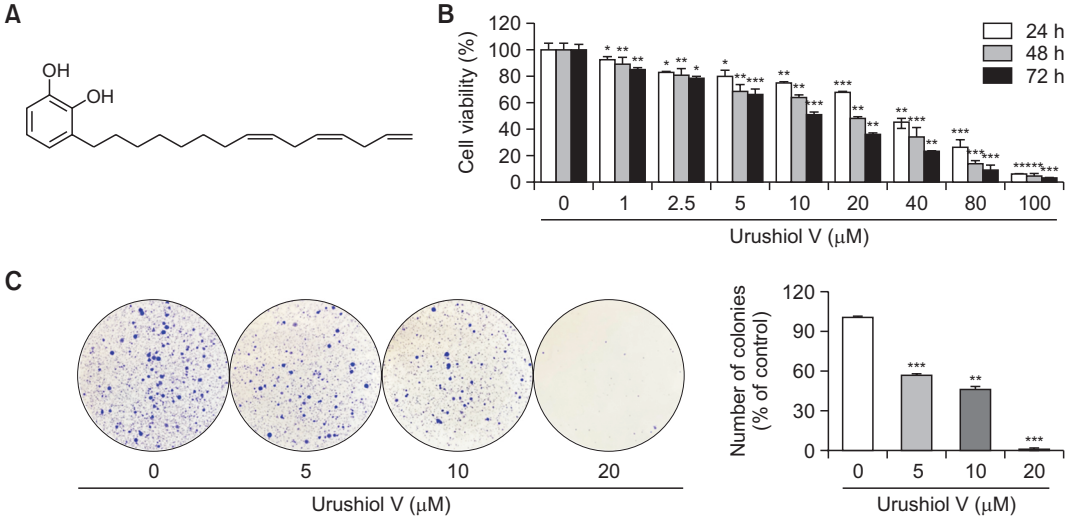

Fig. 1. Effect of urushiol V on cell proliferation of SW480 colon cancer cells. (A) Chemical structure of urushiol V. (B) SW480 cells were treated with urushiol V at indicated concentrations for 24, 48 or 72 h and cell viability was assessed by MTT assay. (C) Colony formation assay was assessed after 12 days of urushiol V treatment for SW480 cells. Wells were stained with crystal violet at the end of the experiment and colonies were counted. All experiments were performed in triplicate and the data represent the mean ± SD. *p<0.05, **p<0.01, ***p<0.001 as compared to vehicle control.

Jose, CA, USA).

RT-PCR analysis

Total RNA was isolated from the cell pellet using TRIzol™ Reagent (Invitrogen, Carlsbad, CA, USA). First-stand cDNA was synthesized using Labopass™ cDNA synthesis kit (Cosmogenetech, Seoul, Korea) and amplified using a PCR thermal cycler (GeneAmp PCR System 2700, Applied Biosystems, Foster City, CA, USA). The primer sequences used for RT-PCR were as follows: FoxM1 forward, 5′-ATGGCAAAT TTTTCGCTCC-3′; FoxM1 reverse, 5′-ATGTCACCAGAAATTCCCAGTT-3′; β-actin forward, 5'-AAGGGACTTCCTGTAACAACG-3'; β-actin reverse, 5'-AGGATGCAGAAGGAGATCACT-3'. Amplified DNA was separated on 2% agarose gels and stained with ethidium bromide.

Western immunoblot analysis

Cell lysis, sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE), and Western blotting were performed as described previously (Jeong and Ryu, 2020). Antibodies used were FoxM1 (A301–533A, Bethyl Laboratories, Montgomery, TX, USA), cyclin E1 (ab71535, Abcam, cambridge, UK), cyclin D1, cyclin B1, survivin, phosphorylated AMPK (p-AMPK), total AMPK, phosphorylated mTOR (p-mTOR), total mTOR, c-Myc (#2922, #4138, #2808, #2535, #5831, #5536, #2972 #9402, Cell Signaling Technology, Danvers, MA, USA), p21, TS (sc-10736, sc-33679, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and β-actin (A2066, Sigma Aldrich, St. Louis, MO, USA).

Statistical analysis

Results are expressed as mean ± standard deviation (SD). The significance of differences among groups was evaluated using Student's t-test. *p* values of less than 0.05 were considered statistically significant.

RESULTS

Urushiol V inhibits proliferation of SW480 colon cancer cells

To assess the effect of urushiol V on cell proliferation, SW480 cells were exposed to different concentrations (0-150 μM) of urushiol V. Cell proliferation was then measured by MTT assay. Urushiol V inhibited the rate of cell proliferation of SW480 with IC_{50} values of 33.1, 14.7, and 10.2 μM after treatment for 24, 48, and 72 h, respectively (Fig. 1B). The suppression effect of urushiol V on cell proliferation was confirmed by colony formation assay. Compared with the control group, groups treated with urushiol V at 5, 10, and 20 μM showed decreased numbers of colony formation by 43.1 ± 1.4, 53.9 ± 0.9, and 98.6 ± 0.1%, respectively (Fig. 1C). Taken together, these results indicate that urushiol V can significantly inhibit SW480 colon cancer cell proliferation.

Urushiol V induces cell cycle arrest at S phase in SW480 colon cancer cells

To further elucidate the anti-proliferative mechanisms of urushiol V in SW480 cells, flow cytometry was performed to analyze the distribution of cell cycle phases. As shown in Fig. 2A, urushiol V reduced the number of cells in the G0/G1 phase with a corresponding accumulation in the S phase. The S phase cell cycle population was 22.1%, 28.6%, and 35.8% at 0, 10, and 30 μM urushiol V, respectively.

Moreover, treatment with urushiol V decreased the levels of cyclin E1 and thymidylate synthase (TS) in a concentration-

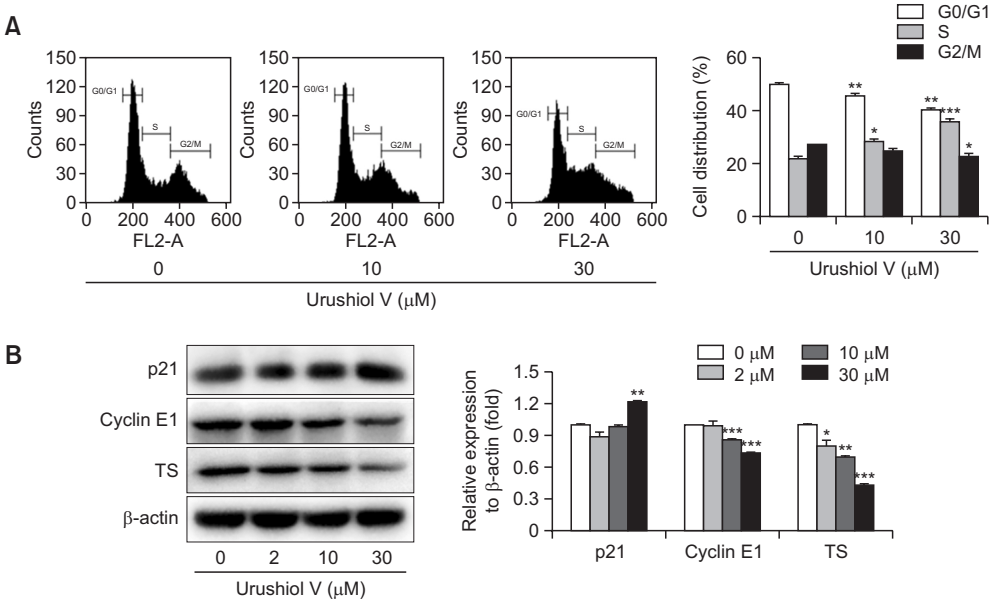

Fig. 2. Effects of urushiol V on cell cycle distribution in SW480 colon cancer cells. (A) SW480 cells were treated with 10 or 30 μM of urushiol V for 24 h. Cell cycle distribution was assessed by flow cytometry. Percentages of cell population in each stage of the cell cycle was determined using BD CellQuest Pro software (version 6.0, BD Biosciences, San Jose, CA, USA). (B) SW480 cells were treated with indicated concentrations of urushiol V for 24 h. Protein levels of p21, cyclin E1, and TS were detected by Western blotting and quantified using Image J software. The data from three independent experiments are presented as the mean ± SD. **p*<0.05, ***p*<0.01, ****p*<0.001 as compared to vehicle control.

dependent manner. On the other hand, the expression level of p21, known as a tumor suppressor (el-Deiry *et al*., 1993), was significantly increased at high (30 μM) concentration of urushiol V (Fig. 2B). These results demonstrate that urushiol V can induce S phase arrest by altering the expression of S phase related genes in SW480 cells.

Urushiol V inhibits FoxM1 protein expression in SW480 colon cancer cells

FoxM1 is known to be closely related to cell proliferation and cell cycle in cancer cells (Zhang *et al*., 2016). FoxM1 was down-regulated in SW480 by treatment with urushiol V at 2, 10 and 30 μM for 24 h. Additionally, several molecules downstream of FoxM1, including c-Myc, cyclin D1, cyclin B1, and survivin, were also decreased by urushiol V treatment in a dose-dependent manner (Fig. 3A).

We next examined effects of urushiol V on mRNA expression of FoxM1 using RT-PCR and found that urushiol V did not affect the mRNA level of FoxM1 (Fig. 3B). These results indicate that urushiol V might inhibit FoxM1 protein expression at the post-transcriptional level.

Urushiol V regulates expression of FoxM1 via AMPK/mTOR signaling pathway

In order to further understand the mechanisms by which urushiol V decreased FoxM1 expression, we examined the effect of urushiol V on FoxM1 stabilization using cycloheximide (CHX) to prevent protein synthesis. SW480 cells were pre-treated with CHX and then exposed to urushiol V for 16 h. As shown in Fig. 4A, protein level of FoxM1 was further reduced by the co-treatment of CHX and urushiol V compared to that by CHX treatment alone, indicating that urushiol V could affect FoxM1 protein stability.

To confirm the effect of urushiol V on FoxM1 protein degradation, SW480 cells were pre-treated with MG132, a proteasome inhibitor, and chloroquine (CQ), a lysosomal inhibitor, and then treated with urushiol V for 16 h. As shown in Fig. 4B, even in the presence of MG132 and CQ, urushiol V decreased FoxM1 protein levels. Proteasome inhibitors such as MG115, MG132, and bortezomib was known to inhibit FoxM1 transcriptional activity and expression (Bhat *et al*., 2009). These data indicate that urushiol V does not induce FoxM1 protein degradation.

It has reported that *Rhus verniciflua* Stokes extract can induce apoptosis of breast cancer cells by activating AMPK signaling (Lee *et al*., 2014). Therefore, we investigated effects of urushiol V on AMPK signaling in SW480 cells. After 24 of treatment with urushiol V, the protein level of p-AMPK was significantly increased. However, the protein level of p-mTOR, a downstream target of AMPK, was decreased by urushiol V in a concentration-dependent manner (Fig. 4C). Taken together, these data indicate that urushiol V can inhibit FoxM1 protein level via the AMPK/mTOR signaling pathway.

Upregulation of FoxM1 confers resistance to 5-FU

Upregulation of FoxM1 has recently been reported to be closely related to 5-FU resistance in CRC (Xie *et al*., 2017). To investigate the expression of FoxM1 in 5-FU resistance, we generated a 5-FU-resistant cell line (SW480/5-FUR). MTT assay was used to confirm the resistance of SW480/5-FUR cells to 5-FU. IC_{50} value of 5-FU was 10.8 μM for SW480 cells and 88.7 μM for SW480/5-FUR cells. Thus, SW480/5-FUR cells were 8-fold more resistant to 5-FU than SW480 cells (Fig. 5A).

We also evaluated levels of drug resistance-related proteins in parental colon cancer cells and their resistant cell lines by western blot. Expression levels of FoxM1 (1.65-fold), free TS (1.64-fold), and complex TS (3.25-fold) in SW480/5-FUR cells were significantly higher than those in parental SW480 cells. Higher TS expression in CRC decreases the efficacy of 5-FU and this is one of the main reasons of resistance of CRC cells to 5-FU (Peters *et al*., 2002).

5-FU is converted to its active metabolite fluoro-deoxyuridine monophosphate (FdUMP) through nucleotide metabolic pathways for thymidine monophosphate (dTMP). It forms a ternary complex with TS and 5,10-methylenetetrahydro-folate (5,10-CH_2THF), leading to inhibition of TS (Drake *et al*., 1993). In SW480/5-FUR cells, the upper band of TS represents the ternary complex form, which correlates with the intracellular concentration of FdUMP (Fig. 5B) (Drake *et al*., 1993; Longley *et al*., 2003). Free TS is responsible for maintaining the thymidylate levels and DNA biosynthesis in cells (Chu *et al*., 1993).

In summary, we confirmed that upregulation of FoxM1 and TS was associated with resistance to 5-FU. Therefore, inhibition of FoxM1 or TS is a therapeutic strategy for enhanc-

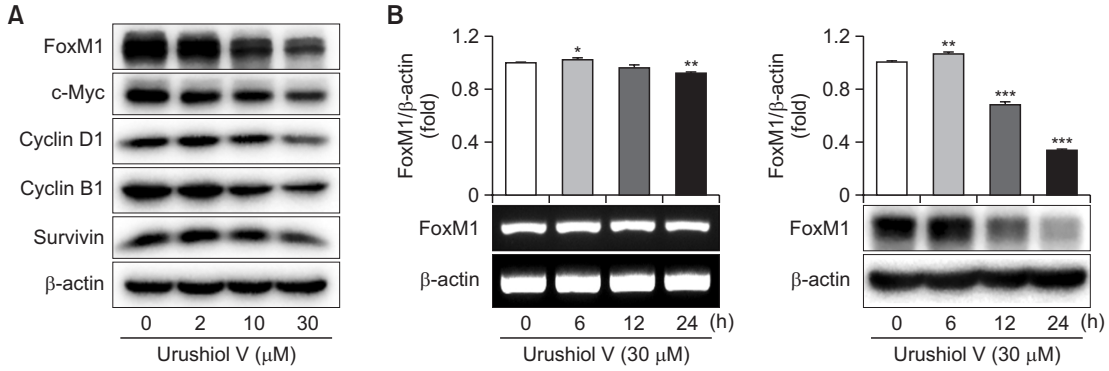

Fig. 3. Effect of urushiol V on the expression of FoxM1 protein. (A) SW480 cells were treated with indicated concentrations of urushiol V for 24 h and protein levels of FoxM1, c-Myc, cyclin D1, cyclin B1, and survivin were detected by Western blotting. (B) SW480 cells were treated with 30 μM urushiol V for the indicated time. FoxM1 mRNA was detected by RT-PCR and FoxM1 protein was detected by Western blotting. Data are shown as the mean ± SD of three independent experiments. **p*<0.05, ***p*<0.01, ****p*<0.001 as compared to vehicle control.

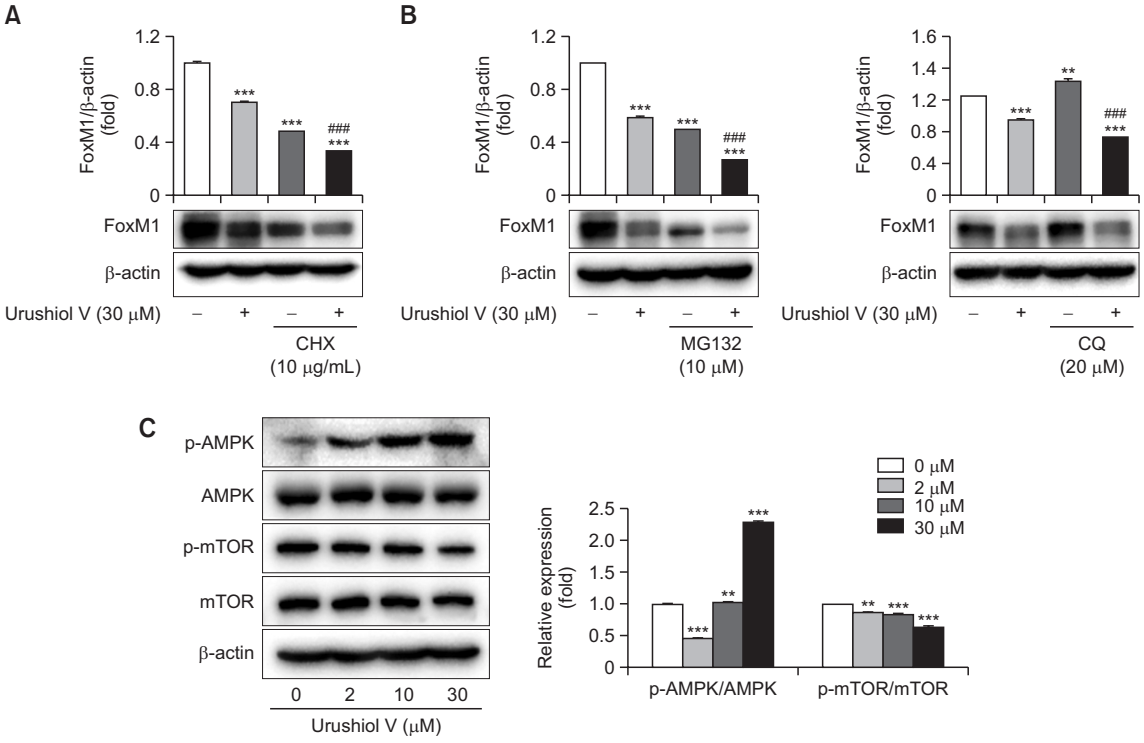

Fig. 4. Effect of urushiol V on AMPK/mTOR signaling pathway. (A) SW480 cells were pre-treated with CHX for 2 h and then further treated with urushiol V for 16 h. FoxM1 protein was detected by Western blotting. Data are shown as the mean ± SD of three independent experiments. ***p<0.001 as compared to vehicle control; $^{###}p$<0.001 as compared to treated with 10 μg/mL CHX. (B) SW480 cells were pre-treated with MG132 or CQ for 2 h and then further treated with urushiol V for 16 h. FoxM1 protein was detected by Western blotting. Data are shown as the mean ± SD of three independent experiments. **p<0.01, ***p<0.001 as compared to vehicle control; $^{###}p$<0.001 as compared to treated with 10 μM MG132 or 20 μM CQ. (C) SW480 cells were treated with indicated concentrations of urushiol V for 24 h, and protein levels of phospho-AMPK and phospho-mTOR were detected by Western blotting. Data are shown as the mean ± SD of three independent experiments. **p<0.01, ***p<0.001 as compared to vehicle control.

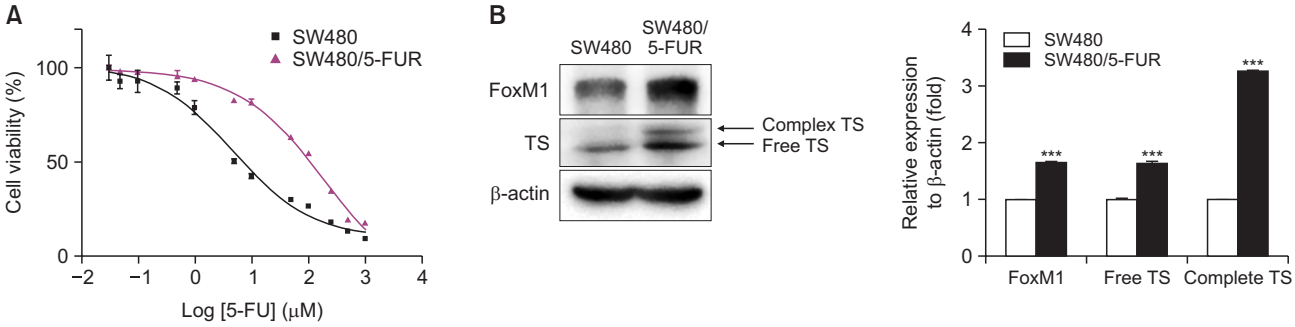

Fig. 5. Establishment of 5-FU-resistant SW480/5-FUR human colon cancer cells. (A) SW480 and SW480/5-FUR cells were cultured in the presence of 5-FU (0-1000 μM) for 72 h and cell viability was determined by MTT assay. (B) Protein levels of FoxM1 and TS were analyzed in 5-FU-sensitive SW480 and 5-FU-resistant SW480/5-FUR cells. The data from three independent experiments are presented as the mean ± SD. ***p<0.001, as compared to those in SW480 cells.

ing 5-FU cytotoxicity and antitumor efficacy in SW480/5-FUR cells.

Urushiol V enhances cytotoxicity of 5-FU in SW480/5-FUR cells by inhibiting FoxM1

To elucidate whether urushiol V could affect levels of FoxM1 and TS in SW480/5-FUR cells, we treated SW480/5-FUR cells with urushiol V at indicated concentrations for 24 h and then performed western blot assays. Results showed that urushiol V dose-dependently inhibited FoxM1 protein expression in SW480/5-FUR cells. The protein levels of free TS and complex TS were also reduced by urushiol V treatment (Fig. 6A). To further examine the effect of urushiol V in the presence of 5-FU, SW480/5-FUR cells were treated with 30 μM urushiol V and 1 μM 5-FU for 24 h. The protein levels of FoxM1, free TS, and complex TS were increased by treatment of 1

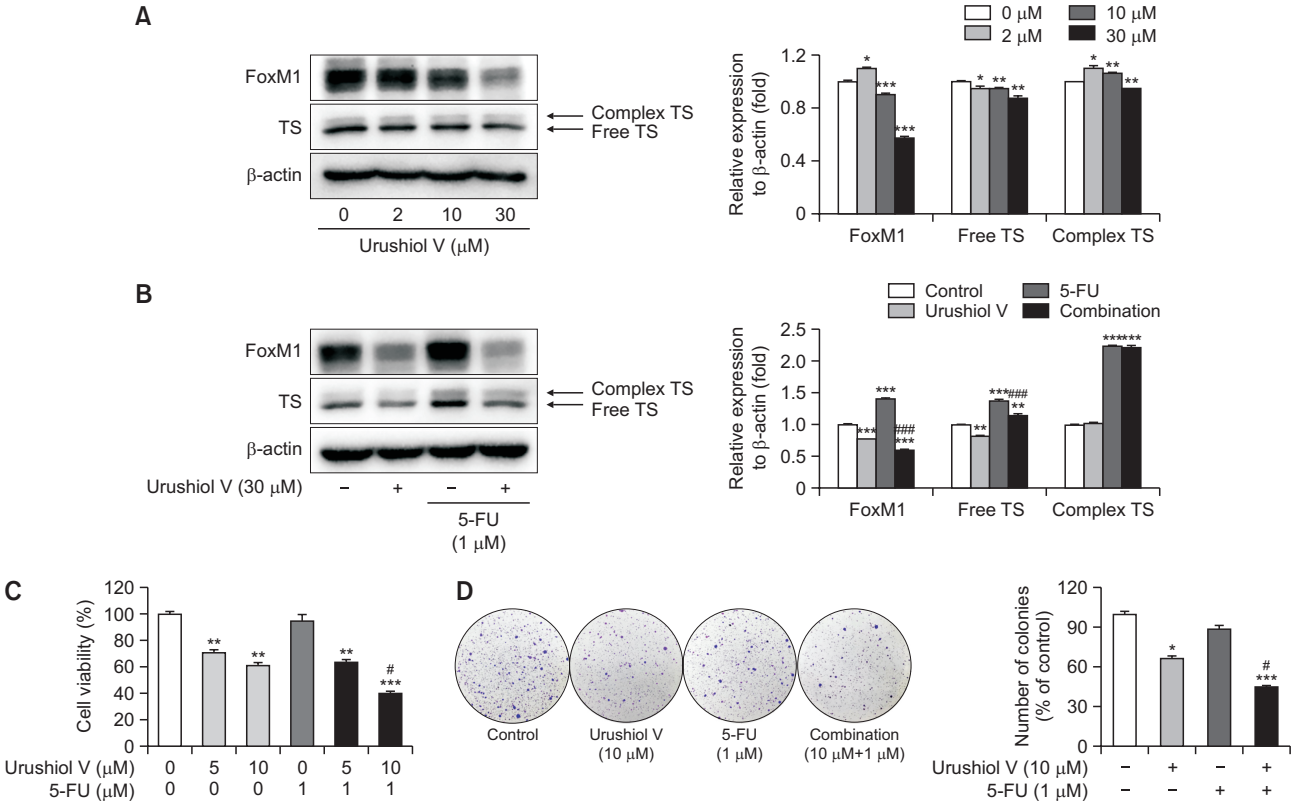

Fig. 6. Combinatorial effect of urushiol V and 5-FU on SW480/5-FUR cells. (A) SW480/5-FUR cells were treated with indicated concentrations of urushiol V for 24 h, and protein levels of FoxM1 and TS were detected by Western blotting. Data are shown as the mean ± SD of three independent experiments. **p*<0.05, ***p*<0.01, ****p*<0.001 as compared to vehicle control. (B) SW480/5-FUR cells were pre-treated with 5-FU (1 μM) for 1 h and then treated with urushiol V for 24 h. Expression levels of FoxM1 and TS were determined by Western blot. Data are shown as the mean ± SD of three independent experiments. ***p*<0.01, ****p*<0.001 as compared to vehicle control; $^{\#\#\#}p$<0.001, as compared to treatment with 1 μM 5-FU. (C) SW480/5-FUR cells were treated with 1 μM 5-FU with or without 5 or 10 μM urushiol V for 72 h. The cell viability was measured by MTT assay. Data are shown as the mean ± SD of three independent experiments. ***p*<0.01, ****p*<0.001 as compared to vehicle control; $^{\#}p$<0.05 as compared to treatment with 10 μM urushiol V. (D) SW480/5-FUR cells were treated with 10 μM urushiol V and 1 μM 5-FU alone or in combination for 72 h, followed by media replacement and culture for 12 days. Wells were stained with crystal violet at the end of the experiment and colonies were counted. The data from three independent experiments are presented as the mean ± SD. **p*<0.05, ****p*<0.001 as compared to vehicle control; $^{\#}p$<0.05 as compared to treatment with 10 μM urushiol V.

μM 5-FU compared with the control group. However, levels of FoxM1 and free TS (but not complex TS) were significantly reduced by combination treatment of 5-FU with urushiol V (30 μM) compared to those by single 5-FU treatment (Fig. 6B). To evaluate whether urushiol V enhanced the cytotoxicity of 5-FU, SW480/5-FUR cells were treated with indicated concentrations of urushiol V and 1 μM 5-FU for 72 h. Treatment with 1 μM 5-FU did not show any significant effect on proliferation of SW480/5-FUR cells. However, co-treatment of 1 μM 5-FU and 10 μM urushiol V significantly reduced proliferation (Fig. 6C) and clonogenic growth (Fig. 6D) of SW480/5-FUR cells as compared with 10 μM urushiol V only. These findings suggest that urushiol V can restore the cytotoxicity of 5-FU in SW480/5-FUR cells by reducing the expression of FoxM1.

DISCUSSION

Colorectal cancer (CRC) is the third-most common cancer in the world (Sung *et al*., 2021). Overexpression of FoxM1 has been observed in CRC (Zhang *et al*., 2016). FoxM1 is a transcription factor that is essential for cell proliferation and cell cycle progression. Numerous studies have documented that down-regulation of FoxM1 can lead to inhibition of cell growth in several cancer types (Yang *et al*., 2013; Wang *et al*., 2019; Xia *et al*., 2019). Here, we investigated the effect of urushiol V on FoxM1 expression in SW480 colon cancer cells. Urushiol can cause allergic skin rash, inflammation, and irritation on contact, known as urushiol-induced contact dermatitis (Wakabayashi *et al*., 2005; Ma *et al*., 2012). Despite an allergic reaction to urushiol, *Rhus verniciflua* Stokes has been traditionally used for the treatment of abdominal masses in Korea (Yoo and Roh, 1977). In addition, numerous studies have reported that urushiol exhibits various beneficial activities such as antioxidant (Kim *et al*., 1997), anti-microbial (Suk *et al*., 2011), and anti-cancer activities (Choi *et al*., 2001; Kim *et al*., 2013). We isolated four urushiols (urushiol I, urushiol II, urushiol III, and urushiol V) from cortex of *Rhus verniciflua* Stokes. Among them, urushiol V showed the most potent cytotoxic effect in SW480 colon cancer cells (data not shown). Urushiol V significantly attenuated cell proliferation and induced S phase arrest in SW480 colon cancer cells by downregulating cyclin E1

and TS and upregulating p21 (Fig. 1, 2). Urushiol V also suppressed the expression of FoxM1 and its target genes such as c-Myc, cyclin D1, cyclin B1, and survivin. Urushiol V inhibited FoxM1 protein level without affecting mRNA level or protein degradation (Fig. 3, 4).

AMPK plays an essential role in cellular energy homeostasis. It controls processes related to tumor development, including cell cycle regulation, cell proliferation, protein synthesis, and survival (Motoshima *et al*., 2006; Mihaylova and Shaw, 2011). Previous research has shown that AMPK activation can attenuate cervical cancer cell growth by suppressing FoxM1 (Yung *et al*., 2013). As shown in Fig. 4C, urushiol V increased phosphorylation of AMPK in SW480 colon cancer cells.

mTOR is one of downstream targets of AMPK. It regulates cell growth, cell survival, metabolism, protein synthesis, and transcription (Tian *et al*., 2019). Activated mTOR can increase 4E-BP1 phosphorylation to release eIF4E, thus initiating cap dependent translation (Gingras *et al*., 2001). Overexpression of eIF4E can enhance the translation of FoxM1 in breast cancer cells (Gong *et al*., 2020). In microarray analysis, FoxM1 is downregulated after rapamycin (mTOR inhibitor) treatment in LG-UC cells (Lee *et al*., 2017). Urushiol V inhibited the phosphorylation of mTOR that might be responsible for blocking FoxM1 protein synthesis (Fig. 4C). Thus, AMPK/mTOR signaling is potentially involved in the action mechanism of urushiol V to inhibit FoxM1 expression. However, further studies are needed to evaluate the effect of urushiol V on mTOR downstream target genes such as p70S6K, 4E-BP1, and eIF4E to disclose the detailed mechanism.

Increased level of FoxM1 is correlated with resistance to 5-FU in many cancers, including CRC. A recent study has shown that FoxM1 can enhance 5-FU resistance through the regulation of TS, a cellular target of 5-FU chemotherapy in CRC (Varghese *et al*., 2019). Siomycin A, a FoxM1 inhibitor, enhances the cytotoxic activity of 5-FU via suppression of both FoxM1 and TS expression in cholangiocarcinoma cancer cell line (Klinhom-On *et al*., 2021). Thus, inhibiting FoxM1 expression in 5-FU-resistant cells might be a potential strategy to sensitize 5-FU activity. In this study, we established 5-FU-resistant colon cancer cells (SW480/5-FUR) in which FoxM1, free TS, and complex TS levels were significantly increased compared to those in SW480 colon cancer cells (Fig. 5B). Urushiol V inhibited FoxM1 expression and consequently decreased levels of free TS and complex TS in SW480/5-FUR cells (Fig. 6A). We also evaluated the combined effect of urushiol V and 5-FU on SW480/5-FUR cells. Levels of FoxM1, free TS, and complex TS were increased by 5-FU treatment. However, levels of FoxM1, and free TS were significantly decreased by combined treatment of 5-FU and urushiol V. Siomycin A increased the level of complex TS protein by binding directly to the ternary complex with TS and 5,10-methylenetetrahydro-folate (5,10-CH_2THF). The binding resulted in the significant reduced free TS levels in cholangiocarcinoma cancer cell line (Klinhom-On *et al*., 2021). However, urushiol V inhibited free TS protein without altering complex TS protein levels in SW480/5-FUR cells. Urushiol V and siomycin A might have different mechanism for reducing free TS and that can be disclosed by further study. Urushiol V also enhanced the growth inhibitory effect of 5-FU in SW480/5-FUR (Fig. 6C, 6D).

Collectively, our findings showed that urushiol V could inhibit the proliferation of SW480 colon cancer cells by downregulating FoxM1. Urushiol V also enhanced anti-proliferative activity of 5-FU by suppressing the levels of FoxM1 and TS in 5-FU resistant colon cancer cells (SW480/5-FUR) (Fig. 7). These results demonstrate that urushiol V has potential therapeutic and/or adjuvant applications for CRC chemotherapy.

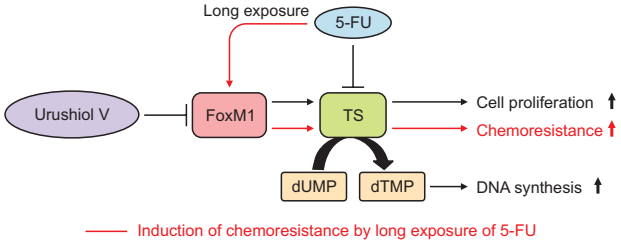

Fig. 7. Schematic diagram of the proposed mechanism of anti-proliferative activity of urushiol V. Urushiol V decreased FoxM1 expression and consequently inhibited cell proliferation of SW480 colon cancer cells. 5-FU exerts an antitumor activity by blocking the activity of thymidylate synthase (TS), an enzyme essential for DNA replication. However, prolonged use of 5-FU leads to drug resistance through increasing expressions of FoxM1 and TS. Urushiol V suppressed expressions of FoxM1 and TS, and enhanced anti-proliferative activity of 5-FU against resistant colon cancer cells (SW480/5-FUR).

CONFLICT OF INTEREST

There are no conflicts of interest.

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