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ORIGINAL ARTICLE

Apoptotic effect of *Polygonum Cuspidatum* in oral cancer cells through the regulation of specificity protein 1

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OBJECTIVES: The aim of this study was to evaluate the growth inhibitory and apoptosis-inducing effects and mechanisms of *Polygonum cuspidatum* root in oral cancer cells.

MATERIALS AND METHODS: The testing materials were separated by normal-phase silica gel liquid chromatography. The effect of *P. cuspidatum* root on apoptotsis and its mechanism were performed using 3-(4, 5-dimethylthiazol-20yl)-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium) (MTS) assay, western blot analysis, RT-PCR, promoter assay, and (4′-6-Diamidino-2-phenylindole) (DAPI) staining.

RESULTS: The methanol extract of *P. cuspidatum* (MEPC) inhibited the proliferation of oral cancer cells by inducing caspase-dependent apoptosis. Protein and mRNA expression levels and the transactivation of Specificity protein 1 (Sp1) were markedly decreased in KB cells treated with MEPC. Ethyl acetate fraction (EA) from MEPC was more potent than aqueous fraction (AQ) from MEPC to induce apoptosis. F2, F3, and F4 from EA differentially inhibited the growth of KB cells, and it depends on the amount of Emodin in F2, F3, and F4. Moreover, Emodin inhibited oral cancer cell growth and induced caspase-dependent apoptosis by decreasing Sp1. MEPC also decreased an apoptosis-related downstream target of Sp1 protein, survivin.

CONCLUSION: The results from this study strongly suggest that MEPC, its fraction, and Emodin may be potential bioactive materials to cause apoptosis mechanism via the down-regulation of Sp1 in oral cancer cells.
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Keywords: oral cancer cells; apoptosis; specificity protein 1; *Polygonum cuspidatum;* emodin; survivin

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Introduction

Oral cancer is the sixth most common cancer in the world and a serious health problem in some parts of the world (Warnakulasuriya, 2009). In spite of rapid development of standard treatment strategies for oral cancer, the 5-year survival rate of patients remains relatively low. As most patients are diagnosed after the disease has reached the advanced stages of oral cancer, the prevention and therapy of oral cancer before developing invasive tumors would be a good solution for inhibiting oral cancer progression. Unfortunately, current strategies have a limitation for the documented toxicity (Papadimitrakopoulou *et al*, 1997; Kim *et al*, 2002).

Specificity protein (Sp) is a transcription factor that is ubiquitously expressed in mammalian cells, and it is critical in a variety of physiological processes, including cell cycle regulation, differentiation and apoptosis (Firestone and Bjeldanes, 2003; Chu and Ferro, 2005; Wong *et al*, 2005). In addition, Sp1 expression may be negatively associated with survival in some cancer patients (Zannetti *et al*, 2000; Wang *et al*, 2003; Hosoi *et al*, 2004; Yao *et al*, 2004). Thus, down-regulation of Sp1 protein expression could be a good strategy for cancer therapy.

The use of naturally occurring products has been a major focus for a long period due to their potential chemotherapeutic activity. *Polygonum cuspidatum* is a perennial species with spreading rhizomes and a number of reddish-brown stems that has been traditionally used in East Asia and used for the treatment of menoxenia, skin burn, gallstone, hepatitis, osteomyelitis and inflammation (Zhou *et al*, 2003; Park *et al*, 2004). It is also the important medicinal herb widely used for the treatment of various inflammatory diseases, hepatitis, diarrhea, and tumors in China, Korea, Taiwan, and Japan (Choi *et al*, 2002). Even though it has been often used to control dental disease in Korea, there are no reports of anti-tumor activity of *P. cuspidatum* and its mechanism in oral cancer.

In this study, the goal is to examine the effect of methanol extracts and its fractions of *P. cuspidatum* on oral cancer cell growth and elucidate the signaling

pathway in mediating *P. cuspidatum*-induced growth inhibition in human oral cancer cells. Our results report that MEPC, ethyl acetate fraction (EA), F2, and its active substance, Emodin clearly induce apoptotic cell death to inhibit the proliferation of oral cancer cells and the down-regulation of Sp1 and its downstream target, survivin mediate apoptotic cell death.

Materials and Methods

Polygonum cuspidatum root was purchased from an herbal drug market (Jeonju, Korea). MEPC was prepared as detailed elsewhere (Song *et al*, 2006). The MEPC was fractionated according to a polarity gradient, using protocols as described by Song *et al* (2007) (Song *et al*, 2007). A part of MEPC obtained was suspended in 70% aqueous methanol and fractioned serially with *n*-hexane and ethyl acetate to gain *n*-hexane fraction, ethyl acetate fraction (EA), and aqueous fraction (AQ). EA was further fractionated by column chromatography over silica gel (Kieselgel 60, 70–230 mesh; Merck, Whitehouse Station, NJ) using *n*-hexane: dichloromethane:ethyl acetate [(1:1:1), (1:1:2)]; *n*-hexane:ethyl acetate:methanol [(1:1:1), (1:1:2)]; *n*-hexane:methanol (1:4) and methanol, successively, as mobile phase. The sub-fractions were collected and monitored by thin layer chromatography (TLC, silica gel 60, F254, Merck, 0.2 mm layer thickness). The sub-fractions were classified into six major substances (F1–F6) according to their TLC profiles. All of the samples were stored at −20°C until tested. They were then dissolved in dimethyl sulfoxide (DMSO; 99.9%) and methanol immediately before each experiments and HPLC analysis, respectively.

Reversed-phase high performance liquid chromatography analysis

The analysis of the samples was performed using Reversed-phase HPLC with a chromatograph equipped with KYA TECH HPLC column HIQ sil C18HS (4.3 μm, 4.6 × 150 mm, KYA TECH corporation, Tokyo, Japan), C18, 4.6 × 12.5 mm guard column (Agilent tech, Palo Alto, CA, USA), and UV/VIS detector. A portion of each sample and standard reference compounds were redissolved in HPLC grade methanol (100–200 μg ml^{-1}), and then injected (20 μl, respectively). The gradient mobile phase consisted of 0.4% formic acid in deionized water (solvent A) and acetonitrile (solvent B). The gradient programme was as follows: 15–20% B over 0–20 min, 20–40% B over 20–40 min, 40–100% B over 40–60 min followed by 100% B for 60–65 min (Qian *et al*, 2006). The flow rate was 1 ml min^{-1}, and the column temperature was maintained at 30°C. To determine the appropriate wavelength for HPLC analysis, the full UV/VIS spectra (200–800 nm) were obtained using a spectrophotometer (Jasco V-630, Tokyo, Japan). Based on the UV/VIS spectra (data not shown), the UV/VIS detector for the chromatograms was set to 290 and 306 nm. The standard reference compounds, emodin, resveratrol, physcion, and polydatin, were purchased from Sigma-Aldrich (St. louis, MO, USA), Chroma Dex (Santa Ana, CA, USA), or ALEXIS Corporation (Lausen, Switzerland).

Reagents

Dulbecco's modified essential medium (DMEM), fetal bovine serum (FBS), 100× antibiotic solution, Trypsin, and D-PBS was obtained from WelGENE Inc. (Dae-gu, Korea). The Poly (ADP-ribose) polymerase (PARP) antibody was purchased from BD Pharmingen^{TM} (San Jose, CA, USA). Antibodies for Sp1 and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody for cleaved caspase3 was supplied by CALBIOCHEM (Gibbstown, NJ, USA). CellTiter 96® Aqueous One Solution Cell Proliferation assay kit for 3-(4,5-dimethylthiazol-20yl)-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium) (MTS) assay was purchased from Promega (Madison, WI, USA). The easy-BLUE^{TM} Total RNA Extraction kit was supplied by iNtRON Biotechnology, Inc. (Seoul, Korea).

Cell Culture and Drug Treatment

KB cells were obtained from the American Tissue Culture Collection. (Manassas, VA, USA). HEp-2 cells were given by School of Dentistry, Kyungpook National University (Daegu, Korea), and YD-15 cells were kindly given by School of Dentistry, Younsei University (Seoul, Korea). Three different oral cancer cells were maintained in DMEM (KB and Hep-2) or RPMI1640 (YD-15) supplemented with 5% FBS and 100 U ml^{-1} each of penicillin and streptomycin (WelGENE Inc., Dae-Ku, Korea) in a humid atmosphere of 5% CO_2. KB cells at passage 15–30, HEp-2 cells at passage 22–27, YD-15 cells at passage 20–30 were used for all the experiments. Cells were trypsinized and suspended in medium, and cell numbers were determined with a HEMACYTOMETER (Thermo Fisher Scientific Inc., Waltham, MA, USA). Equal number of cells were seeded and allowed to attach overnight. The cells were treated with vehicle (DMSO) or MEPC (15,30,40,60,80 μg ml^{-1}), EA (7.5,15,30 μg ml^{-1}), AQ (7.5,15,30 μg ml^{-1}), F2 (1,2,3 μg ml^{-1}), F3 (25,50,100 μg ml^{-1}), F4 (25,50,100 μg ml^{-1}), and Emodin (0.5,1,2,4,6 μM) diluted in DMEM supplemented with 2.5% FBS for 6 h, 12 h, 24 h, 48 h, and 72 h depending on experimental designs.

MTS assay

KB, HEp-2, and YD-15 cells (8×10^4 cells ml^{-1}) were seeded in 96-well plates and then incubated for 24 h with different doses of MEPC, EA, AQ, F2, F3, F4, and Emodin for 24 h, 48 h, and 72 h. Their effect on cell proliferation was estimated using the CellTiter 96® (Promega) Aqueous One Solution Cell Proliferation assay kit according to the manufacturer's instructions. The assay solution was added to each well, and the plates were incubated at 37°C in humidified 5% CO_2 atmosphere for 2 h. The absorbance at 490 nm was recorded using ELISA plate reader.

Western blot analysis

Whole-cell lysates were extracted with lysis buffer and quantified with *DC* Protein Assay (Bio-RAD, Hercules.

CA, USA). Equal amount of protein from each treatment group was separated by electrophoresis using a sodium dodecyl sulfate-polyacrylamide gel, and the separated proteins were transferred to Immun-Blot™ PVDF membrane (Bio-RAD). The membrane was blocked with 5% skim milk in TBS-T for 2 h at RT and maintained overnight at 4°C with the primary antibody, followed by maintaining it with horseradish peroxidase-conjugated secondary antibodies. The antibody-bound proteins were detected using an ECL Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Inc.).

Detection of nuclear morphological changes

Cell death was measured by DAPI staining (Sigma Chemical Co, MO, USA). After an experimental treatment with 15, 30, and 60 μg ml^{-1} of MEPC for 72 h, KB cells were harvested by trypsinization and fixed in 4% paraformaldehyde at RT for 20 min. The cells were resuspended in PBS, deposited them on poly-L-lysin-coated slides, stained with a DAPI solution (2 μg ml^{-1}), and viewed under a fluorescence microscope.

Reverse transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNAs were extracted from the cells using the easy-BLUE™ Total RNA Extraction Kit (iNtRON), and one microgram of RNA was used to synthesize cDNA using ImProm-II™ Reverse Transcription System (Promega). The PCR conditions of GAPDH and Sp1 were as follows: (30 cycles: 1 min at 94°C, 1 min at 57°C, and 1 min 30 s at 72°C), and the PCR condition of Survivin was as follows: (30 cycles: 1 min at 94°C, 1 min at 60°C, and 1 min 30 s at 72°C). The primer sequences used for GAPDH: 5′-CGG AGT CAA CGG ATT TGG TCG TAT-3′ (S), 5′-AGC CTT CTC CAT GGT GGT GAA GAC-3′ (AS), Sp1 : 5′-ATG GGG GCA ATG GTA ATG GTG G-3′ (S), 5′-TCA GAA CTT GCT GGT TCT GTA AG-3′ (AS), Survivin : 5′- ATG GCC GAG GCT GGC TTC ATC-3′ (S), 5′-ACG GCG CAC TTT CTT CGC AGT T-3′ (AS). PCR products were analyzed by 1% agarose gel electrophoresis.

Transfection and Luciferase assay

KB cells (7×10^4 cells ml^{-1}) were seeded in 48-well plates and allowed to attach overnight. Transient transfection was performed using jetPEI™ (POLYPLUS-TRANSFECTION Inc., New York, NY, USA), and assays for the activity of firefly luciferase and galactosidase were conducted according to the manufacturer's manual (Promega). Briefly, human Sp1 and Survivin luciferase reporter plasmid transfected into cell and cultured for 24 h. Cells were treated with MEPC for 48 h. Cells were lysed with 200 μl of 1× passive lysis

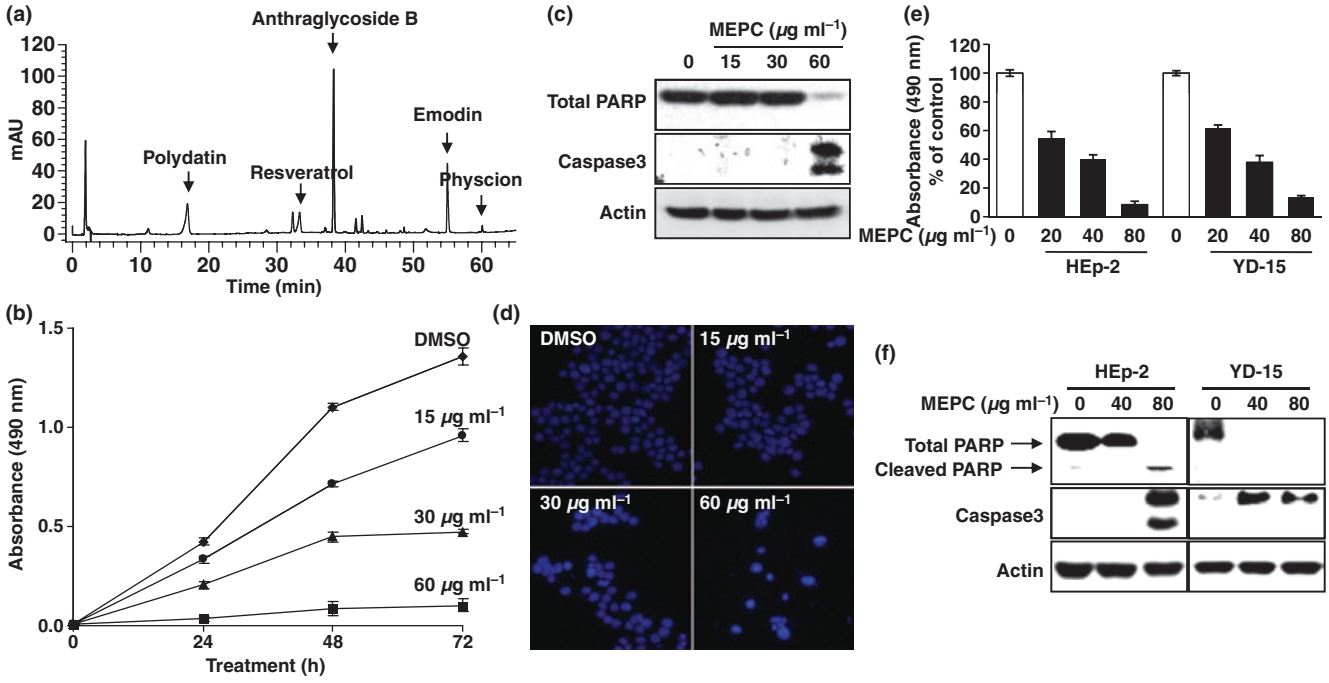

Figure 1 (**a**) Representative HPLC chromatograms of the methanol extract from *Polygonum cuspidatum* root (MEPC) at 290 nm. (**b**) Effect of MEPC on cell proliferation in KB human oral cancer cells. KB cells were treated with DMSO or various dose of MEPC for 24, 48, and 72 h. Cell proliferation was estimated using a MTS assay. The results are reported as the mean ± s.d. of three independent experiments. (**c**) Apoptotic effect of MEPC in KB cells. Western blot analysis for PARP and caspase 3 using whole lysates from DMSO and MEPC-treated KB cells. Actin was probed to determine the evenness of the loading protein extract from each treatment. The results are representative for two independent experiments. (**d**) Induction of apoptosis in KB cells treated with DMSO or MEPC for 72 h was determined by DAPI staining. White arrows show nuclear condensation and fragmentation. (**e**) Effect of MEPC on cell proliferation in HEp-2 and YD-15 human oral cancer cells. HEp-2 and YD-15 cells were treated with DMSO or various dose of MEPC for 72 h. Cell proliferation was estimated using a MTS assay. The results are reported as the mean ± s.d. of three independent experiments. (**f**) Apoptotic effect of MEPC in HEp-2 and YD-15 cells. Western blot analysis for PARP and caspase 3 using whole lysates from DMSO and MEPC-treated oral cancer cells

buffer, 50 μl of cell extract was subjected to gal assay, and 100 μl of cell extract was subjected to firefly luciferase assay. Firefly luciferase was measured with Perkin Elmer Microbeta Trilux 1450 LSC (Turku, Finland) Luminescence Counter, and luciferase activities were normalized to β-gal activities.

Statistical Analysis
Statistical significance was assessed using a Student's t-test. A value of $P < 0.05$ compared with the solvent control was considered statistically significant.

Results

The inhibitory effect of methanol extract of Polygonum cuspidatum on the proliferation of KB cells
MEPC was tested *in vitro* for its potential human oral cancer cell growth inhibitory effect on KB cell lines using MTS assay, which was widely used to quantify cell viability. The results, summarized in Figure 1b, showed that MEPC exhibited an inhibitory effect on KB cells in a dose-dependent manner. At higher dose (60 μg ml^{-1}) for 72 h, the cell viability was 7.2%.

MEPC causes apoptotic cell death of oral cancer cells
To understand why MEPC inhibits KB cell proliferation, the level of apoptosis in the MEPC-treated KB cells was determined using western blot analysis with antibodies for PARP and caspase 3 (Figure 1c) and DAPI staining (Figure 1d). Western blot analysis also showed that the decrease of total PARP and the activation of caspase 3 were observed in KB cells treated with 60 μg ml^{-1} MEPC. Nuclear staining with DAPI revealed that nuclear condensation and fragmentation were observed in KB cells exposed to 60 μg ml^{-1} MEPC for 72 h. The results suggest that MEPC induced apoptotic cell death to inhibit the growth of KB cells. We also examined the role of cell context in the induction of apoptosis by MEPC. The treatment of HEp-2 and YD-15 oral cancer cell lines with 40 and 80 μg ml^{-1} for 72 h inhibited cell proliferation and induced apoptosis (Figure 1e, f).

MEPC down-regulates Specificity protein 1 (Sp1) to induced apoptosis in oral cancer cells.
As Sp1 protein plays an important role in the growth of cancer (Black *et al*, 2001), experiments were carried out to determine whether MEPC treatment affects its expression level. The results in Figure 2a, b show the down-regulation of Sp1 protein and mRNA by 60 μg ml^{-1} of MEPC. In all, 60 μg ml^{-1} of MEPC also inhibited the transactivation of Sp1 promoter in KB cells (Figure 2c). The time-course effects of MEPC on Sp1 protein and PARP cleavage were investigated. The results showed that the time-course inhibition of Sp1 protein and cleavage of PARP by MEPC were observed after treatment for 24 h, and this response remains down-regulated for up to 72 h (Figure 2d). We also found that MEPC decreased the expression levels of Sp1 protein in HEp-2 and YD-15 cells (Figure 2e).

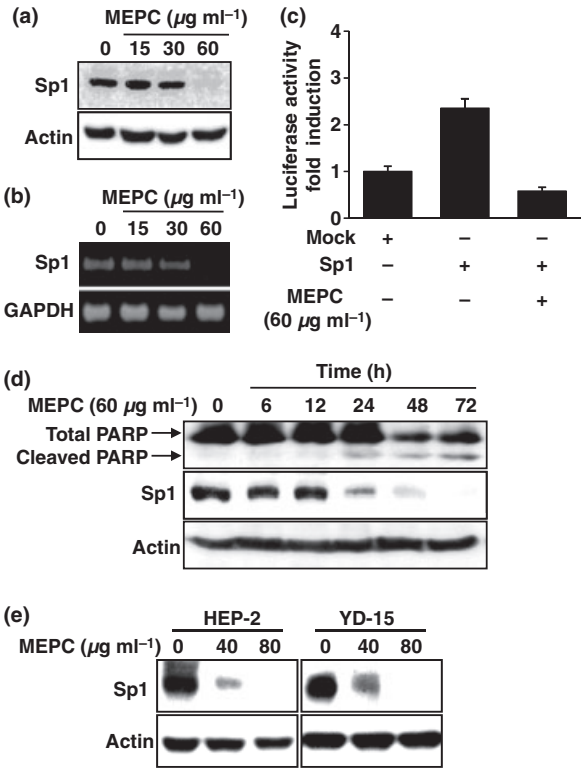

Figure 2 Specificity protein 1 (Sp1) protein (**a**) and mRNA (**b**) expression. KB cells were treated with DMSO and various dose of MEPC for 72 h. Protein or mRNA was extracted to analyze Sp1 protein and mRNA expression by RT-PCR or Western blot analysis, respectively. mRNA and protein levels were normalized to GAPDH and actin. (**c**) Transactivation of Sp1 promoter inhibited by MEPC in KB cells. (**d**) Time-dependent induction of Sp1 protein and apoptosis in KB cells. KB cells were treated with 60 μg ml^{-1} of MEPC for 6, 12, 24, 48, and 72 h, and whole-cell lysates were analyzed by Western blot analysis. (**e**), Sp1 protein expression in MEPC-treated HEp-2 and YD-15 oral cancer cells for 72 h

Ethyl acetate fraction of MEPC (EA) is more efficient than aqueous fraction of MEPC (AQ) to inhibit tumor cell progression in KB cells
Next, we extracted EA and AQ from MEPC to find the active substances, and KB cells were treated with DMSO, 7.5, 15 and 30 μg ml^{-1} of EA or AQ for 72 h. The results showed that EA exhibited the dose-dependent inhibition of KB cell growth, but AQ did not (Figure 3b). To confirm these results, the cleavage of PARP and caspase 3 activation induced by both fractions were evaluated. The results showed that EA induced a great deal of cleaved PARP and activated caspase 3, but AQ did not (Figure 3c). In addition, EA clearly inhibited Sp1 protein.

F2, F3, and F4 extracted from EA differentially inhibits the proliferation of KB cells
F2, F3, and F4 were separated from EA by normal phase column chromatography, and Figure 4a represented their HPLC chromatograms. For the inhibitory activities of F2, F3, and F4 on the growth of KB cells, the cells were exposed to F2 (1, 2, 3 μg ml^{-1}), F3 (25, 50, 100 μg ml^{-1}), and F4 (25, 50, 100 μg ml^{-1}) for 24, 48,

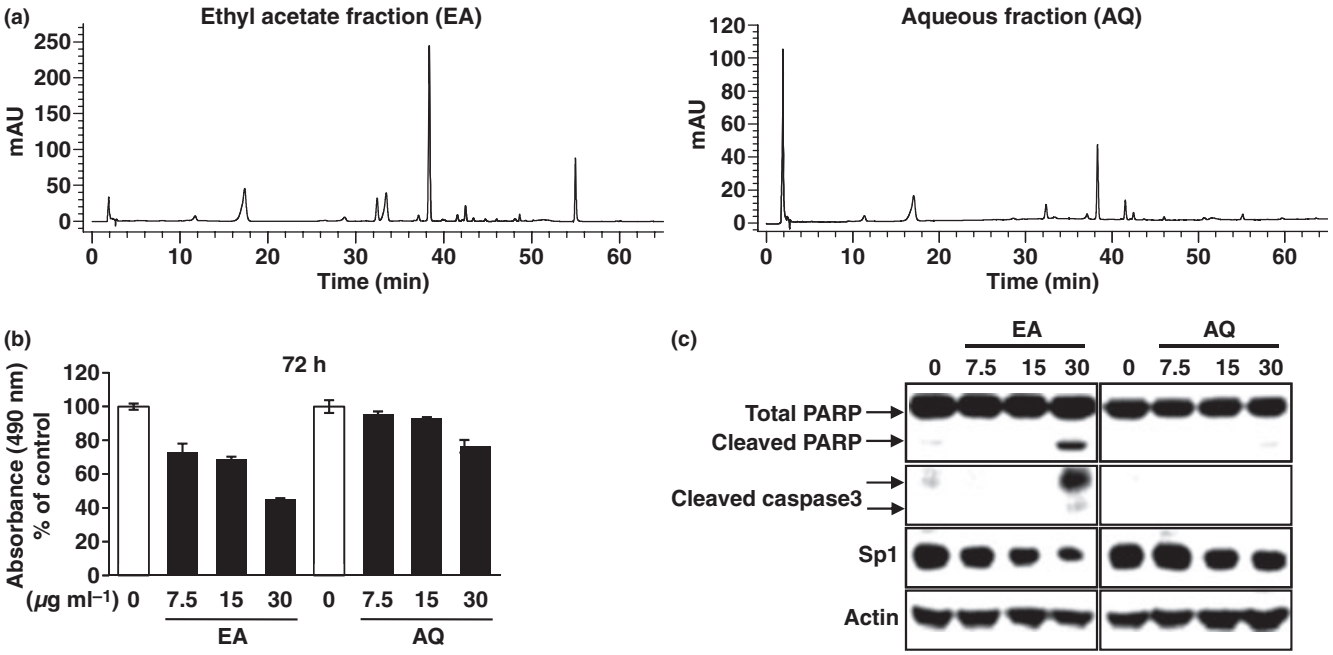

Figure 3 (**a**) Representative HPLC chromatograms of the ethylacetate fraction (EA) and aqueous fraction (AQ) of ME from *P. cuspidatum* root at 290 nm. (**b**) Comparison on the effects of EA and AQ on cell proliferation in KB cells. KB cells were treated with DMSO or various dose of EA and AQ for 24, 48, and 72 h. Cell proliferation was estimated using a MTS assay. The results are reported as the mean ± s.d. of three independent experiments. (**c**) Comparison on the effects of EA and AQ on Sp1 protein and apoptosis in KB cells using Western blot analysis

and 72 h. The results showed that F2 had stronger anti-proliferative effect on KB cells than F3 and F4 did (Figure 4b), even though lower dose was used for F2. Western blot analysis also showed that F2 induced stronger apoptotic cell death than F3 and F4 by the down-regulation of Sp1. To compare F2 with F3 and F4 at the same experimental condition, KB cells were treated with same dose (3 μg ml^{-1}) of F2, F3, and F4 for 72 h. The results showed that F2 induced the cleavage of PARP and inhibited Sp1 protein, whereas F3 and F4 did not affect them (Figure 4d).

Emodin has the inhibitory effect on the growth of oral cancer cells.

As F2 contains high amount of Emodin compared with F3 and F4, it was assumed that the inhibitory effect of F2 on cell proliferation may be due to Emodin. To test this hypothesis, KB, HEp-2, and YD-15 cells were treated with various concentrations of Emodin for 72 h. The results showed that Emodin inhibited the growth of oral cancer cells in a concentration-dependent manner, and it induced caspase-dependent apoptosis and down-regulated Sp1 protein (Figure 5a, b).

Anti-apoptotic molecule, survivin is regulated by MEPC in KB cells.

To connect the inhibition of Sp1 protein to MEPC-induced apoptosis, a mediating factor between the two processes must be identified. Several studies have reported that the Sp1 protein regulates survivin protein as typical anti-apoptotic protein (Chintharlapalli *et al*, 2007; Chadalapaka *et al*, 2008). MEPC inhibited the expression levels of survivin protein and mRNA and decreased its promoter activity in a dose-dependent manner (Figure 6a, b and c). We also found that MEPC decreased survivin in a time-dependent manner correlated with down-regulation of Sp1 protein and cleavage of PARP shown in Figure 2d (Figure 6d). We also found that MEPC decreased the expression levels of survivin protein in HEp-2 and YD-15 cells (Figure 6e).

Discussion

Our previous study demonstrated that *P. Cuspidatum* has anti-acidogenic activity of *Streptococcus mutants* (*S. mutants*) to control dental caries formation (Song *et al*, 2006, 2007). In case of cancer therapy with *P. Cuspidatum*, the methanolic extract from the roots of *P. Cuspidatum* was found to enhance the proliferation of MCF-7, an estrogen-sensitive breast cancer cell line, because it has the estrogenic activity (Matsuda *et al*, 2001). On the other hand, it inhibited the proliferation of many cancer cells but not human normal liver cell (Feng *et al*, 2006). However, anti-cancer activity of *P. Cuspidatum* has not been well established in cancer cells, especially oral cancer.

In this study, we focused on three primary objectives in relation to the anti-cancer effect of *P. Cuspidatum* in oral cancer. The first was to examine the effect of *P. Cuspidatum* on the growth and apoptosis of human oral cancer cells. The second was to determine what kind of key molecular factor is involved in *P. Cuspidatum*-induced apoptosis. The last objective is to identify what is the critical bioactive component in *P. Cuspidatum* for its apoptotic activity. For this study, *P. Cuspidatum* was extracted with methanol (MEPC). MEPC decreased the

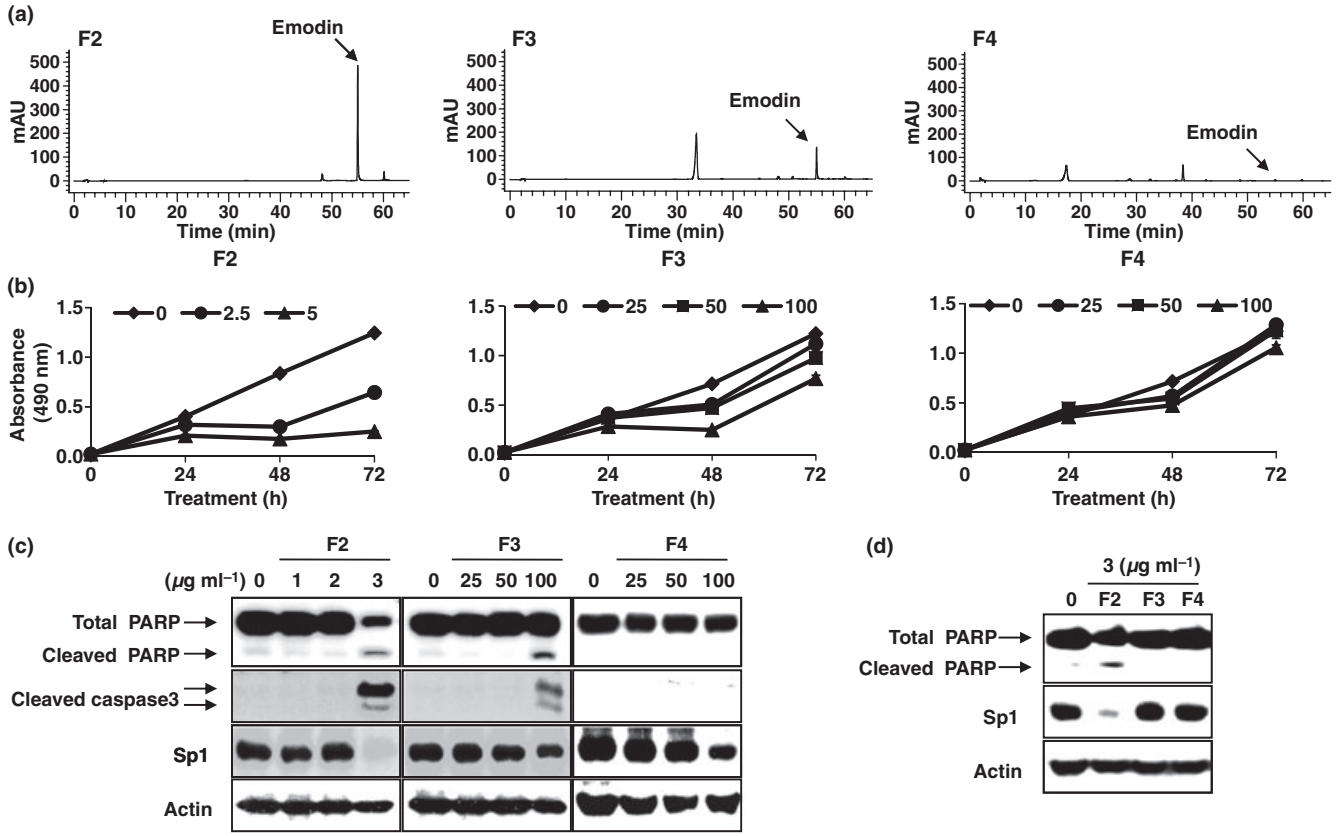

Figure 4 (**a**) Representative HPLC chromatograms of F2, F3, and F4 fraction from EA at 290 nm. (**b**) The effects of F2, F3, and F4 fraction on cell proliferation in KB cells. KB cells were treated with DMSO or various dose of F2, F3, and F4 fraction for 24, 48, and 72 h. (**c**) The effects of F2, F3, and F4 fraction on Sp1 and apoptosis in KB cells using Western blot analysis. (**d**) Comparison on the effects of 3 μg ml^{-1} of F2, F3, and F4 fraction on cell proliferation and apoptosis

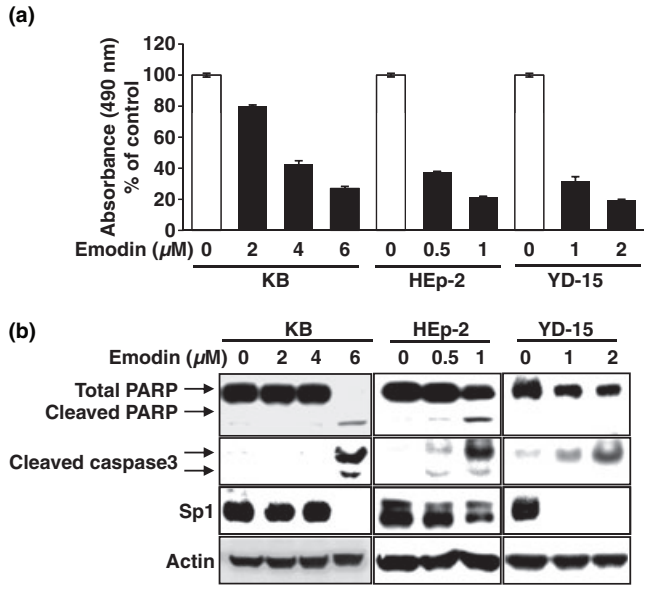

Figure 5 (**a**) The effects of emodin on cell proliferation in KB, HEp-2, and YD-15 cells. Oral cancer cells were treated with DMSO or various concentration of emodin for 72 h. (**b**) The effects of emodin on caspase-dependent apoptosis and Sp1 protein in oral cancer cells using Western blot analysis

number of viable cells in a dose-dependent manner and induced dramatic apoptosis as evidenced by PARP cleavage, the activation of caspase 3, and nuclear condensation and fragmentation (Figure 1). These results show that MEPC has the inhibitory effect on cell proliferation in KB oral cancer cells. We also found that MEPC inhibited the proliferation of HEp-2 and YD-15 oral cancer cells. These indicate that MEPC is highly effective in multiple oral cancer cell lines. Sp 1 protein is overexpressed in a variety of human tumors and cancer cell lines (Zannetti *et al*, 2000; Chiefari *et al*, 2002; Wang *et al*, 2003; Hosoi *et al*, 2004; Yao *et al*, 2004). It was also reported that transformation of fibroblasts resulted in higher expression of Sp1 protein than normal fibroblast, and these transformed cells made malignant tumors in xenograft animal model, whereas normal fibroblasts did not (Lou *et al*, 2005). Therefore, Sp1 is a potential target for the development of drugs for cancer therapy. For these reasons, we determined whether MEPC shows a reduced activity in Sp1 expression to have apoptotic activity in oral cancer cells. The western blot analyses and RT-PCR showed the significant inhibition of Sp1 protein and mRNA expression in KB cells (Figure 2a, b). We also found that MEPC reduced the expression level of Sp1 protein

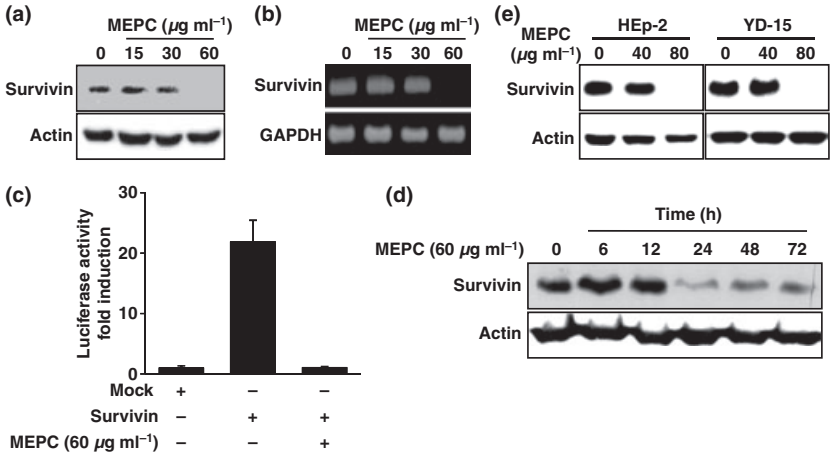

Figure 6 Survivin protein (**a**) and mRNA (**b**) expression. KB cells were treated with DMSO and various dose of MEPC for 72 h. Protein or mRNA was extracted to analyze survivin protein and mRNA expression by RT-PCR or Western blot analysis, respectively. mRNA and protein levels were normalized to GAPDH and actin. (**c**), Transactivation of survivin promoter inhibited by MEPC in KB cells. (**d**) Time-dependent induction of survivin in KB cells. KB cells were treated with 60 μg ml^{-1} of MEPC for 6, 12, 24, 48, and 72 h and whole-cell lysates were analyzed by Western blot analysis. (**e**) Survivin protein expression in MEPC-treated HEp-2 and YD-15 oral cancer cells for 72 h

in HEp-2 and YD-15 cells (Figure 2e). MEPC also decreased Sp1 promoter activity suggesting that it inactivated Sp1 gene expression at the transcriptional level (Figure 2c). We also examined the time-course effects of MEPC on apoptosis of KB cells and Sp1 expression level. MEPC induced apoptotic cell death in KB cells and decreased Sp1 expression (Figure 2d). Apoptosis induced by MEPC was consistent with the parallel down-regulation of Sp1 protein in KB cells. These data indicate that Sp1 may be associated with MEPC-induced apoptosis in oral cancer cells. As MEPC has the growth inhibitory effect on KB oral cancer cells, EA and AQ were separated from MEPC to isolate bioactive components in *P. cuspidatum*. Their inhibitory effects on the growth of KB cells were tested. It was found that EA is more efficient than AQ to inhibit tumor cell progression in KB cells suggesting that bioactive components may be in EA but not AQ (Figure 3b, c). IC_{50} values for MEPC and EA were 28.25 μg ml^{-1} and 25.27 μg ml^{-1}, respectively, and the effect of EA on the growth inhibition in KB cells is much stronger than MEPC. This difference is because all the components identified in EA were more concentrated than MEPC, even though the composition of EA was similar to that of MEPC (unpublished data).

Next, F2, F3, and F4 were separated from EA using normal phase column chromatography, and their anti-proliferative activities were examined. The results showed that they exhibited anti-proliferative activities in KB cells differentially. The anti-cancer effect of F2 was the strongest, F3 was the second, and F4 did not inhibit the growth of KB cells anymore (Figure 4b, c). Our previous unpublished data revealed that the amount of Emodin in F2, F3, and F4 is 476, 146, 12.2 μg mg^{-1}, respectively. These suggest that the differential anti-tumor effects of F2, F3, and F4 may be dependent on the amount of Emodin. Several studies revealed that Emodin was extracted and purified from *P. Cuspidatum*, and these results are totally in agreement with our study (Wang *et al*, 2008; Zhuang *et al*, 2008). It has demonstrated that Emodin possesses variously biological function, such as anti-bacterial, anti-inflammatory, a potent inhibitor of the casein kinase (Chang *et al*, 1996; Wang and Chung, 1997; Yim *et al*, 1999). Recently, Emodin has been reported to exhibit anti-tumor effects in various cancer cells including lung pancreatic cancer (Cai *et al*, 2008; Guo *et al*, 2009; Lai *et al*, 2009; Li *et al*, 2009). Thus, the anti-tumor activity of Emodin in several oral cancer cells was tested. As expected, Emodin has a strong anti-tumor activity in oral cancer cells indicating that the inhibitory effects of *P. Cuspidatum* and its franctions on oral cancer cell proliferation are due to Emodin in *P. Cuspidatum*. Expression of survivin in some cancer cells lines is regulated by Sp protein interactions with GC-rich promoter sites (Finkenzeller *et al*, 1997; Wu *et al*, 2005). Therefore, the effect of MEPC on decreased survivin was further investigated (Figure 6). The results showed that MEPC decreased survivin mRNA, protein, and its transactivation suggesting that MEPC inhibits survivin expression through the regulation of Sp1 to induce apoptosis in KB, HEp-2, and YD-15 cells. Now, the deep mechanistic study how *P. Cuspidatum* and its fractions inhibit Sp1 protein to exhibit anti-apoptotic activity in oral cancer cells is in progress.

In summary, our data showed *P. Cuspidatum* and its fractions mediate their proapoptotic and anti-proliferative effects in oral cancer cells. The Sp1 and survivin play important signaling roles for *P. Cuspidatum*-induced apoptosis. We also demonstrated that Emodin in *P. Cuspidatum* is a bioactive component to mediated *P. Cuspidatum*-induced anti-cancer activity. Therefore, we suggest that *P. Cuspidatum* and its fraction may be as a promising compound for the effective treatment of oral cancer.

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Author contributions

Ji-Ae Shin, Jung-Hyun Shim, Jae-Gyu Jeon, Kyeong-Hee Choi, and Eun-Sun Choi performed experiments and laboratory analyses. Syng-Ook Lee, Gu Kong, and Nam-Pyo Cho performed data analyses. Sung-Dae Cho designed research plan and prepared manuscript. All authors participated in the editing and final preparation of the manuscript.

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