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Expression of Survivin and p53 Modulates Honokiol-Induced Apoptosis in Colorectal Cancer Cells

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ABSTRACT

Honokiol is a small biphenolic compound, which exerts antitumor activities; however, the precise mechanism of honokiol-induced apoptosis in the human colorectal cancer cells remains unclear. Here, we show that survivin and p53 display the opposite role on the regulation of honokiol-induced apoptosis in the human colorectal cancer cells. Honokiol induced the cell death and apoptosis in various colorectal cancer cell lines. Moreover, honokiol elicited the extrinsic death receptor pathway of DR5 and caspase 8 and the intrinsic pathway of caspase 9. The common intrinsic and extrinsic downstream targets of activated caspase 3 and PARP protein cleavage were induced by honokiol. Interestingly, honokiol reduced anti-apoptotic survivin protein and gene expression. Transfection with a green fluorescent protein (GFP)-survivin-expressed vector increased the colorectal cancer cell viability and resisted the honokiol-induced apoptosis. Meantime, honokiol increased total p53 and the phosphorylated p53 proteins at Ser15 and Ser46. The p53-wild type colorectal cancer cells were exhibited greater cytotoxicity, apoptosis and survivin reduction than the p53-null cancer cells after treatment with honokiol. Together, these findings demonstrate that the existence of survivin and p53 can modulate the honokiol-induced apoptosis in the human colorectal cancer cells. J. Cell. Biochem. 115: 1888–1899, 2014.

KEY WORDS: HONOKIOL; SURVIVIN; p53; APOPTOSIS; COLORECTAL CANCER

Honokiol, a small biphenolic compound, is a promising natural flavonoid for cancer prevention and treatment. It has been shown that honokiol induced apoptosis in various human cancer cells [Battle et al., 2005; Ishitsuka et al., 2005; Hahm and Singh, 2007; Hahm et al., 2008; Raja et al., 2008; Crane et al., 2009; Chen et al., 2010; Chae et al., 2013; Singh et al., 2013; Wang et al., 2013]. Honokiol induced the apoptotic pathways that may be involved in the mitochondrial dysfunction [Chen et al., 2010], interfering Rb function and E2F1 transcriptional activity [Hahm and Singh, 2007], and inhibiting PI3K/mTOR pathway [Crane et al., 2009], down-regulation of Sp1 [Chae et al., 2013], and suppression of histone deacetylases [Singh et al., 2013]. Furthermore, the anticancer activity of honokiol was demonstrated as a single agent or in combination with other chemotherapy agents [Liu et al., 2008].

Survivin, an inhibitor of apoptosis protein, is a 16.5 kDa protein encoded by a single gene located on the human 17q25 chromosome [Ambrosini et al., 1997]. Survivin is expressed in a variety of human cancer cells, but it is undetectable in most normal adult cells. It has been shown that survivin may exhibit anti-apoptotic effect and inhibit the activity of caspases in cancer cells [Ambrosini et al., 1997; Li et al., 1998; Mita et al., 2008]. For example, survivin can inhibit caspase 9 activation to suppress apoptosis via the mitochondria/cytochrome *c* pathway [Marusawa et al., 2003; Dohi et al., 2004]. Survivin has become a promising target for cancer therapy.

p53 is a tumor suppressor protein that is encoded by the TP53 gene in human [Hofseth et al., 2004]. Activation of p53 is involved in the regulation of cell cycle arrest, DNA repair, and apoptosis [Harris, 1996; Hofseth et al., 2004]. p53 can be phosphorylated at multiple sites by several different protein kinases and mediates various

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physiological functions [Milne et al., 1992; Shieh et al., 1999; Taira and Yoshida, 2012]. DNA damage induces the phosphorylation of p53 at Ser15 and Ser20 that can reduce the interaction between p53 and its negative regulator, MDM2 [Dumaz and Meek, 1999; Shieh et al., 1997]. Moreover, the phosphorylation of p53 at Ser46 mediates the transcriptional activation of apoptosis-related genes [Oda et al., 2000]. Bax and PUMA are the downstream proteins of p53 [Fridman and Lowe, 2003]. It has been shown that p53 can directly regulate mitochondrial permeabilization and cytochrome *c* release through the intrinsic apoptotic pathway [Mihara et al., 2003]. In addition to the intrinsic apoptotic pathway, p53 can transactivate the extrinsic apoptotic pathway of DR5 gene through an intrinsic sequence-specific DNA-binding site [Takimoto and El-Deiry, 2000].

Colorectal cancer is one of the leading causes and deaths in the world [Hemminki and Li, 2001; Sanoff et al., 2008]. Development of colorectal cancer prevention and therapy by dietary flavonoids is highly desired. In this study, the role of p53 and survivin on the regulation of honokiol-induced apoptosis in the human colorectal cancer cells was investigated. Honokiol inhibited survivin expression in colorectal cancer cells; furthermore, the ectopic protein expression of survivin increased the colorectal cancer cell survival and resisted the honokiol-induced cell death. In contrast, p53 mediated the honokiol-induced apoptosis and survivin inhibition. The existence of survivin and p53 may display the opposite role in modulation of honokiol-induced apoptosis in the human colorectal cancer cells.

MATERIALS AND METHODS

CHEMICALS AND ANTIBODIES

Honokiol, Hoechst 33258, and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). BODIPY FL phallacidin, SuperScript™ III reverse transcriptase, oligo-dT12-18 primer and Lipofectamine™ 2000 were purchased from Invitrogen (Carlsbad, CA). Anti-survivin, anti-p53, anti-Bax, anti-Bcl-2, anti-PUMA, anti-GFP, goat anti-rabbit IgG horseradish peoxidase, and goat anti-mouse IgG horseradish peoxidase antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-caspase 3 antibody was purchased from BioVision, Inc. (San Francisco, CA). Anti-PARP, anti-phospho-p53 (Ser15), and anti-phospho-p53 (Ser46) antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Anti-caspase 9 was purchased from Upstate Biotech, Inc. (Lake Placid, NY). Anti-actin was purchased from Millipore Corp. (Beverly, MA).

CELL CULTURE

The RKO and HCT116 cells were colorectal carcinoma cell lines. The HCT116 p53 (−/−) colorectal carcinoma cell line was kindly provided by Dr. B. Vogelstein of Johns Hopkins University (Baltimore, MD). RKO cells were cultured in DMEM medium (Gibco, Life Technologies, Grand Island, NY). HCT116 cells were cultured in McCoy's 5A medium (Sigma Chemical). The complete medium was supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, and L-glutamine (0.03%, w/v). These cells were incubated at 37°C and 5% CO_2.

CELL VIABILITY ANALYSIS

The cell viability was analyzed by MTT assay. Briefly, 1×10^4 cells were seeded into each well in 96-well plates for overnight. The cells were treated with or without 10–60 μM of honokiol for 24 h. Then the cells were washed with phosphate-buffered saline (PBS) and recovered in fresh medium for 48 h. Finally, the cells were incubated with 0.5 mg/ml of MTT in the medium for 4 h. The survival cells would convert MTT to formazan which is purple when dissolved in dimethyl sulfoxide. The intensity of formazan was measured at 565 nm using a plate reader (VERSAmax, Molecular Dynamics, Sunnyvale, CA). The relative percentage of cell viability was calculated by dividing the absorbance of treatment by that of the control in each experiment. Moreover, the cell viability following honokiol treatment was observed by living cell images. After the cells were plated at a density of 1×10^6 cells/60-mm Petri dish for overnight, the cells were treated with or without 20–60 μM honokiol for 24 h. And then the cell morphology was observed an inverted phase contrast microscope (Olympus, Tokyo, Japan).

ANNEXIN V-PROPIDIUM IODIDE ANALYSIS

The annexin V-PI staining kit (BioVision, Mountain View, CA) was used to stain cells with fluorescein isothiocyanate (FITC)-conjugated annexin V and PI according to the manufacturer's instructions. After honokiol treatment, the cells were centrifuged at 1,500 rpm for 5 min. Cells were then resuspended in 500 μl of annexin V-PI labeling solution (containing 5 μl of annexin V-FITC and 5 μl of PI) at room temperature in the dark for 5 min and analyzed using a flow cytometer (FACSCalibur, Becton Dickinson, San Jose, CA).

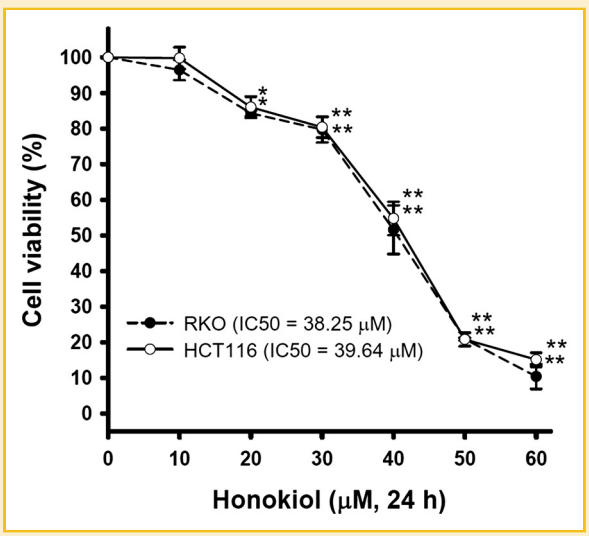

Fig. 1. Honokiol reduces the cell viability via a concentration-dependent manner in human colorectal cancer cells. RKO or HCT116 colorectal cancer cells were treated with or without 10–60 μM honokiol for 24 h. At the end of treatment, the cells were re-cultured in fresh medium for 2 days. The cell viability was measured by MTT assay. The results were obtained from four independent experiments and the bar represents the mean ± SE. $^*P < 0.05$ and $^{**}P < 0.01$ indicate significant differences between the control and honokiol treated samples. The IC_{50} values were calculated by simple linear regression.

Subsequently, the samples were analyzed using CellQuest software. Annexin V (+)/PI (−) and annexin V (+)/PI (+) represent early and late apoptosis, respectively.

WESTERN BLOTTING

Cells were lysed in ice-cold whole cell extraction buffer combined with the protease inhibitors. The whole cell extraction buffer (pH 7.6) containing 0.5 mM DTT, 0.2 mM EDTA, 20 mM HEPES, 2.5 mM $MgCl_2$, 75 mM NaCl, 0.1 mM Na_3VO_4, 50 mM NaF, 0.1% Triton X-100, and the protease inhibitors including 1 μg/ml aprotinin, 0.5 μg/ml leupeptin, and 100 μg/ml 4-(2-aminoethyl) benzenesulfonyl fluoride. Protein concentration was measured by BCA protein assay kit (Pierce, Rockford, IL). Equal amounts of proteins were separated on 10–12% sodium dodecyl sulfate–polyacrylamide gels, and transferred electrophoretically onto polyvinylidene difluoride membranes. The membranes were sequentially blocked overnight at 4°C using blocking buffer (5% non-fat dried milk in solution containing 50 mM Tris/HCl (pH 8.0), 2 mM $CaCl_2$, 80 mM sodium chloride, 0.05% Tween 20, and 0.02% sodium azide). Then, hybridized with primary antibody for 2 h at room temperature and

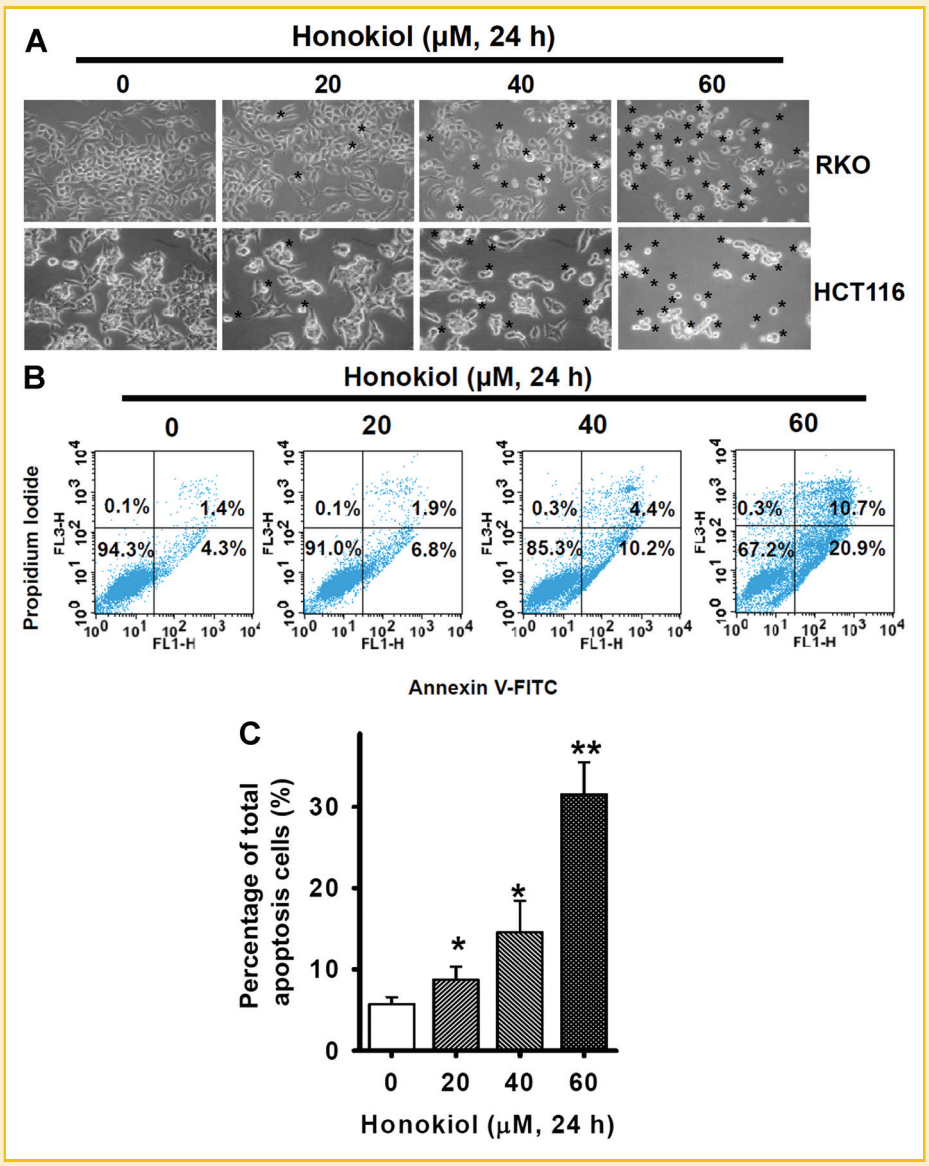

Fig. 2. Honokiol induces apoptosis in human colorectal cancer cells. A: RKO or HCT116 colorectal cancer cells were treated with or without 20–60 μM honokiol for 24 h. The cell morphology was observed by inverted microscope. The representative cell morphology data were shown from one of three separate experiments with similar findings. The stars indicated the round-up cells during cell death following honokiol treatment at 24 h observation following honokiol treatment. B: Apoptosis was determined by Annexin V-PI staining using flow cytometry analysis. The population of Annexin V^+/PI^- cells represents cells undergoing early apoptosis (lower right), whereas the fraction of Annexin V^+/PI^+ cells are those undergoing late apoptosis (upper right). Populations of apoptosis cells were quantified using CellQuest software. Data were shown from one of seven separate experiments with similar findings. C: The percentage of total apoptotic cells (late and early apoptosis) was quantified from average of the seven individual experiments. The bar represented the mean ± SE. $^*P < 0.05$ and $^{**}P < 0.01$ indicate significant differences between the control and honokiol treated samples.

followed with a horseradish peroxidase conjugated secondary antibody for 1 h at room temperature. Thereafter, the protein bands were visualized on the X-ray film using the enhanced chemiluminescence detection system (PerkinElmer Life and Analytical Sciences, Boston, MA). The protein intensities of scanned images were semi-quantified by using Un-Scan-It gel software (ver. 5.1; Silk Scientific, Inc., Orem, UT).

REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR)

Total cellular RNA was purified by ZR RNA MicroPrep™ kit according to the manufacturer's protocol (Murphy Ave, Irvine, CA). RNA concentrations were determined by a spectrophotometer (Eppendorf, Hamburg, Germany). cDNAs were synthesized by SuperScript™ III reverse transcriptase with oligo-dT 12–18 primer. Each reverse transcript was amplified with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control. The following primer pairs were used for amplification: survivin, forward primer: 5′-GGCATGGGTGCCCCGACGTTG-3′ and reverse primer: 5′-CAGAGGCCTCAATCCATGGCA-3′; GAPDH, forward primer: 5′-CGGAGTCAACGGATTTGGTCGTAT-3′ and reverse primer: 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′. RT-PCR was performed by a DNA thermal cycler, Mastercycler gradient (Eppendorf), 56°C for 30 s, and 72°C for 40 s; and 72°C for 5 min. The PCR products were visualized on 1.5% agarose gels with ethidium bromide staining under UV transillumination with a digital camera system (DH27-S3, Medclub, Taoyuan, Taiwan).

IMMUNOFLUORESCENCE STAINING AND CONFOCAL MICROSCOPY

At the end of treatment, the cells were fixed with 4% paraformaldehyde solution in PBS for 1 h at 37°C. Thereafter, the cells were washed three times with PBS, and non-specific binding sites were blocked with PBS containing 10% FBS, 0.3% Triton X-100 for 1 h at 37°C. The cells were incubated with rabbit anti-survivin (1:50) antibody in PBS containing 10% FBS for overnight at 4°C. Thereafter, the cells incubated with goat anti-rabbit Cy3 (1:50) in PBS containing 10% FBS for 1 h at 37°C. The actin filament (F-actin) and nuclei were stained with BODIPY FL phallacidin and Hoechst 33258, respectively, for 30 min at 37°C. The samples were examined under a Leica TCS SP5X/MP confocal laser scanning microscope (Leica, Heidelberg, Germany).

TRANSFECTION

The pCT-GFP, pCT-GFP-survivin, and pCT-GFP-p53 vectors were employed for transfection using Lipofectamine™ 2000 according to the manufacturer's recommendations. The cells were plated in 60-mm Petri dish at a density of 2×10^6 in complete medium for overnight, then transfected with 20 μg of control, survivin, and p53 expressed vectors in serum-free medium for 6 h at 37°C in a CO_2 incubator according to the manufacturer's recommendations. Then, the equal amount medium with 20% fetal bovine serum was added without removing the transfection mixture, and incubation proceeded for an additional 24 h. After transfection, the cells were subjected to MTT assay or Western blot analysis as described above.

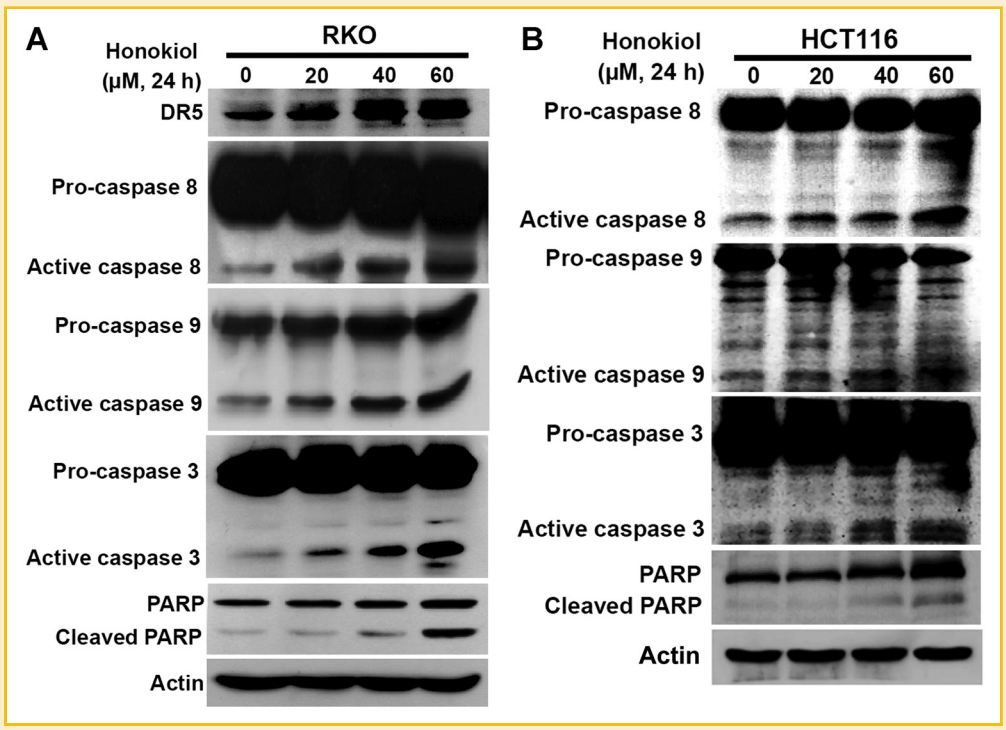

Fig. 3. Honokiol induces the death receptor DR5 and cleaved protein forms of caspase 9, caspase 8, caspase 3 and PARP in human colorectal cancer cells. A: RKO or (B) HCT116 colorectal cancer cells were treated with or without 20–60 μM honokiol for 24 h. The total cellular protein extracts were subjected to Western blot analysis using anti-DR5, anti-caspase 8, anti-caspase 9, anti-caspase 3, anti-PARP, and anti-actin antibodies. Actin protein was the internal control. The representative data were shown from one of three independent experiments with similar findings.

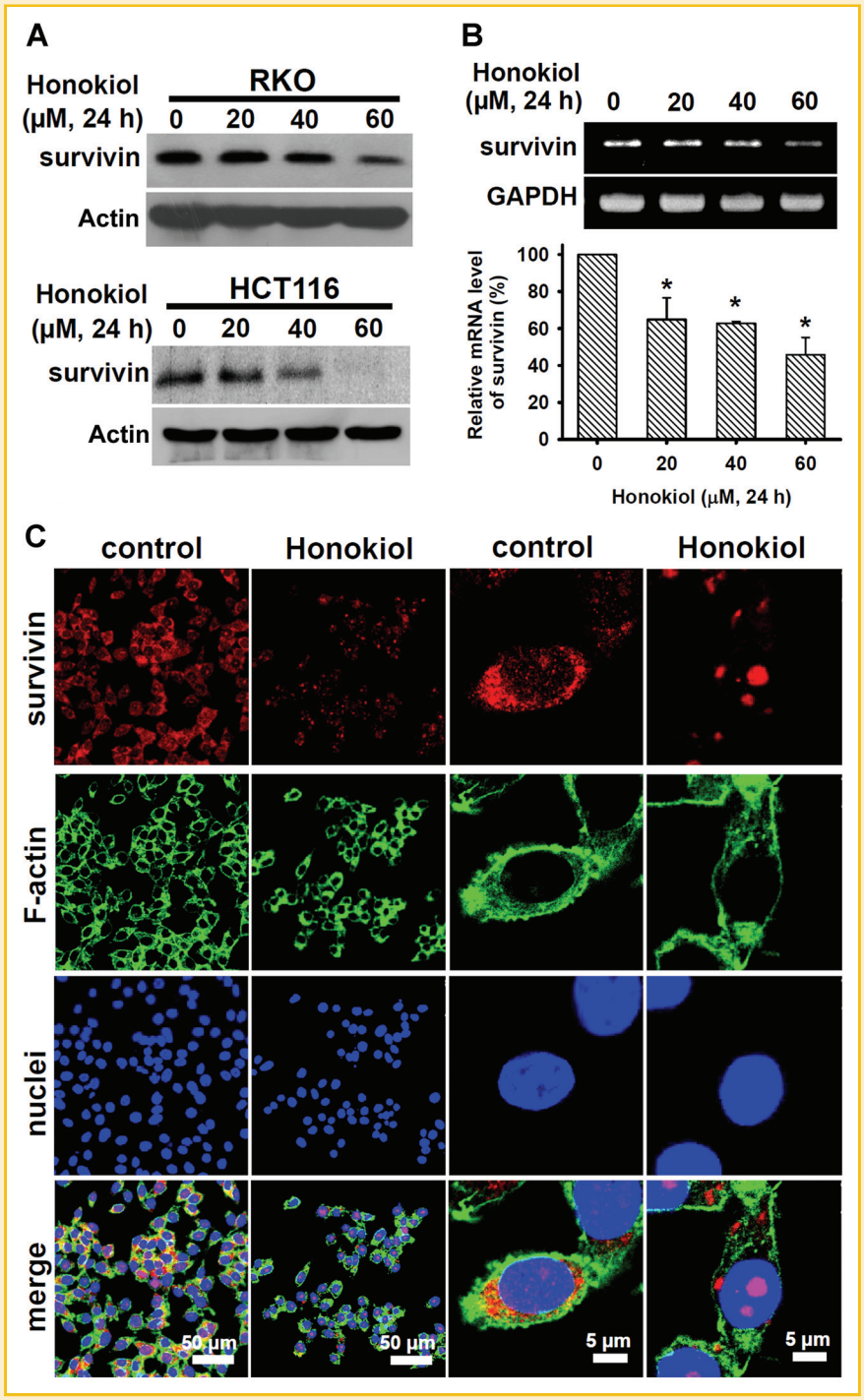

Fig. 4. Honokiol inhibits the survivin expression of human colorectal cancer cells. A: RKO or HCT116 colorectal cancer cells were treated with or without 20–60 μM honokiol for 24 h. The total cellular protein extracts were subjected to Western blot analysis using anti-survivin and anti-actin antibodies. Actin protein was the internal control. The representative data of Western blot were shown from one of three independent experiments with similar findings. B: Total RNA was isolated from each sample and then converted to cDNA. The survivin mRNA levels were performed by RT-PCR. GAPDH was an internal control. The bands of RT-PCR were quantified by UN-SCAN-IT. Relative survivin mRNA expression was normalized to GAPDH. Results were from three independent experiments and the bar represents the mean ± SE. $*P < 0.05$ indicates significance between control and honokiol treatment. C: RKO cells were treated with or without 60 μM honokiol for 24 h. The survivin proteins were stained with anti-survivin-Cy3, which displayed red fluorescence. The actin filament (F-actin) was stained with BODIPY FL phallacidin, which displayed green fluorescence. The nuclei were stained with Hoechst 33258 that displayed blue fluorescence.

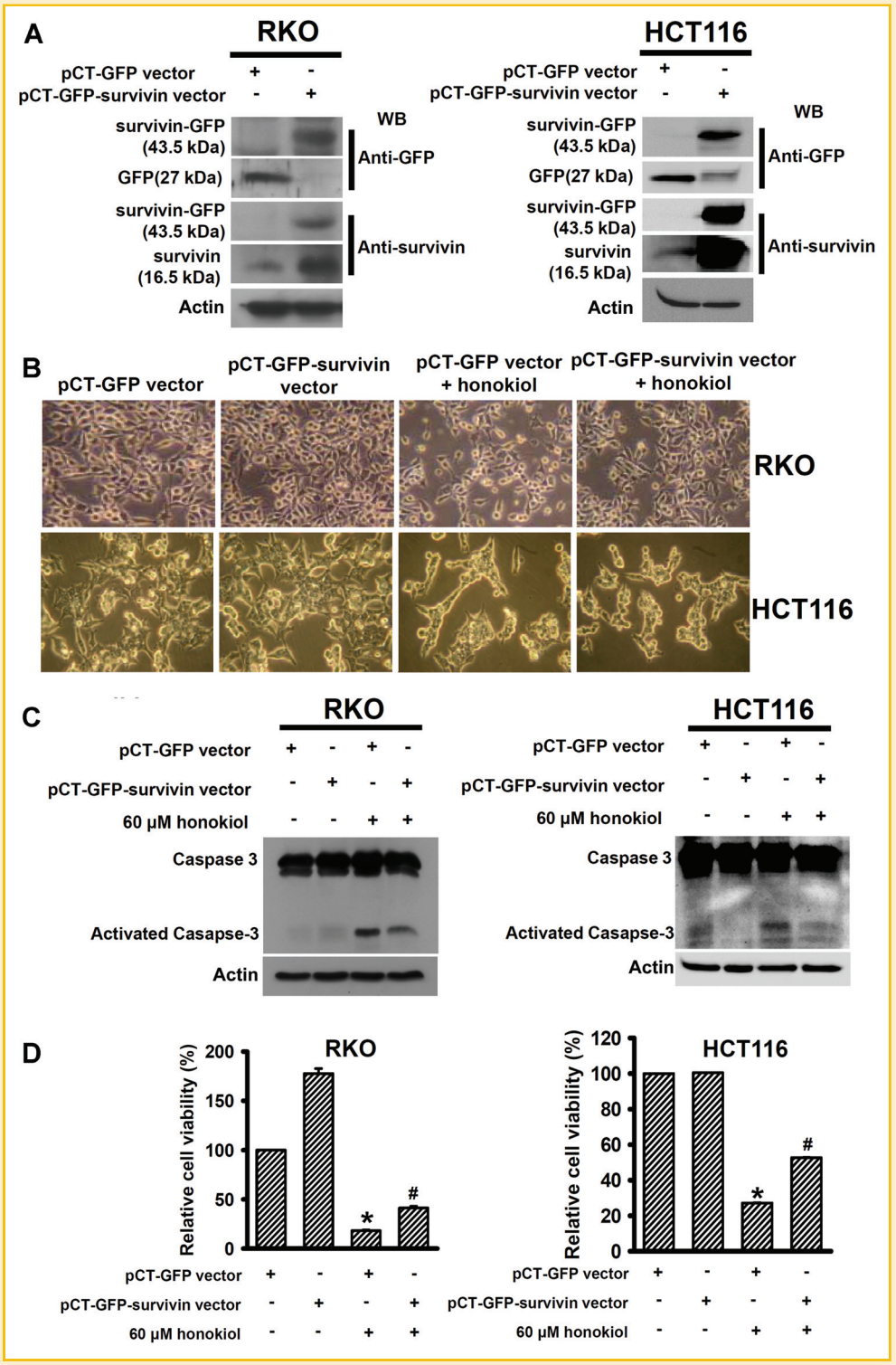

Fig. 5. Expression of survivin proteins by a survivin-expressed vector increases cell viability and reduces the honokiol-induced apoptosis in human colorectal cancer cells. A: The pcDNA3.1/CT-GFP-TOPO® plasmid was used to construct a survivin-expressed vector. Full length survivin sequence was inserted into the vector. RKO or HCT116 colorectal cancer cells were transfected with 20 μg control vector (pCT-GFP) or GFP-survivin expressed vector (pCT-GFP-survivin) for 24 h. Total protein extracts were subjected to Western blot analysis using anti-survivin, anti-GFP, and anti-actin antibodies. B: The cells were transfected with 20 μg pCT-GFP or pCT-GFP-survivin for 24 h and then treated with or without 60 μM honokiol for 24 h. The cell morphology was observed by inverted microscope. C: Total protein extracts were subjected to Western blot analysis using anti-caspase 3 and anti-actin antibodies. The above data were shown from one of three separate experiments with similar findings. D: After transfection and honokiol treatment, the cell viability was measured by MTT assays. Results were obtained from three separate experiments. The bar represents the mean ± SE. $^{*}P < 0.05$ indicates significant difference between control and honokiol-treated samples. $^{\#}P < 0.05$ indicates significant difference between control vector and GFP-survivin vector following honokiol treatment.

STATISTICAL ANALYSIS

Each experiment was repeated at least three times. Differences between control and honokiol-treated samples were analyzed using Student's *t*-test. Differences between HCT116 p53 (+/+) and p53 (−/−) cells (control, survivin, or p53 vectors) form control and the honokiol-treated samples were compared by two-way ANOVA with Bonferroni post-tests. A *P*-value of <0.05 was considered statistically significant in the experiments.

RESULTS

HONOKIOL INDUCES CYTOTOXICITY IN HUMAN COLORECTAL CANCER CELLS

To examine the cell viability following treatment with honokiol in human colorectal cancer cells, the RKO and HCT116 colorectal cancer cell lines were analyzed by MTT assays. Figure 1 shows that treatment with 10–60 μM honokiol reduced the cell viability via a concentration-dependent manner in both RKO and HCT116 colorectal cancer cells. The IC_{50} values (the concentration of 50% inhibition cell viability) of honokiol were 38.25 μM in RKO and 39.64 μM in HCT116. The RKO and HCT116 colorectal cancer cells had similar the IC_{50} values after treatment with honokiol.

HONOKIOL INDUCES APOPTOSIS IN HUMAN COLORECTAL CANCER CELLS

To determine the induction of cell death by honokiol in the human colorectal cancer cells, these cells were analyzed by the morphological analysis and Annexin V-PI staining. Treatment with 20–60 μM of honokiol for 24 h induced the abnormal cellular morphology, which detached from cultured dishes that observed by an inverted phase microscope in both RKO and HCT116 colorectal cancer cells (Fig. 2A, stars). To further determine the apoptosis levels by treatment with honokiol, the human colorectal cancer cells were analyzed by Annexin V-PI staining. The Annexin V^+/PI^- cells indicated the cells were undergoing early apoptosis, and the Annexin V^+/PI^+ cells indicated the cells were undergoing late apoptosis (Fig. 2B). Treatment with 20–60 μM honokiol induced apoptosis via a concentration-dependent manner in RKO colorectal cancer cells (Figs. 2B and C). The total apoptotic levels increased >30% with statistic significance following 60 μM of honokiol treatment (Fig. 2C). HCT116 cell line was similar result in apoptosis induction (data not shown).

HONOKIOL INDUCES BOTH THE INTRINSIC AND EXTRINSIC APOPTOTIC PATHWAYS IN HUMAN COLORECTAL CANCER CELLS

To examine the induction of apoptotic pathways following honokiol treatment, the cells were analyzed the protein levels of DR5, caspase 3, caspase 8, caspase 9, and PARP by Western blot assays. The active forms of caspase 3, caspase 8, and caspase 9 were increased following treatment with 20–60 μM of honokiol for 24 h in both RKO and HCT116 cells (Figs. 3A and B). Moreover, DR5 and the cleaved form of PARP proteins were increased by exposure to honokiol.

HONOKIOL INHIBITS THE SURVIVIN PROTEIN AND GENE EXPRESSION IN HUMAN COLORECTAL CANCER CELLS

As shown in Figure 4A, treatment with 20–60 μM of honokiol for 24 h reduced the survivin protein levels in both RKO and HCT116 cells. We have further investigated the survivin gene expression

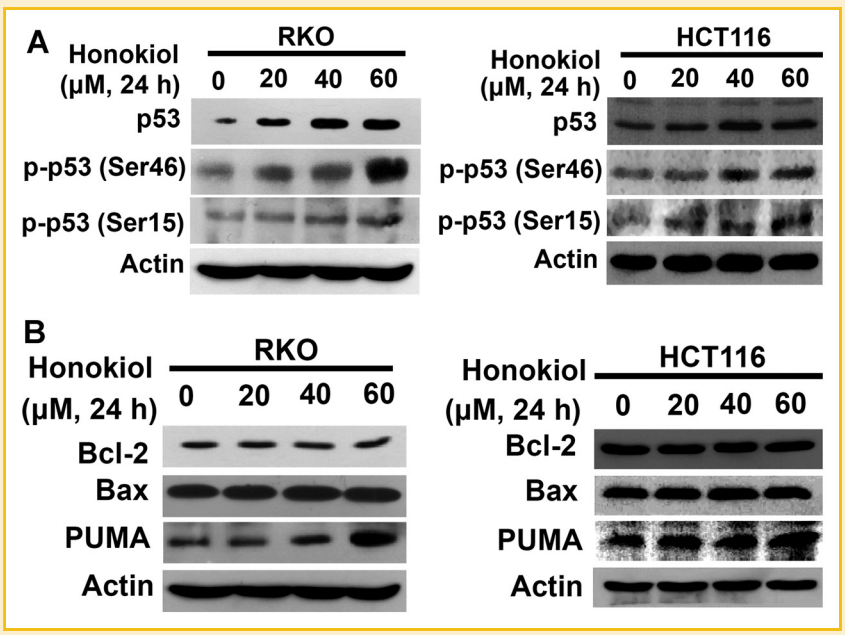

Fig. 6. Honokiol increases the protein levels of total p53 and phospho-p53 (Ser15 and Ser46) in human colorectal cancer cells. A: RKO or HCT116 colorectal cancer cells were treated with or without 20–60 μM honokiol for 24 h. The total cellular protein extracts were subjected to Western blot analysis using anti-p53, anti-phospho-p53 (Ser15), anti-phospho-p53 (Ser46), and anti-actin antibodies. B: The total cellular protein extracts were subjected to Western blot analysis using anti-Bcl-2, anti-Bax, and anti-PUMA antibodies. Actin protein was the internal control. The representative data were shown from one of three independent experiments with similar findings.

levels by RT-PCR. Treatment with honokiol significantly reduced survivin mRNA levels in colorectal cancer cells (Fig. 4B). The semi-quantified data of RT-PCR showed that honokiol significantly inhibited the mRNA levels of survivin. We confirmed that the effect of honokiol on the survivin protein expression by immunofluorescence staining and confocal microscope analysis. The fluorescence intensities of survivin proteins were markedly inhibited following treatment with honokiol (Fig. 4C).

OVEREXPRESSION OF SURVIVIN RESISTS THE HONOKIOL-INDUCED CASPASE 3 ACTIVATION AND CELL DEATH IN HUMAN COLORECTAL CANCER CELLS

To further determine the role of survivin in regulating the honokiol-induced apoptosis, the RKO or HCT116 cells were transfected with a survivin-expressed vector (pCT-GFP-survivin). Western blot analysis showed that transfection with pCT-GFP-survivin vector expressed a GFP-survivin-fusion protein (43.5 kDa), which was recognized by anti-GFP or anti-survivin antibodies in both RKO and HCT116 cells (Fig. 5A). The control pCT-GFP vector expressed the GFP proteins (27 kDa) in these cells (Fig. 5A). The endogenous survivin was recognized as a 16.5 kDa protein. Transfection of pCT-GFP-survivin vector rescued the cell number in the honokiol-treated cells (Fig. 5B). Moreover, the activated caspase 3 proteins induced by honokiol were reduced by transfection with pCT-GFP-survivin vector in both RKO and HCT116 cells (Fig. 5C). Besides, the transfection of pCT-GFP-survivin vector significantly reduced to the honokiol-induced cell death (Fig. 5D).

HONOKIOL INCREASES PHOSPHORYLATED P53 (SER15 AND SER46) AND TOTAL P53 PROTEINS TO INDUCE CELL DEATH IN HUMAN COLORECTAL CANCER CELLS

Treatment with 20–60 μM honokiol for 24 h increased the phosphorylated p53 (Ser15 and Ser46) and total p53 proteins in both RKO and HCT116 cells (Fig. 6A). Besides, honokiol significantly increased PUMA protein levels; however, the protein levels of Bcl-2, and Bax were not significantly altered by treatment with honokiol (Fig. 6B). Actin protein was used as an internal control that did not affect after treatment with honokiol.

THE EXISTENCE OF P53 ENHANCES THE HONOKIOL-INDUCED CELL DEATH AND INHIBITION OF SURVIVIN

To investigate the role of p53 in the honokiol-induced cell death, the HCT116 p53 (+/+) and p53 (−/−) cells were compared by treatment with honokiol. Figure 7A shows that both HCT116 p53 (+/+) and p53 (−/−) cells reduced the cell viability following treatment with honokiol. However, HCT116 p53 (+/+) cells exhibited a greater susceptibility to the cell death than HCT116 p53 (−/−) cells when exposed to 30–60 μM of honokiol (Fig. 7A). Moreover, we constructed a p53-expressed vector (pCT-GFP-p53) for investigating the role of p53 in the honokiol-treated cells. Transfection of pCT-GFP-p53 vector enhanced the honokiol-induced cell death than control vector in HCT116 p53 (−/−) cells (Fig. 7B). We have further confirmed the role of p53 on the apoptosis induction by treatment with honokiol. Honokiol induced apoptosis in both HCT116 p53 (+/+) and p53 (−/−) cells (Fig. 8A). However, HCT116 p53 (+/+) cells increased higher the apoptotic levels than HCT116 p53 (−/−) cells after treatment with honokiol (Fig. 8B). In addition, HCT116 p53 (+/+) cells were more significant on the decrease of survivin proteins than HCT116 p53 (−/−) cells following treatment with honokiol (Fig. 8C).

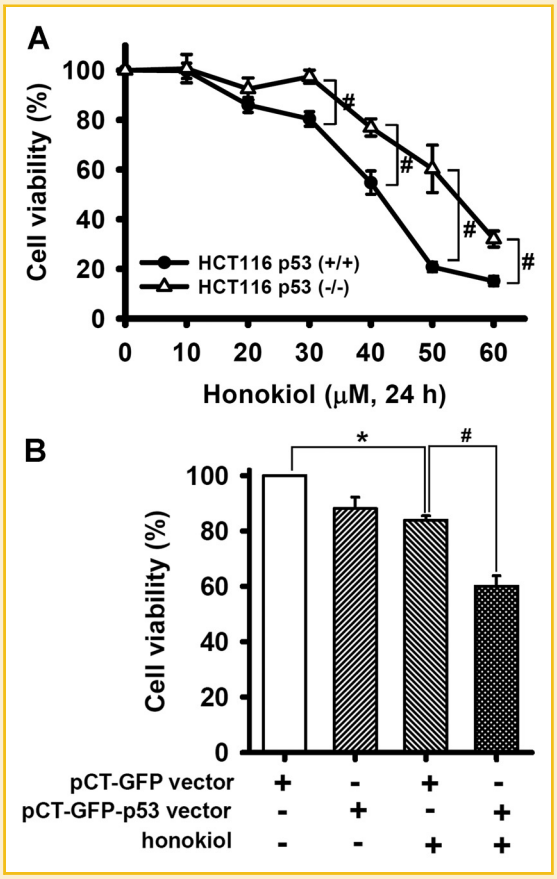

Fig. 7. The expression of p53 increases the honokiol-induced cell death in human colorectal cancer cells. A: HCT116 p53 (+/+) and p53 (−/−) cells were treated with or without 10–60 μM honokiol for 24 h. After drug treatment, the cells were re-cultured in fresh medium for 2 days. The cell viability was measured by MTT assay. The results were obtained from four independent experiments and the bar represents the mean ± SE. $^{\#}P < 0.05$ indicates significant difference between HCT116 p53 (+/+) and p53 (−/−) cells by honokiol treatment. B: The pcDNA3.1/CT-GFP-TOPO® plasmid was used to construct a p53-expressed vector. Full-length p53 sequence was inserted into the vector. HCT116 p53 (−/−) cells were transfected with 20 μg control or pCT-GFP-p53 vectors for 24 h and then treated with or without 40 μM honokiol for 24 h. Cell viability was measured by MTT assays. Results were obtained from three separate experiments. The bar represents the mean ± SE. $^{*}P < 0.05$ indicates significant difference between untreated and honokiol treated samples. $^{\#}P < 0.05$ indicates significant difference between control vector and GFP-p53 vector following treatment with honokiol.

DISCUSSION

Honokiol is a major active component from the bark of *Magnolia officinalis*, which has been used clinically in traditional oriental medicine for treatment of various indications. Several studies demonstrated that honokiol exerted anti-tumor effects [Battle et al., 2005; Ishitsuka et al., 2005; Raja et al., 2008; Mannal et al., 2011].

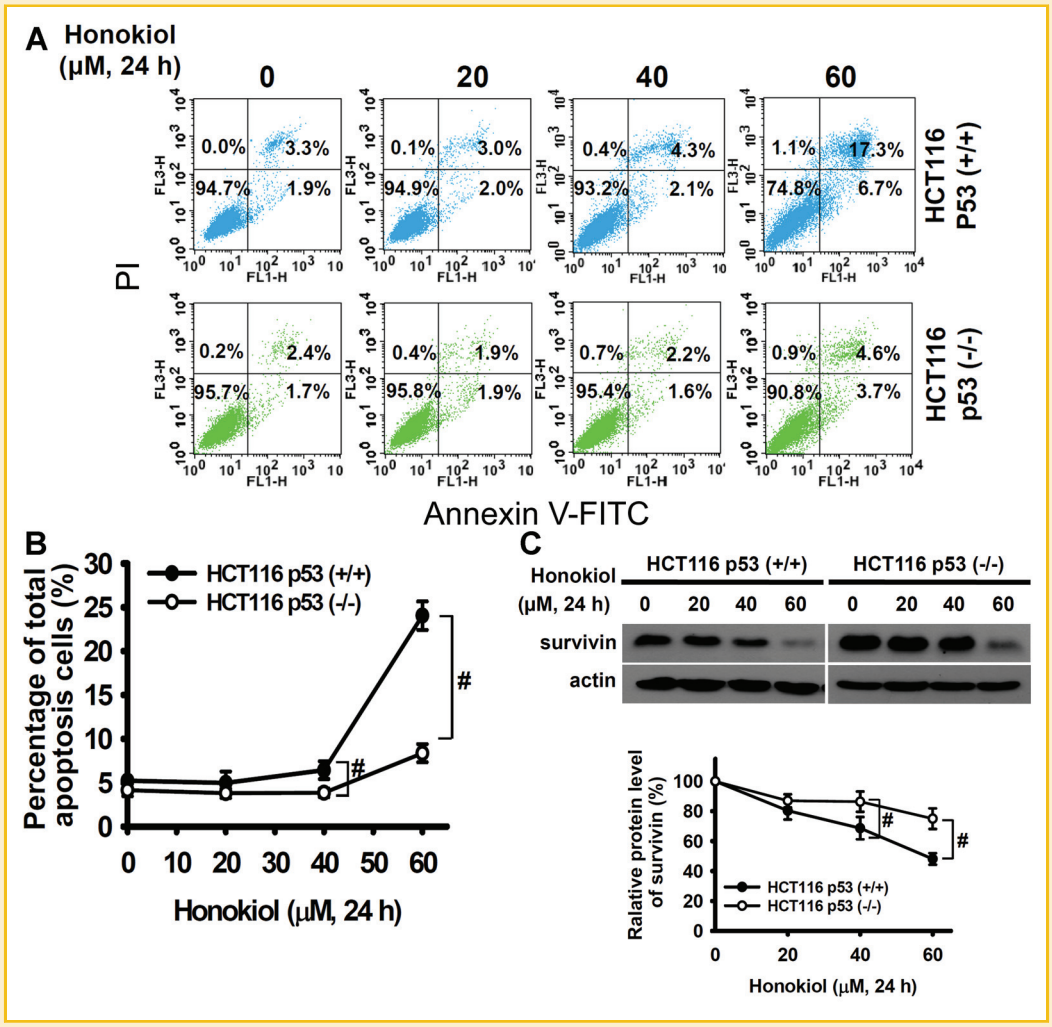

Fig. 8. The existence of p53 reduces survivin protein expression and increases apoptosis following honokiol treatment in human colorectal cancer cells. A: HCT116 p53 (+/+) and p53 (−/−) cells were treated with or without 20–60 μM of honokiol for 24 h. Apoptosis was determined by Annexin V-PI staining using flow cytometry analysis. The population of Annexin V^+/PI^- cells represents cells undergoing early apoptosis (lower right), whereas the fraction of Annexin V^+/PI^+ cells are those undergoing late apoptosis (upper right). Populations of apoptosis cells were quantified using CellQuest software. Data were shown from one of three separate experiments with similar findings. B: The percentage of total apoptotic cells was quantified from average of the three individual experiments. $^{\#}P < 0.05$ indicates significant difference between HCT116 p53 (+/+) and p53 (−/−) cells by treatment with honokiol. C: The total cellular protein extracts were subjected to Western blot analysis using anti-survivin and anti-actin antibodies. Actin protein was the internal control. The Western blots were quantified by UN-SCAN-IT and the protein expression of survivin was normalized to actin. The bar represents the mean ± SE. $^{\#}P < 0.05$ indicates significant difference between HCT116 p53 (+/+) and p53 (−/−) cells by treatment with honokiol.

However, the precise mechanism of honokiol-induced apoptosis in human colorectal cancer remains unclear. Colorectal cancer is one of the leading causes and deaths in the world [Hemminki and Li, 2001; Sanoff et al., 2008]. Development of colorectal cancer prevention and therapy by dietary flavonoids is highly desired. Interestingly, we found that honokiol increased tumor suppressor p53 expression but conversely reduced anti-apoptotic survivin expression in human colorectal cancer cells. Survivin has been proposed as a promising target for cancer therapy. Overexpression of survivin by a survivin-expressed vector could increase the colorectal cancer cell survival and resisted the honokiol-induced cell death. Furthermore, the p53-wild-type colorectal cancer cells were exhibited greater cytotoxicity, apoptosis and survivin inhibition than the p53-null cancer cells by treatment with honokiol. These findings demonstrate for the first time that survivin and p53 display the opposite role on the regulation of honokiol-induced apoptosis in the human colorectal cancer cells.

Honokiol has been shown to induce apoptosis through the intrinsic or extrinsic apoptotic pathways [Ishitsuka et al., 2005; Hahm et al., 2008; Raja et al., 2008; Wang et al., 2013]. Honokiol-induced apoptosis is characterized by the activation of caspases and cleavage of PARP [Battle et al., 2005]. We found that honokiol elicited the extrinsic death receptor pathway of DR5 and active form of caspase 8 in the human colorectal cancer cells. Apoptosis can be mediated by the extrinsic pathway of DR5 [Takeda et al., 2007]. The death receptors can recruit FADD and further stimulates caspase 8 activation that activate caspase 3 to induce apoptosis [Chinnaiyan et al., 1995; Ashkenazi and Dixit, 1998]. We found that honokiol potentiated the death receptor pathway DR5-caspase 8 activation in

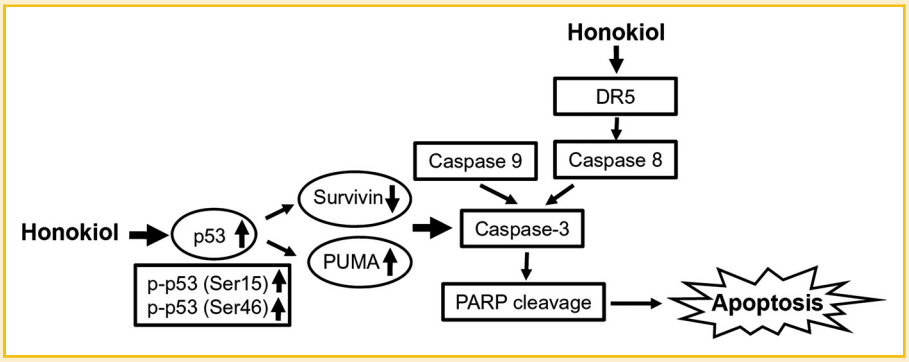

Fig. 9. The model of honokiol-induced apoptosis regulated by p53 and survivin in the human colorectal cancer cells.

colorectal cancer cells. In addition to extrinsic apoptotic pathway, the intrinsic pathway of caspase 9 was activated by honokiol. The activated caspase 3 and PARP protein cleavage were induced by honokiol in colorectal cancer cells. It has been reported that honokiol induced mitochondrial dysfunction for apoptosis induction [Battle et al., 2005; Ishitsuka et al., 2005; Chen et al., 2010]. In this study, we provide that honokiol can induce both the intrinsic and extrinsic apoptotic pathways in the human colorectal cancer. Furthermore, the ectopic expression of survivin by a survivin-GFP expressed vector reduced the caspase-3 activation and apoptosis. These findings indicate that survivin plays an important role in the honokiol-induced apoptosis of colorectal cancer cells.

Survivin has been reported as a crucial role for anti-apoptosis in human cancer cells by treatment with bioactive flavonoids (e.g., baicalein) [Chao et al., 2007]. It has been reported that survivin gene expression can be transcriptionally repressed by wild-type p53 [Hoffman et al., 2002; Mirza et al., 2002]. We found that honokiol reduced both the survivin gene and protein expression. Moreover, HCT116 p53 (+/+) colorectal cancer cells were higher on the decrease of survivin mRNA levels than HCT116 p53 (−/−) cells by treatment with honokiol (data not shown). Thus, we suggest that p53 can down-regulate the survivin expression in the honokiol-treated colorectal cancer cells.

p53 is a key tumor suppressor for controlling apoptosis in cancer cells [Bates and Vousden, 1996; Levine, 1997; Hofseth et al., 2004]. Bax and PUMA are transcriptionally regulated by p53 [Fridman and Lowe, 2003]. p53 mediated apoptosis by proapoptotic proteins through translocation from the cytosol to mitochondria membranes, which caused cytochrome *c* release from mitochondria and caspase 9 activation, followed by the activation of caspases 3, 6, and 7 [Shen and White, 2001]. The p53 up-regulated modulator of apoptosis (PUMA) was identified a p53 downstream protein for promoting apoptosis [Nakano and Vousden, 2001]. We found that honokiol increased PUMA proteins but not altered Bax protein expression. These findings suggest that honokiol induces apoptosis may be through p53-PUMA pathway in the human colorectal cancer cells. PUMA encodes a BH3-only protein member of the BCL-2 family that is a key mediator to trigger apoptosis via a p53-dependent pathway [Nakano and Vousden, 2001]. It has been shown that PUMA can sensitize cancer cells to chemotherapeutic agents [Yu et al., 2006a,b]. Thus, the activation of p53-PUMA pathway by honokiol can contribute to anti-cancer ability in human colorectal cancer.

The diverse phosphorylation sites of p53 have been shown to play important roles in the responses of various cellular stresses [Meek, 1999; Oda et al., 2000; Saito et al., 2003]. The phosphorylation of p53 at Ser15 enhances the stability of p53 but the phosphorylated site of Ser46 is related to apoptotic induction [Dumaz and Meek, 1999; Oda et al., 2000]. We found honokiol increased both the phosphorylated p53 proteins at Ser15 and Ser46 in colorectal cancer cells. The phosphorylation of p53 at Ser15 can mediate its stabilization and activation [Shieh et al., 1997; Dumaz and Meek, 1999]. The phosphorylation of p53 at Ser46 triggers the transcriptional activation of apoptosis-inducing genes [Oda et al., 2000]. HCT116 p53 (+/+) colorectal cancer cells were higher on the induction of apoptosis than HCT116 p53 (−/−) cells by honokiol treatment. These findings suggest that honokiol-induced apoptosis is mediated the stabilization and activation of p53 in the human colorectal cancer cells.

As shown in Figure 9, we conclude that survivin and p53 display the opposing role of honokiol-induced apoptosis in the human colorectal cancer cells. Honokiol can induce apoptosis through the intrinsic and extrinsic apoptotic pathways. Understanding the mechanisms by which p53 and survivin mediate apoptotic pathways by honokiol may provide novel therapeutic strategies for the colorectal cancer prevention and therapy.

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