

***Houttuynia cordata* Thunb Extract Inhibits Cell Growth and Induces Apoptosis in Human Primary Colorectal Cancer Cells**

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Abstract. *It is reported that Houttuynia cordata Thunb. (HCT), a traditional Chinese herbal medicine, has many biological properties such as antiviral, antibacterial and antileukemic activities. However, the molecular mechanisms of cytotoxicity and apoptosis in human primary colorectal cancer cells are not clear. In this study, whether HCT induced cytotoxicity in primary colorectal cancer cells obtained from three patients was investigated. The results indicated that HCT inhibited growth of cancer cells in a dose-dependent manner. After treatment with HCT (250 µg/ml) for 24 h, cells exhibited chromatin condensation (an apoptotic characteristic). HCT increased reactive oxygen species (ROS) production and decreased the mitochondrial membrane potential ($\Delta\Psi_m$) in examined cells. Mitochondria-dependent apoptotic signaling pathway was shown to be involved as determined by increase in the levels of cytochrome c, Apaf-1, and caspase-3 and -9.*

The decrease in the level of $\Delta\Psi_m$ was associated with an increase in the BAX/BCL-2 ratio which led to activation of caspase-9 and -3. Based on our results, HCT induced apoptotic cell death in human primary colorectal cancer cells through a mitochondria-dependent signaling pathway.

Colorectal cancer (CRC) is one of the most prevalent types of cancer worldwide and a common cause of morbidity and mortality in humans (1). According to the reports of the Department of Health, Taiwan (ROC) in 2009, colorectal cancer is the third leading cause of cancer-related death in Taiwan, accounting for 11.4% of all cancer deaths. In clinical practice, surgery, radiotherapy and chemotherapy are main strategies for treating colon cancer patients. Significant improvements in patient survival rates have been achieved in recent years due to successful treatment (2). However, most patients still die of their disease. Approximately 20% of patients have distant metastatic disease at the time of presentation and are rarely cured (3). The median overall survival duration for patients with metastatic disease is currently about 20 months (4). Chemotherapy resistance in cancer is one of the most serious obstacles. Therefore, in this study, we focus on identifying a new agent to treat colorectal cancer.

Apoptosis is the process of programmed cell death *via* a highly regulated mechanism. There are three major signaling pathways leading to apoptosis. Firstly, the death receptor pathway is triggered by the binding of extrinsic signals to surface receptors including CD95/Fas, tumor necrosis factor (TNF), and death receptors. This ligand receptor interaction initiates death signals from the extracellular micro-environment

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Key Words: *Houttuynia cordata* Thunb. (HCT), chinese herb, apoptosis, human primary colorectal cancer cells.

Table I. Patient information.

| | Patient 1 | Patient 2 | Patient 3 |
|---------------------|---|---|---|
| TNM | T3N1M0 | T3N2M0 | T3N1M1 |
| Location | Cecum | Sigmoid | Sigmoid |
| Familial history | Non-contributory | Non-contributory | Non-contributory |
| Histology | Adenocarcinoma, moderately differentiated | Adenocarcinoma, moderately differentiated | Adenocarcinoma, moderately differentiated |
| Pre-op chemotherapy | None | None | None |
| Pre-op radiotherapy | None | None | None |
| Pre-op CEA | 5.90 | 14.1 | 3.03 |

Patient background and diagnosis were determined by the histopathologist. CEA: Carcinoembryonic antigen.

to the cytoplasm, then inducing apoptosis (5). Secondly the mitochondrial pathway is triggered by various stimuli including DNA damage, cellular distress, hypoxia, cytotoxic agent damage inside the cell and increased intracellular calcium concentration. This pathway is involved in mitochondrial function and BCL-2 family. BCL-2 exerts anti-apoptotic effects, but BAX induces pro-apoptotic responses. When an excess of pro-apoptotic over anti-apoptotic signals occurs, it initiates mitochondrial dysfunction and leads to apoptosis (6). To date, induction of apoptosis in cancer cells has been considered as a possible strategy in cancer treatment (7).

Hottuynia cordata Thunb. (HCT), a traditional Chinese medicine, is a perennial herb that is native to Japan, Korea, southern China and Southeast Asia. It has been reported that *Houttuynia cordata* has many biological properties such as anti-allergy (8), antioxidant (9), antiviral (10) and antibacterial activities (11). Furthermore, Chang *et al.* demonstrated that HCT has antileukemic activities in various leukemia cell lines including L1210, U937, K562 and P3HR1 (12). However, the molecular mechanisms of its exerting cytotoxicity in cancer cells are not well understood. Our previous study also showed that HCT induced apoptosis through a mitochondria-dependent pathway in human colorectal adenocarcinoma HT-29 cells (13), but there are no reports showing the effects of HCT on human primary colorectal cancer cells. In this study, we evaluated the possible molecular signaling of HCT-induced apoptosis in human primary colorectal cancer cells from three patients *in vitro*.

Materials and Methods

Preparation of HCT. HCT 50% ethanol extracts (yield: 6.73% of dry wt.) were obtained by 48 h maceration of plant material at room temperature. The ethanol extract was filtered through a 0.45 µm filter (Osmonics, Minnetonka,

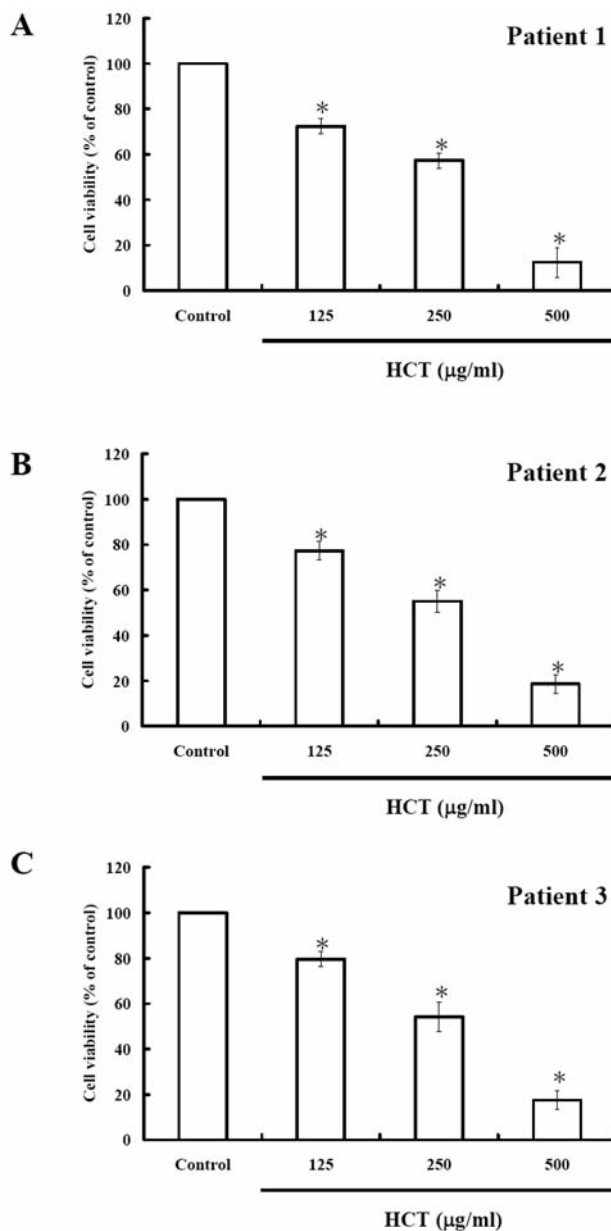


Figure 1. Effects of HCT on cell viability in human primary colorectal cancer cells. After treatment with different concentrations of HCT for 24 h, the cell viability of three human primary colorectal cancer cells significantly decreased. Patient 1, $IC_{50}=289.62\pm 10.36$ µg/ml (A) patient 2, $IC_{50}=321.09\pm 15.34$ µg/ml (B) and patient 3, $IC_{50}=296.41\pm 9.68$ µg/ml (C). Data from three independent experiments are presented (* $p < 0.05$ as compared with control treatments).

MN, USA), lyophilized, and kept at 4°C. The dried extract was resolubilized in distilled water before use.

Chemicals and reagents. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI) and 4',6-diamidino-2-phenylindole (DAPI) were purchased

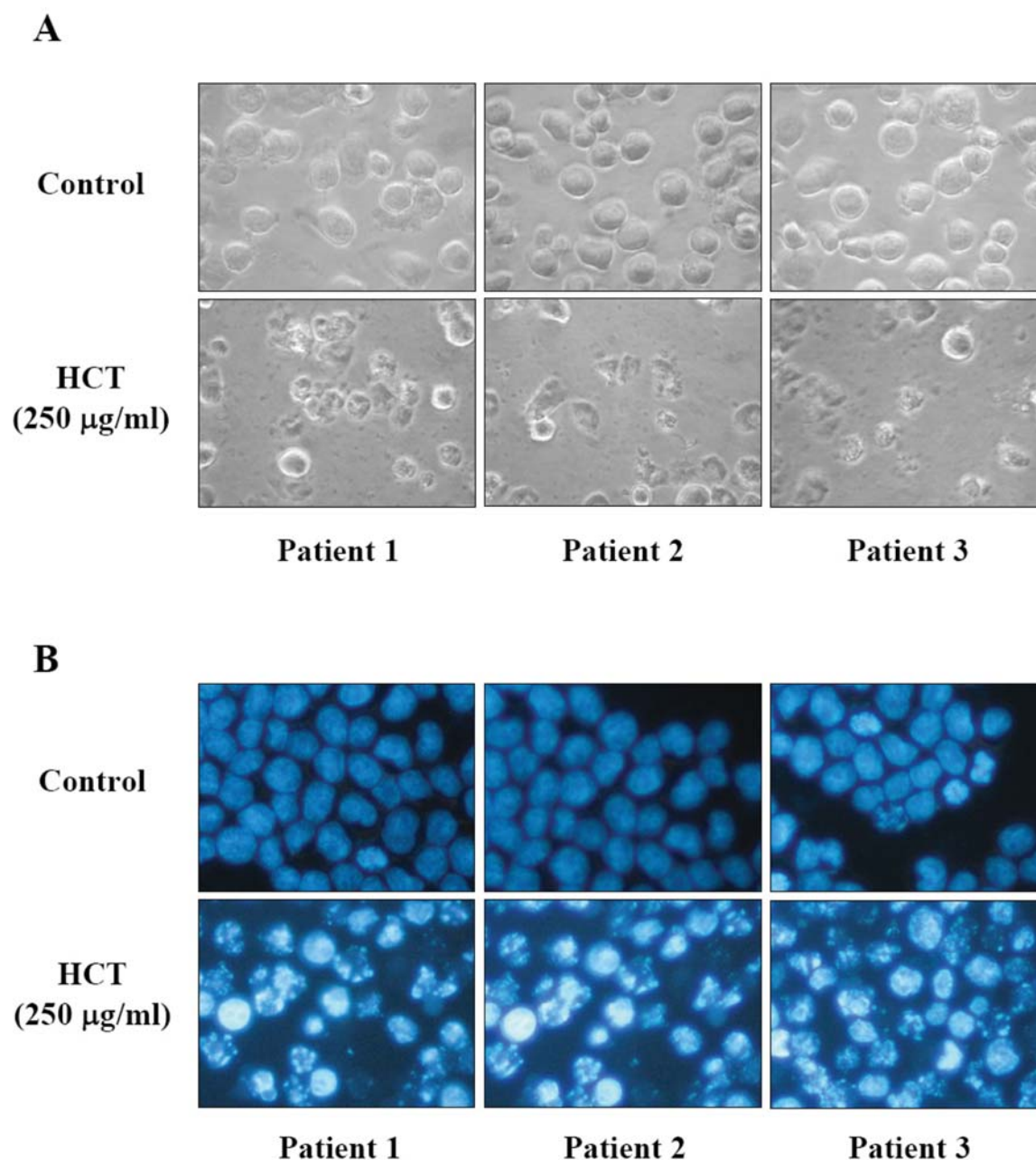


Figure 2. Effects of HCT on cell morphological changes and DNA condensation in human primary colorectal cancer cells. After incubation with HCT (250 µg/ml) for 24 h, cells exhibited nuclear shrinkage and chromatin condensation, whereas those of the control group were well spread with flattened morphology (A). DNA condensation was determined by DAPI staining (B). Cells were examined and photographed under fluorescence microscopy ($\times 200$) as described in the Materials and Methods.

from Sigma-Aldrich Corp. (St. Louis, MO, USA). RPMI-1640, fetal bovine serum (FBS), trypsin-EDTA, and penicillin/streptomycin were obtained from GIBCO BRL/Invitrogen Corp. (Grand Island, NY, USA). Proteinase K was purchased from Roche Diagnostics (GmbH, Mannheim, Germany). 2,7-Dichlorodihydrofluorescein

diacetate (DCFH-DA) and 3,3'-dihexyloxycarbocyanine iodide (DiOC6) were purchased from Molecular Probes (Invitrogen, Eugene, OR, USA). The Bio-100™ DNA Ladder marker was obtained from PROtech Technology Enterprise Co. (Taipei, Taiwan). All other chemicals used were of analytical grade.

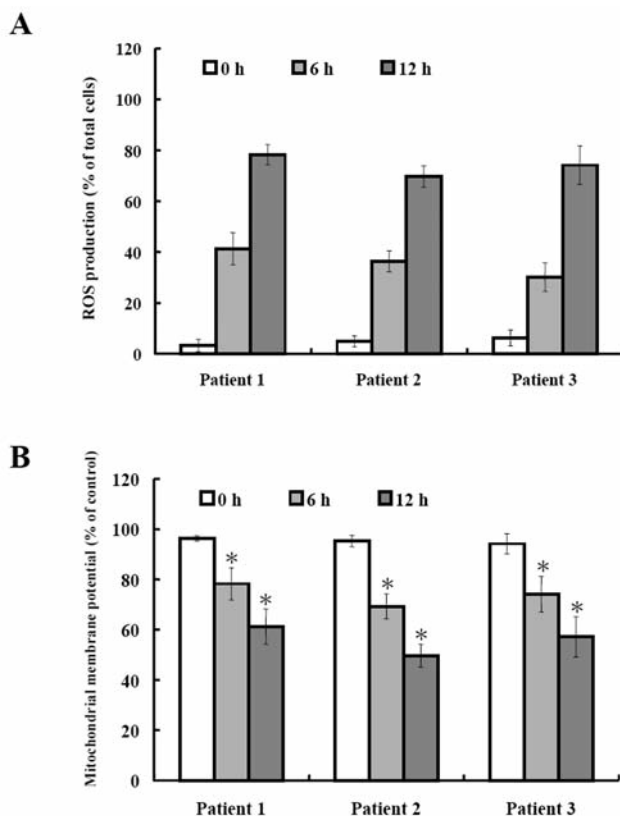


Figure 3. Effects of HCT on the levels of reactive oxygen species (ROS) production and mitochondria membrane potential ($\Delta\Psi_m$) in human primary colorectal cancer cells. Three human primary colorectal cancer cells were treated with 250 $\mu\text{g/ml}$ of HCT for 0, 6 and 12 h, and the data from flow cytometric analysis showed that ROS production increased (A) and these was loss of $\Delta\Psi_m$ (B). Data from three independent experiments are presented (* $p < 0.05$ as compared with control treatments).

Human primary colorectal cancer cells isolation. Colorectal carcinoma specimens from three patients were obtained around 2008 to 2009 from the Department of Surgery, China Medical University Hospital, Taichung, Taiwan after approval of the study by the hospital's Ethical Committee, and with written and informed consent from patients (IRB NO: DMR-96-IRB-72) (Table I). Specimens were dissected into 1 mm^3 pieces, immersed in a ten-fold volume of 0.25% trypsin solution (Sigma-Aldrich Corp), kept at 4°C overnight and then incubated at 37°C for 1 h. After stopping trypsin activity with FBS, the solution containing released cells was centrifuged at 150 \times g for 5 min. The precipitated cells were re-suspended with culture medium, and seeded into a 10 cm culture dish. The remaining undigested tissue was immersed in collagenase solution (500 Units/ml in RPMI-1640 medium with 10% serum) (Sigma-Aldrich Corp.) and incubated at 37°C for 1 h. The cells released were collected, centrifuged, re-suspended with culture medium,

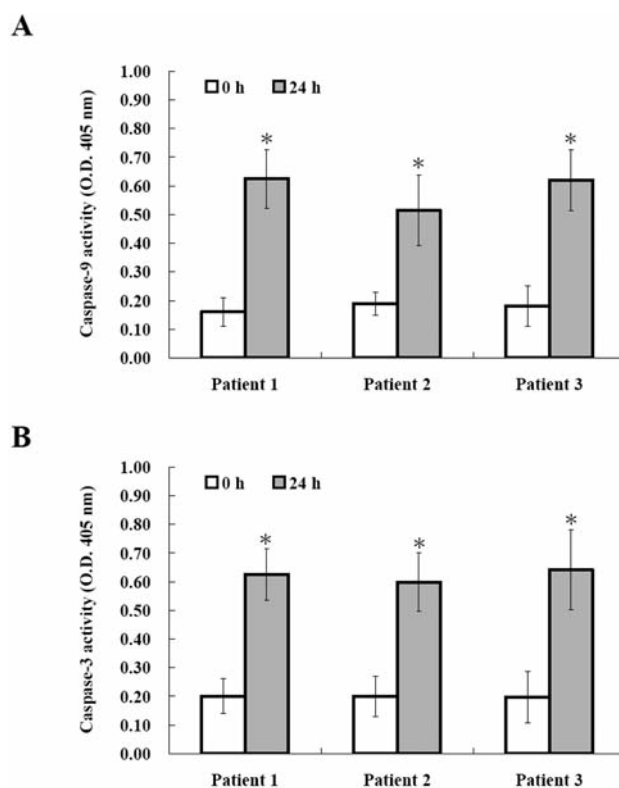


Figure 4. Effects of HCT on the caspase-9 and caspase-3 activity in human primary colorectal cancer cells. After incubation with HCT (250 $\mu\text{g/ml}$) for 24 h, 50 μg protein were incubated with caspase-3- and -9-specific substrates (Ac-DEVD-pNA and Ac-IETD-pNA) with reaction buffer in a 96-well plate at 37°C for 1 h. The release of pNA was measured at 405 nm by a spectrophotometer. Caspase-9 (A) and caspase-3 (B) activities are significantly increased. Data from three independent experiments were presented (* $p < 0.05$ as compared with control treatments).

and seeded into a culture flask. The culture medium was RPMI-1640 medium supplemented with 10% FBS. After primary cultures became confluent, cells were detached by trypsin (0.25%)–ethylenediaminetetraacetic acid (EDTA) (0.02%) solution (Sigma-Aldrich Corp.), counted, centrifuged, re-suspended and seeded into new culture flasks (14).

Cell viability assay. Human primary colorectal cancer cells were plated onto 96-well plates at a density of 2×10^4 cells/well and exposure to 0, 125, 250 or 500 $\mu\text{g/ml}$ of HCT for 24 h. MTT was then added to each well and plates were then incubated for an additional 4 h in the dark at 37°C. The medium was then aspirated from the wells and the blue formazon product was dissolved in 100 μl of DMSO. The plates were analyzed at OD 570 nm using a spectrophotometric plate reader (Bio-Rad, Tokyo, Japan). Each data point was replicated in triplicate. Percentage of

cell viability was calculated as (OD of HCT-treated sample/OD of none treated sample) \times 100% (15-16).

DAPI staining. Approximately 2×10^5 cells/well were treated with HCT (250 μ g/ml) for 24 h, and were then fixed in 4% paraformaldehyde for 30 min. Cells were added 0.1% Triton-X 100 for 10 min and then incubated with 1 μ g/ml of DAPI staining solution for 30 min in the dark. Apoptotic cells were observed through fluorescence microscopy (Zeiss, Oberkochen, Germany) as previously described (17).

Reactive oxygen species (ROS) production assay. ROS were measured after staining human primary colorectal cancer cells with DCFH-DA. About 2×10^5 cells/well was treated with 250 μ g/ml of HCT for 24 h, and then cells were collected and washed with PBS. One ml of PBS containing 20 μ M H₂DCF-DA was added, and the cells were incubated for 30 min at 37°C. The fluorescence emission from DCF was analyzed by flow cytometry (FACSCalibur; Becton-Dickinson, San Jose, CA, USA) as previously described (18).

Mitochondrial membrane potential ($\Delta\Psi_m$) determination. Changes of $\Delta\Psi_m$ were monitored after staining with DiOC₆. About 2×10^5 cells/well were treated with 250 μ g/ml of HCT for 24 h, cells were collected, trypsinized and washed in PBS, then they were stained with DiOC₆ (50 nmole/l) for 30 min at 37°C. The percentage of green fluorescence was estimated by flow cytometry as previously described (19).

Western blotting analysis. About 1×10^6 cells/well was treated with 250 μ g/ml of HCT for 24 h, and then cells were collected for evaluating the apoptosis associated protein levels. Assay kits for total proteins (Calbiochem/EMD Biosciences Inc., San Diego, CA, USA) were used to assess the release of cytochrome *c*, Apaf-1, caspase-9, caspase-3, BCL-2 and BAX in cytosol and the purity of the fractions estimated. Fifty μ g of proteins were resolved on SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (PVDF; Millipore, Billerica, MA, USA). After blocking with 5% non-fat milk, the blots were incubated with an appropriate dilution of specific monoclonal antibodies for cytochrome *c*, Apaf-1, caspase-9, caspase-3, BCL-2 and BAX (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 12 h. Blots were washed three times and then incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Inc.). The specific proteins were detected by using enhanced chemiluminescence kits (Amersham ECL kits, Piscataway, NJ, USA) as previously described (20-21).

Caspase activity assay. Approximately 5×10^5 cells/well were treated with 250 μ g/ml of HCT for 24 h, after which human primary colorectal cancer cells were collected in lysis buffer

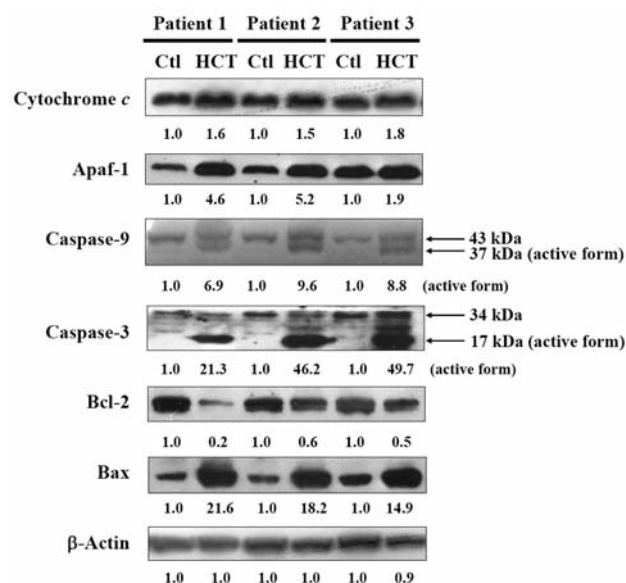


Figure 5. Effects of HCT on apoptosis-associated protein levels in human primary colorectal cancer cells. Cells were treated with 250 μ g/ml of HCT for 24 h, and whole cell lysates were prepared and subjected to Western blotting. The resulting blots were probed for cytochrome *c*, Apaf-1, caspase-9, caspase-3, BCL-2 and BAX. β -Actin served as the loading control.

(50 mM Tris-HCl, 1 mM EDTA, 10 mM EGTA, 10 mM digitonin and 2 mM DTT) on ice for 10 min. The lysates were centrifuged at $15,000 \times g$ at 4°C for 10 min. Cell lysates (50 μ g) were incubated with caspase-3- and -9-specific substrates AC-ASP-GLU-VAL-ASP-*p*-nitroanilide (Ac-DEVD-*p*NA); AC-ILE-GLU-THR-ASP-nitroanilide; (Ac-IETD-*p*NA) with reaction buffer in a 96-well plate at 37°C for 1 h. The release of *p*NA was measured at 405 nm by a spectrophotometer as previously described (22).

Statistical analysis. Data are presented as the mean \pm standard deviation (SD) for the indicated number of separate experiments. Statistical analyses of data were carried out with one-way ANOVA followed by Student's *t*-test between HCT-treated and control groups, and $p < 0.05$ was considered significant.

Results

Effects of HCT on cell viability in human primary colorectal cancer cells. Cells were treated with HCT at 0, 125, 250 and 500 μ g/ml for 24 h. The viabilities were measured by MTT method. As shown in Figure 1, the cell viability significantly decreased in HCT-treated human primary colorectal cancer cells. The concentration required to inhibit growth by 50% (IC₅₀) of the three patients' primary cells were 289.62,

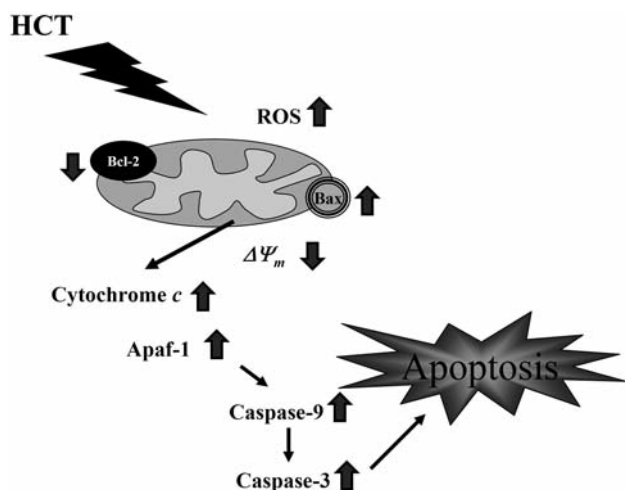


Figure 6. A proposed model illustrates the molecular mechanism and the overall possible signaling pathways of HCT-induced apoptosis in human primary colorectal cancer cells.

321.09 and 296.41 $\mu\text{g/ml}$, respectively. The data indicated that HCT reduced the proportion of viable cells in a dose-dependent manner.

Effects of HCT on cell morphological changes and apoptosis in human primary colorectal cancer cells. To investigate the occurrence of morphological changes and chromatin condensation in HCT-treated human primary colorectal cancer cells, we assessed the nuclear morphological changes by phase-contrast microscopy and DAPI staining. As shown in Figure 2A and B, after incubation with HCT (250 $\mu\text{g/ml}$) for 24 h, cells exhibited nuclear shrinkage and chromatin condensation, whereas the untreated group were well spread with flattened morphology. The results demonstrated that HCT induced morphological changes and chromatin condensation in human primary colorectal cancer cells.

Effects of HCT on the levels of ROS and $\Delta\Psi_m$ in human primary colorectal cancer cells. To elucidate whether HCT could cause cell apoptosis through induction of mitochondrial dysfunction in human primary colorectal cancer cells, we measured the intracellular ROS levels and the change of $\Delta\Psi_m$. As shown in Figure 3A and B, cells were treated with 250 $\mu\text{g/ml}$ of HCT for 0, 6 or 12 h and the data showed a significant increase in the level of ROS and a decrease of $\Delta\Psi_m$ in a time-dependent manner. The results suggest that mitochondria dysfunction may be involved in HCT-induced apoptosis, and ROS generation is also involved.

Effects of HCT on the caspase-9 and caspase-3 activity in human primary colorectal cancer cells. In order to confirm that HCT-induced apoptosis was mediated by caspase-

dependent pathway, we investigated the caspase-9 and -3 activities by fluorogenic enzymatic assay. As shown in Figure 4A and B, both caspase-9 and caspase-3 activities significantly increased after incubation with 250 $\mu\text{g/ml}$ of HCT for 24 h. The results suggest caspase-dependent signaling pathway is involved in HCT-induced apoptosis of human primary colorectal cancer cells.

Effects of HCT on levels of apoptosis-associated proteins in human primary colorectal cancer cells. To elucidate the possible molecular mechanisms of HCT-induced apoptosis, we investigated the protein levels of cytochrome *c*, Apaf-1, caspase-9, caspase-3, BCL-2 and BAX by Western blotting analysis. The levels of cytochrome *c*, Apaf-1, caspase-9 and caspase-3 significantly increased after HCT 24-h treatment (Figure 5A). Furthermore, the pro-apoptotic protein level of BAX was up-regulated, whereas the anti-apoptotic protein level of BCL-2 was down-regulated. The results suggest that the HCT-induced apoptotic response is mediated by the mitochondria-dependent apoptotic pathway.

Discussion

HCT has been used as a traditional Chinese herbal medicine in eastern and southern Asia for a long time. The biological activities of HCT include antioxidant, anti-bacterial, anti-viral effects and inhibition of anaphylactic reaction and mast cell activation (23). In addition, Chen *et al.* reported the anti-mutagenic effects of HCT under oxidized frying oil feeding-induced oxidative stress in Sprague-Dawley rats (24). Chang *et al.* demonstrated that HCT inhibited the growth of five leukemia cell lines (L1210, U937, K562, Raji and P3HR1, IC_{50} was between 478 $\mu\text{g/ml}$ to 662 $\mu\text{g/ml}$) but is well tolerated by healthy human cells ($\text{IC}_{50} > 1000 \mu\text{g/ml}$). (12) Although HCT has been reported to be active against tumors, its anti-cancer effect is not very clear. Our previous study reported that HCT extract induced apoptosis through a mitochondria-dependent pathway in human colon adenocarcinoma HT-29 cells, and the IC_{50} in HT-29 cells was 435 $\mu\text{g/ml}$. In this study, we firstly investigated the anticancer effects of HCT and the associated molecular mechanisms in human primary colorectal cancer cells *in vitro*.

Cells undergoing apoptosis exhibited cytoplasmic blebbing, irregularity in shape apoptotic bodies, nuclear condensation and DNA fragmentation. DNA fragmentation into well defined fragments is linked to the activation of endonucleases, leading to apoptosis (25). In Figure 2, the results showed the morphological change and strand breaks of human primary colorectal cancer cells that were detected by DAPI staining. In the HCT-treated group, cells were detached from the surface and contained some debris, whereas cells of the control group were well spread with flattened morphology. For further investigations of the molecular mechanism

involved in apoptosis was caused by HCT. We measured the intracellular ROS level and change of $\Delta\Psi_m$ by flow cytometry and investigated the apoptotic-related protein expression by Western blotting analysis. It was reported that the exogenous and endogenous ROS, such as hydrogen peroxide, cause apoptosis through mitochondrial permeability transition (26). In Figure 3, our results indicated that HCT induced ROS production and depolarization of $\Delta\Psi_m$. BCL-2 family members were reported in the regulation of mitochondria-mediated apoptotic pathways, including pro-apoptotic proteins (BAX, BAD and BAK) and anti-apoptotic proteins (BCL-2 and BCL-XL). (27) When an excess of pro-apoptotic over anti-apoptotic signal exists, it initiates mitochondrial outer membrane permeabilization (6). Figure 5 indicates that HCT promoted the pro-apoptotic protein level of BAX and inhibited the anti-apoptotic protein level of BCL-2, which led to a change of the ratio of BAX/BCL-2, resulting in loss of $\Delta\Psi_m$. Additionally, the results also showed that HCT induced up-regulation of cytochrome *c*, Apaf-1, caspase-9, caspase-3. Chalah *et al.* and Guicciardi *et al.* reported that cytochrome *c*, Apaf-1 and pro-caspase-9 are released from the mitochondrial membrane into the cytosol on the loss of $\Delta\Psi_m$. Subsequently, cytosolic cytochrome *c* binds to Apaf-1, ATP, and pro-caspase-9, creating a protein complex (apoptosome) which then activates caspase-3 and leads to apoptosis (28-29). These results indicated that the HCT-induced apoptotic response of human primary colorectal cancer cells was mediated *via* a mitochondria-dependent pathway. Furthermore, as shown in Figure 4, we investigated the caspase-9 and -3 activities by fluorogenic enzymatic assay, and it was shown that both caspase-9 and caspase-3 activities were significantly increased. Activation of caspases is the major mechanism that promotes apoptotic cell death in response to death-inducing signals from cell surface receptors and mitochondrial stress (30).

In conclusion, our results once again confirmed the molecular mechanism in HCT-treated HT-29 cells and human primary colorectal cancer cells. We demonstrated that HCT induced cytotoxicity in human primary colorectal cancer cells by the induction of apoptosis through the mitochondria-dependent pathway. The proposed signaling pathways are shown in Figure 6. This is in agreement with our previous study, the IC_{50} of three human primary colorectal cancer cells was lower than that for HT-29 cells (three human primary colorectal cancer cells: 289.62, 321.09 and 296.41 $\mu\text{g/ml}$, HT-29 cells: 435 $\mu\text{g/ml}$). Human primary colorectal cancer cells seem more sensitive to HCT treatment, and the reason for this is currently under investigation. Taken together, these findings provide important possible molecular mechanisms for the activity of HCT towards primary colorectal cancer cells and confirm that HCT may be useful in anticancer therapy in the future.

Acknowledgements

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