Research Article

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Solanum nigrum L. polyphenolic extract inhibits hepatocarcinoma cell growth by inducing G_2/M phase arrest and apoptosis

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Abstract

BACKGROUND: Hepatocellular carcinoma (HCC) is a rapidly progressive cancer with poor prognosis. However, there have been no significant new developments in treating liver cancer. To search for an effective agent against HCC progression, we prepared a polyphenolic extract of *Solanum nigrum* L. (SNPE), a herbal plant indigenous to Southeast Asia and commonly used in oriental medicine, to evaluate its inhibitive effect on hepatocarcinoma cell growth. The growth inhibition of HepG2 cells *in vitro* and *in vivo* was determined in the presence of SNPE.

RESULTS: We found 1 μg mL^{-1} SNPE-fed mice showed decreased tumor weight and tumor volume by 90%. Notably, 2 μg mL^{-1} SNPE resulted in almost complete inhibition of tumor weight as well as tumor volume. In line with this notion, SNPE reduced the viability of HepG_2 cells in a dose-dependent manner. HepG_2 cells were arrested in the G_2/M phase of the cell cycle; meanwhile, the protein levels of cell CDC25A, CDC25B, and CDC25C were clearly reduced. Moreover, sub-G_1 phase accumulation and caspases-3, 8, and 9 cleavages were induced by SNPE.

CONCLUSION: This study shows that SNPE is a potent agent for HCC treatment through targeting G_2/M arrest and apoptosis induction, achieving cell growth inhibition.

Keywords: apoptosis; caspases; G_2/M arrest; hepatocellular carcinoma; *Solanum nigrum* L. polyphenolic extract

INTRODUCTION

Liver cancer, of which about 95% are hepatocellular carcinoma (HCC), is the third most common cause of death from cancer in the world. Owing to a high rate of relapse, HCC carries a very poor prognosis, with a 5-year survival rate of 3–5%.[1,2] Thus it is very important to develop a new agent which effectively treats and prevents recurrence of HCC.

Solanum nigrum L., a herbal plant indigenous to Southeast Asia, is commonly used in oriental medicine. A previous study showed that ripe fruits of *S. nigrum* L. induce growth inhibition and apoptosis in breast cancer cells.[3] In addition, the extract from the whole plant of *S. nigrum* L. results in hepatoma cell death through autophagy and apoptosis.[4,5] This evidence suggests that *S. nigrum* L. could exert its antineoplastic activity as a cancer chemopreventive agent. Steroidal glycoalkaloid and glycoprotein, isolated from *S. nigrum* L., exert cytotoxic and apoptotic effects in human cancer cells[6,7] and this may explain the antineoplastic activity of *S. nigrum* L.

Since *S. nigrum* L. is rich in polyphenolic compounds derived from naturally occurring substances, it has attracted increasing interest in preventing carcinogenesis.[4–6,8–12] It is interesting to speculate whether *S. nigrum* L. polyphenolic extract (SNPE) has therapeutic potential in treating HCC. Aberrations in cell cycle progression and apoptosis dysregulation are the cause of oncogenic transformation.[13] Therefore the purpose of this work was to evaluate whether SNPE could be an effective chemopreventive agent through regulating the cell cycle and apoptosis in hepatocarcinoma cells. In this study, we provided evidence supporting the contention that SNPE significantly induces G_2/M arrest and apoptosis in HepG_2 cells in a dose-dependent manner, thus providing its therapeutic potential in treating HCC.

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Table 1. Characterization of phenolic compounds of SNPE

Peak no.	Retention time (min)	Assigned identity	Recovery (%)	UV λ_{max} (nm)	$[M-H]^-$ (*m/z*)	LC-ESI-MS/MS[a] (*m/z*)
1	8.18	GA	1.100 ± 0.32	270, 225	168.9	125.0
2	14.43	PCA	4.555 ± 1.44	222, 259	153.1	108.9
4	22.07	GC	1.378 ± 0.63	272	305.1	216.6
5	24.52	CA	7.179 ± 1.13	256, 354	178.9	135.0
6	26.08	GCG	4.738 ± 0.93	276	457.1	338.3
7	32.64	R	3.001 ± 0.87	256, 354	609.2	301.1, 343.1
8	50.27	Q	2.304 ± 0.48	266, 368	301.3	151.0, 179.0
9	54.87	N	4.539 ± 1.45	231, 288	271.1	150.9, 177.1

[a] LC-ESI-MS/MS, liquid chromatography–electrospray ionization–tandem mass spectrometry; MS/MS run with 30% collision energy.
GA, gallic acid; PCA, protocatechuic acid; GC, gallocatechin; CA, caffeic acid; GCG, gallocatechin gallate; R, rutin; Q, quercetin; N, naringenin.

MATERIAL AND METHODS

Preparation and characterization of SNPE

The whole plant of *S. nigrum* L. was collected from a mountain region in Miaoli country, located in central Taiwan. *Solanum nigrum* L. water extract (SNWE) was prepared as described previously[4,14] and then used for SNPE preparation. Briefly, the sun-dried *S. nigrum* L. was mixed with water for 30 min and subjected to continuous hot extraction at 100 °C for 40 min. The resulting water extract was filtered and subsequently concentrated in a water bath at 90 °C until it became creamy, and then dried in an oven at 70 °C. The analysis of SNWE revealed that it contained 20.4% ± 0.97% polyphenol with gallic acid and quercetin as the standards, 14.9% ± 1.3% polysaccharide and 4.8% ± 0.4% protein. For preparing the polyphenol extract of *S. nigrum* L. (SNPE), 100 g dried SNWE powders was mixed with 300 mL ethanol and heated at 50 °C for 3 h. The extract was filtered and then lyophilized under reduced pressure at room temperature. The powder was resuspended in 500 mL of 50 °C distilled water, followed by extraction with 180 mL ethyl acetate three times, redissolved in 250 mL distilled water, and stored at −70 °C overnight and lyophilized. Finally, the resulting powder was resuspended in distilled water and filtered with a 0.22 μm filter for use in the following experiments. The presence and proportion of the main constituents of SNPE were identified as gallic acid (1.10%), protocatechuic acid (4.56%), gallocatechin (1.38%), caffeic acid (7.18%), gallocatechin gallates (4.74%), rutin (3.00%), quercetin (2.30%), naringenin (4.54%) and unknown components (Fig. 1). Further identification of the nine components was established from recorded mass spectra (Table 1). The polyphenol content of SNPE was estimated to be about 58.86% ± 0.19% with gallic acid and quercetin as the standards.

Cell culture

$HepG_2$, a human liver cancer cell line, was cultured in Dulbecco's modified Eagle's medium supplemented with 100 μL mL^{-1} bovine serum, 2 mmol L^{-1} glutamine, 100 U mL^{-1} penicillin, 100 mg mL^{-1} streptomycin sulfate, 0.1 mmol L^{-1} MEM nonessential amino acids, and l mmol L^{-1} sodium pyruvate. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2. Cell lysates were further subjected to immunoprecipitation assays and western blot analysis, performed as described previously.[4,14]

Cell viability assay

Cells were seeded in 24-well plates at a density of 4×10^4 per well and treated with 0–1 mg mL^{-1} SNPE for 24 h. Cells were then incubated with medium containing 100 μmol L^{-1} 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for another 4 h. The viable cell number was directly proportional to formazan production and was measured by spectrophotometry at 563 nm.[4]

Flow cytometry analysis

Cell cycle progression was analyzed as described previously.[15] Briefly, cells treated with or without SNPE were harvested for propidium iodide (PI) staining. The cell cycle distribution was presented as the number of cells *versus* the amount of DNA as shown by the intensity of fluorescence, and the extent of apoptosis was determined by counting the cells with DNA content below the sub-G_1 peak.

Immunoprecipitation and western blot analysis

$HepG_2$ cells were lysed directly in radioimmunoprecipitation assay (RIPA) buffer (50 mmol L^{-1} Tris-HCl (pH 7.8), 150 mmol L^{-1} NaCl, 5 mmol L^{-1} ethylenediaminetetraacetic acid (EDTA), 5 μL mL^{-1} Triton X100, 5 μL mL^{-1} Nonidet-P40, 1 μL mL^{-1} sodium deoxycholate). Cell lysates were mixed with antiserum against cyclin B (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and the immunocomplexes were collected on protein A/G-Sepharose beads (Amersham Pharmacia Biotech, Little Chalfont, UK). The immunoblotting of predicated proteins were performed as described previously.[4,14] Anti-CDK1 antibody was purchased from BD Biosciences, San Jose, CA, USA. Anti-CDC25A antibody, anti-CDC25B antibody, anti-CDC25C antibody, anti-caspase 9 antibody, anti-caspase 3 antibody, anti-caspase 8 antibody, anti-Fas antibody, and anti-Bcl-2 antibody were purchased from Santa Cruz Biotechnology, Inc. (CDC25 is cell division cycle 25; CDK is cyclin-dependent kinase.) Anti-β-actin antibody was purchased from Chemicon (Temecula, CA, USA).

Xenograft tumor assay

Five-week-old athymic nude mice were obtained from the National Laboratory Animal Center, Taiwan, housed in cages and maintained at a temperature of 22 ± 2 °C and humidity 65% ± 5% in a controlled animal facility with a 12 h light–dark cycle and allowed *ad libitum* access to water. First, 5×10^6 $HepG_2$ cells in 400 μL matrigel were implanted into the right flank of nude mice, resulting in tumor formation. Mice were then randomly divided into three groups (five mice per group), and fed with 5 g of daily basal diets containing 1 or 2 μg mL^{-1} (w/v) SNPE for 35 days,

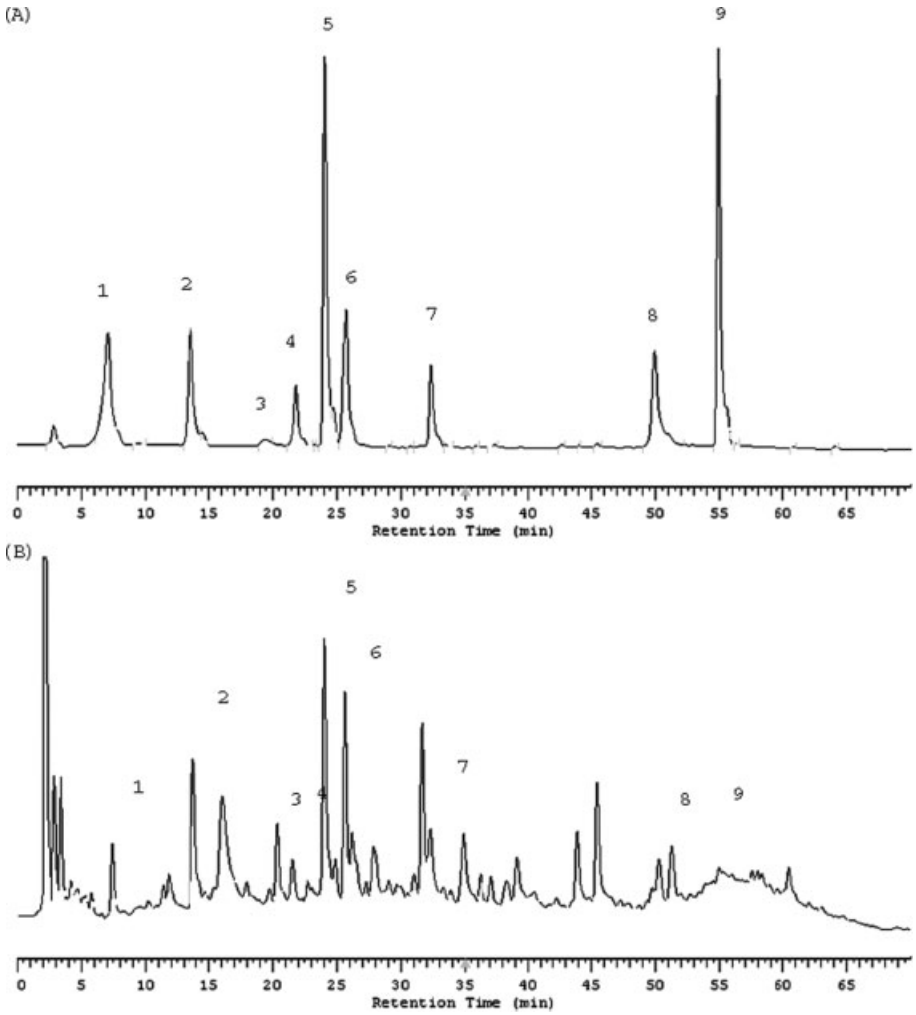

Figure 1. High-performance liquid chromatograms of SNPE. (A) Chromatogram of nine kinds of standard polyphenols (1 mg mL^{-1}; 10 µL). Peaks: 1, gallic acid; 2, protocatechuic acid; 3, catechin; 4, gallocatechin; 5, caffeic acid; 6, gallocatechin gallate; 7, rutin; 8, quercetin; 9, naringenin. (B) Chromatogram of free polyphenols from SNPE (10 mg mL^{-1}, 10 µL).

respectively. Tumor volume was measured every week. On the last day of the observational period, the mice were sacrificed and the tumor xenografts were dissected for final volume and wet weight measurement.

Statistical analysis

All data were analyzed by Student's *t*-test and represented as the mean ± SD. The difference was considered statistically significant at $P < 0.01$.

RESULTS

SNPE inhibits the growth of hepatic tumor xenograft in athymic nude mice

To evaluate the potential preventive effect of SNPE on HCC development, we measured the $HepG_2$ cells growth in athymic mice. The tumor sections were used for analysis of weight and volume. Mice fed 1 µg mL^{-1} SNPE showed more than a 90% decrease in tumor weight as well as tumor volume, compared to the control mice. Notably, treatment with 2 µg mL^{-1} SNPE resulted in almost complete inhibition of both tumor weight and tumor volume (Fig. 2(A–C), $P < 0.01$). The data indicate that SNPE significantly suppresses hepatic tumor growth *in vivo*.

SNPE reduces the viability of hepatocarcinoma cells

To examine whether SNPE could be an anticancer chemotherapeutic agent *in vitro*, we further determined the cytotoxicity of SNPE in $HepG_2$ cells. When $HepG_2$ cells were separately incubated with 0.5, 1.0 and 2.0 mg mL^{-1} SNPE for 24 h, the percentage of viability was 85%, 27% and 6%, respectively. The concentration of SNPE on the inhibition of 50% of $HepG_2$ cell viability (IC_{50}) was 0.75 mg mL^{-1}. As expected, adding SNPE led to a cytotoxic effect in a dose-dependent manner (Fig. 3, $P < 0.01$) and this result is consistent with the above *in vivo* data (Fig. 2).

SNPE induces G_2/M phase arrest through CDK1 and CDC25s regulation

Because of the significant cytotoxic effect of SNPE in $HepG_2$ cells, we considered whether SNPE-mediated reduction of total cell numbers had resulted from its regulation of the cell cycle or from apoptosis. First, to check whether SNPE could inhibit cell growth through cell cycle arrest, $HepG_2$ cells stimulated with SNPE were harvested for analyzing the cell cycle progression by flow

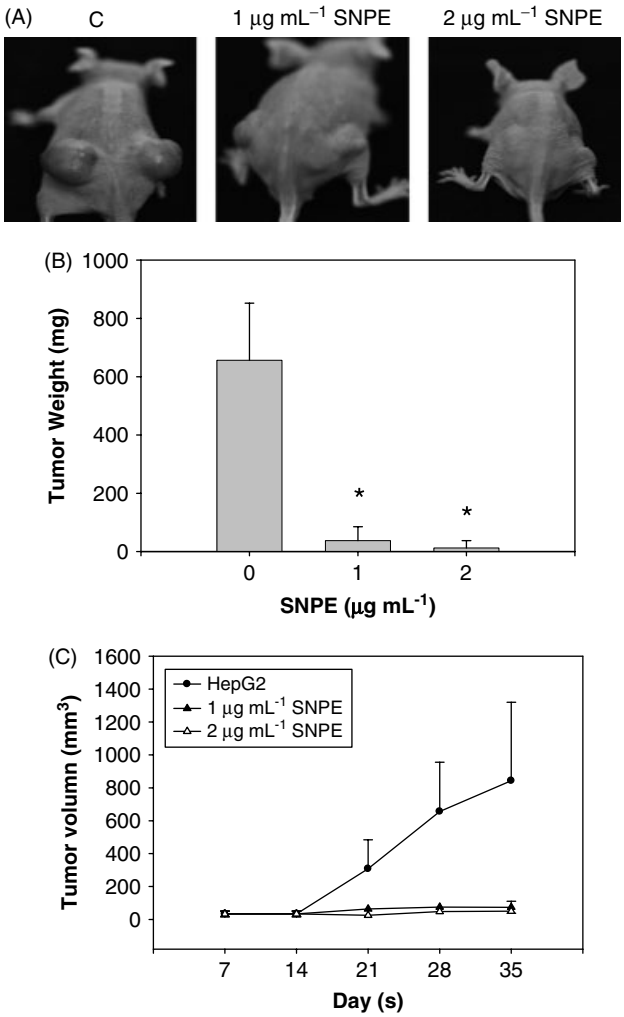

Figure 2. SNPE inhibits hepatocellular tumor growth in athymic nude mice. Athymic nude mice implanted with $HepG_2$ xenografts were treated with SNPE as indicated, for 35 days. (A) Tumor formation was visualized by photography. (B) Tumor weight and (C) tumor volume are represented as mean ± SD, $n = 5$; $^*P < 0.01$ when compared with control. C, control group.

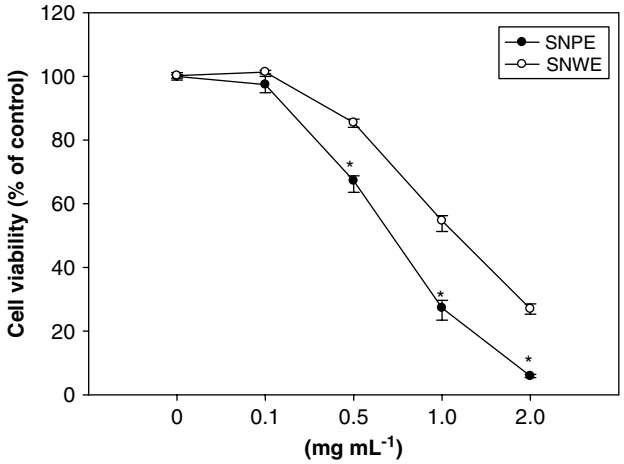

Figure 3. Cytotoxic effect of SNPE in hepatocarcinoma cells. $HepG_2$ cells were stimulated with SNPE or SNWE for 24 h and then subjected to MTT assay. Cell viability was represented as percentage of control cells. Data are expressed as mean ± SD from three independent experiments; $^*P < 0.01$ when compared with control. SNPE, *Solanum nigrum* L. polyphenolic extract; SNWE, *Solanum nigrum* L. water extract.

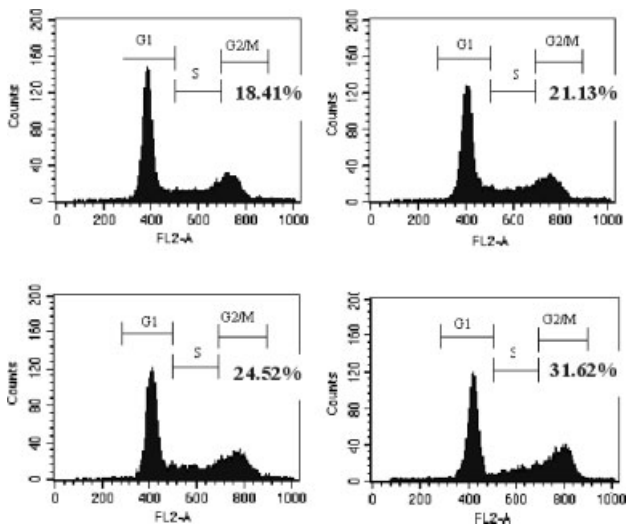

Figure 4. SNPE arrests $HepG_2$ cells in G_2/M phase. $HepG_2$ cells were treated with indicated concentration of SNPE for 24 h. The DNA content of cells was labeled with propidium iodide (PI) and analyzed by flow cytometry. The representative images show the PI staining profile of $HepG_2$ cells from three independent experiments.

cytometry. With increased amounts of SNPE, $HepG_2$ cells showed a gradually increased cell population in the G_2/M phase. The percentage of arrested cells increased to 21.13, 24.53, and 31.62, respectively, compared with the control (Fig. 4).

To progress from the G_2 to M phase, CDK1 needs to be activated through coupling with its cofactor, cyclin B. The above data showing SNPE leading $HepG_2$ cells to arrest in the G_2/M phase led us to examine whether SNPE could interrupt CDK1/cyclin B complex formation by co-immunoprecipitation experiments. The interaction between CDK1 and cyclin B apparently decreased with increased amounts of SNPE (Fig. 5(A)). The data imply that the ability of SNPE-mediated G_2/M phase arrest is due its downregulation of CDK1 activity. Also, CDC25, being a CDK1 activator which in turn regulates G_2/M transition, was considered a putative target of SNPE. The result showed that with increased amounts of SNPE, the protein level of the CDC25 family, including CDC25A, CDC25B, and CDC25C, is clearly reduced (Fig. 5(B)). Together, this evidence suggests SNPE delays G_2/M transition through regulating the activity of CDC25 family and CDK1.

SNPE triggers apoptosis through caspase activation and Bcl-2 family regulation

Next, we further consider whether the apoptotic trigger was the cause of the reduced viability of hepatocarcinoma cells by SNPE. Analyzing the fractional DNA content of cells clearly demonstrated that the sub-G_1 fraction of $HepG_2$ cells significantly ascended stepwise with increasing amounts of SNPE (Fig. 6(A, B), $P < 0.01$). Further, caspase activation, another early event of apoptosis, was measured by immunoblotting. Following cleavage, activated forms of cysteine aspartyl-specific protease (caspase) 8, 9 and 3 from pro-caspase 8, 9, and 3 were generated in a dose-dependent manner (Fig. 7(A), lower panel, upper panel and middle panel, respectively). In line with this notion, the Fas-mediated apoptotic signaling pathway upon binding to the death receptor showed

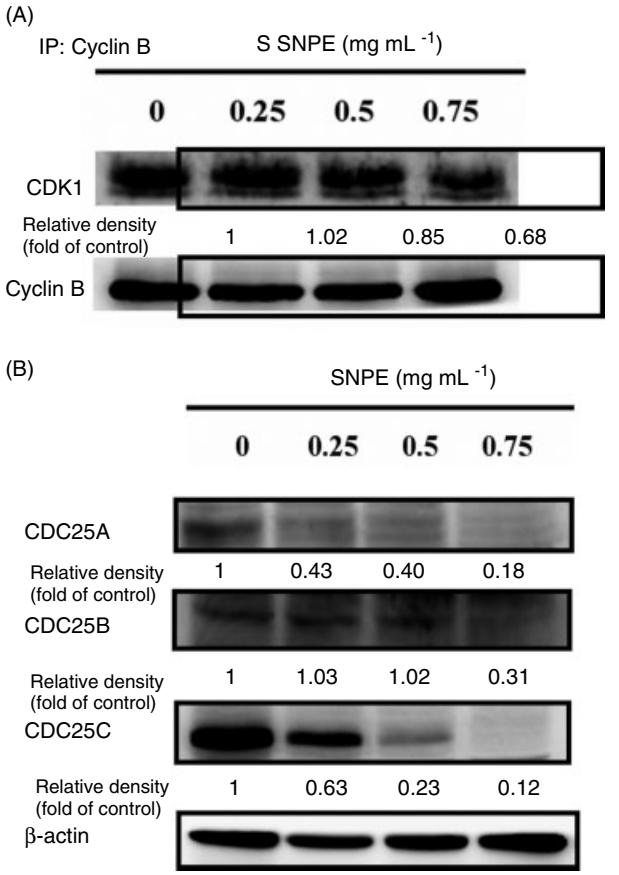

Figure 5. Effect of SNPE on CDK1/cyclin B complex formation and CDC25s protein level. (A) $HepG_2$ cells were stimulated with indicated concentration of SNPE for 24 h. Cells were lysed and subjected to immunoprecipitation experiments with anti-cyclin B antibody. The immunoprecipitates were further analyzed by western blot with anti-CDK1 and anti-cyclin B antibodies as indicated. (B) Cells treated as described in (A) were lysed and subjected to western blot with anti-CDC25A, anti-CDC25B, and anti-CDC25C antibodies. β-Actin was used as a loading control. Data are representative of three independent experiments and quantified by densitometric analysis. Expression levels were normalized to β-actin protein level.

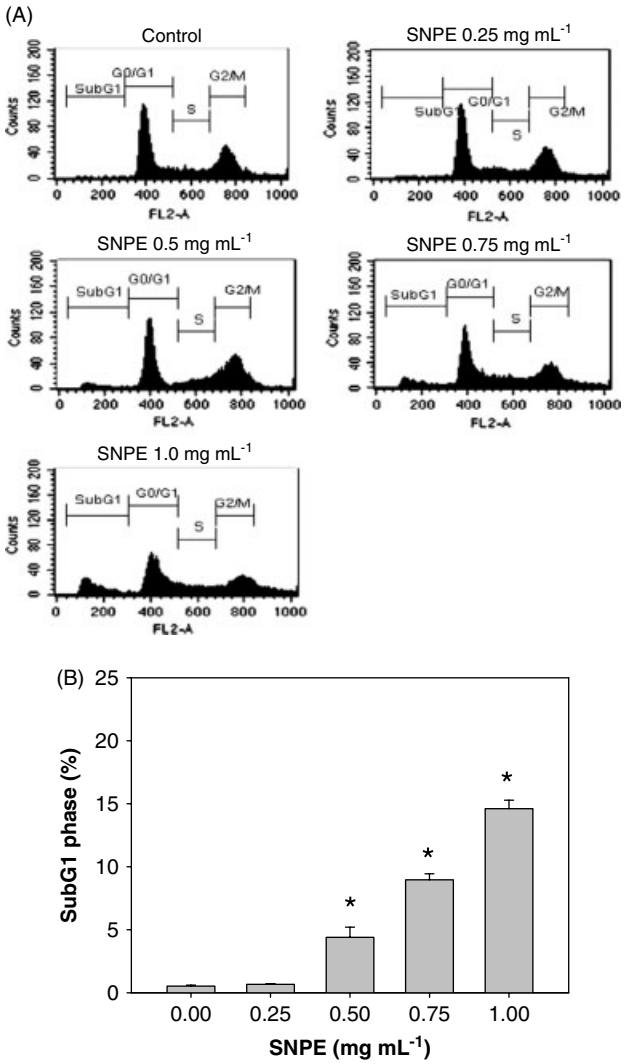

Figure 6. SNPE induces sub-G_1 fraction accumulation in $HepG_2$ cells. (A) $HepG_2$ cells were treated with indicated concentration of SNPE for 24 h. The DNA content of cells was labeled with propidium iodide (PI) and analyzed by flow cytometry. The representative images show the PI staining profile of $HepG_2$ cells. (B) Quantification of sub-G1 fraction was represented as mean $\pm$ SD from three independent experiments; $^*P < 0.01$ when compared with control.

a significant increase in protein levels in the presence of SNPE (Fig. 7(B), upper panel). Bcl-2 and Bid, apoptosis-related proteins, represented a significant decrease in protein expression, indicating that SNPE is involved in the apoptosis signaling pathway (Fig. 7(B), second and third panels). Collectively, the above evidence strongly supports the notion that SNPE blocks hepatocarcinoma cell growth by arresting the G_2/M phase as well as undergoing apoptosis.

DISCUSSION

A wide range of naturally occurring substances present in our daily diet have cancer-preventing properties, turning attention from chemoprevention toward diet-based intervention.[16,17] In this regard, polyphenolic compounds are plentifully found in fruits, vegetables and some beverages. These have been reported to inhibit growth and induce apoptosis in various cancer cell lines.[4–6,8–12,16,17] Phenolic acid and polyphenol, two major representative polyphenolic compounds identified in *S. nigrum* L. with gallic acid and quercetin as standards, have been extensively studied as chemopreventive agents in several cancer models because of their antioxidant, anti-inflammation and antitumor activities. Our *in vivo* data showed a dramatic effect on antitumor activity compared with others reports (Fig. 2). According to the study by Raina *et al.*,[18] 3 $\mu g\ mL^{-1}$ gallic acid feeding inhibits prostate cancer growth in mice by suppressing cell cycle progression and cell proliferation as well as increased apoptosis. Also, Jeong *et al.* showed that physiologically relevant doses of quercetin (0–10 $\mu mol\ L^{-1}$, responding to 0–3 $\mu g\ mL^{-1}$) have chemopreventive efficacy through inhibiting cell cycle progression.[19] Actually, quercetin and quercetin metabolites were widely distributed in rat tissue and exerted their actions *in vivo* even with 1 $\mu g\ mL^{-1}$ quercetin diet administration.[20] Because we characterized several types of polyphenols other than gallic acid and quercetin in *S. nigrum* L., the notion of effective antitumor activity arising from a low-dose SNPE diet may be due to the combined effects of the multiple polyphenolic compounds rather than any single compound alone. Indeed, the study of comparing

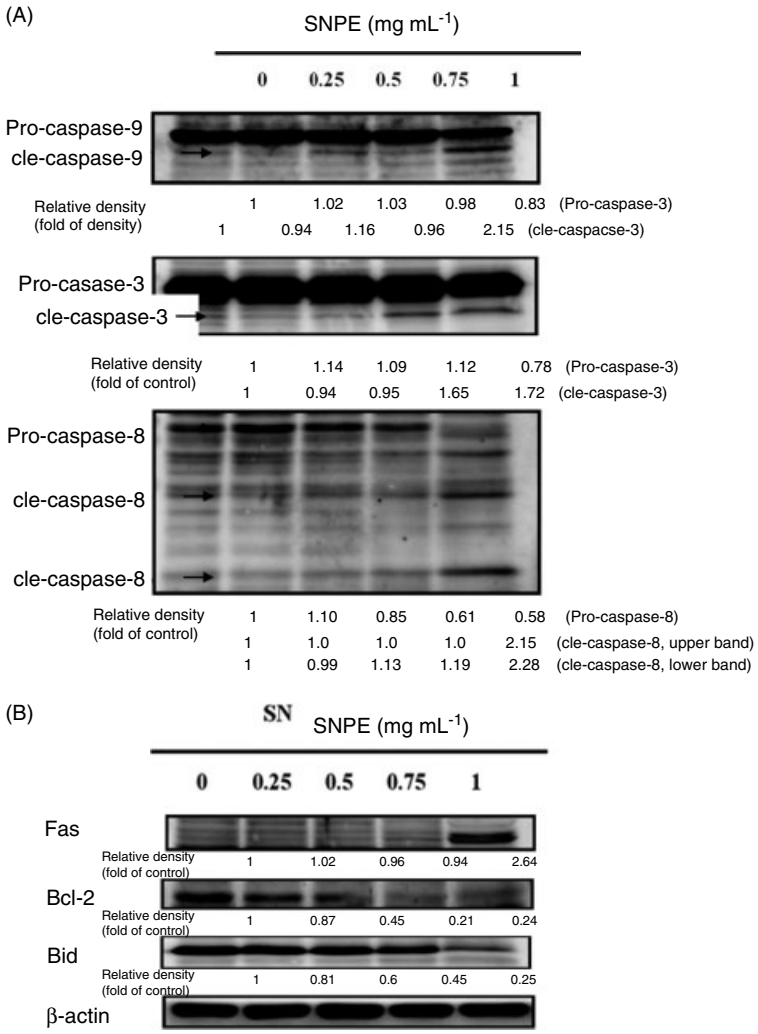

Figure 7. SNPE triggers caspase cascade activation, leading to apoptotic morphology. HepG$_2$ cells were stimulated with indicated concentration of SNPE for 24 h. Cells were lysed and subjected to western blot with (A) anti-caspase 3, 8, and 9 antibodies, and (B) anti-Fas, anti-Bid, and Bcl-2 antibodies as indicated. β-Actin was used as a loading control. Arrowhead and arrow denote the pro and cleaved form of caspases, respectively. Data are representative of three independent experiments and quantified by densitometric analysis. Expression levels were normalized to β-actin protein level.

the ability of four flavonoids and one whole herb mixture to suppress aberrant crypt foci and apoptosis in rat colon cancer model could support this conclusion. Based on this previous report, it has been shown the most effective concentrations for quercetin alone and one whole herb mixture containing quercetin were 30 000 and 50 mg L^{-1}, respectively,[21] indicating that the combinative effects of the polyphenolic compounds is much better than any single compound alone. Although several studies have pointed out that polyphenolic compounds show their anti-proliferation abilities in hepatocellular carcinoma mediated by either apoptosis or cell cycle arrest,[19,22–25] whether the antineoplastic activity of SNPE is mediated through the same mechanism(s) needs to be confirmed by further animal experiments.

Nowadays, the search for novel agents to treat human malignancies has tended to focus on targeting the cell cycle and apoptotic pathway. Our findings have provided the first demonstration that SNPE could greatly benefit HCC treatment because of its significant growth inhibition of hepatocarcinoma cells through G$_2$/M arrest as well as the apoptotic pathway. Our previous study demonstrated that the extract of *S. nigrum* L. selectively kills hepatocarcinoma cells rather than normal cells.[4] Additionally, the polyphenolic extract of *S. nigrum* L. was more cytotoxic to hepatocarcinoma cells than the entire plant, implying that polyphenolic extract has better therapeutic potential in treating HCC (Fig. 3).

Cell cycle progression is tightly controlled by cyclical activation of the cyclin-dependent kinase complexes. The complexes are composed of CDKs and cofactors such as cyclins, thus suggesting that CDKs and cofactors are potential targets for cancer therapeutics.[26] Complex formation between CDK1 and cyclin B was decreased in response to SNPE (Fig. 5(A)). Whether SNPE manipulates CDK1 activity remains to be examined. Elevated CDC25 protein levels could probably contribute to the progression of hepatocarcinoma cancers through dysregulation of the cell division cycle.[27–29] Corresponding to this observation, decreased CDC25 protein expression increases the potential of SNPE as a promising therapeutic agent for treating HCC (Fig. 5(B)).

There are two main caspase cascades responsible for apoptotic morphology. One is the extrinsic pathway characterized by the initial activation of death receptors and the resulting activation of caspase-8. The other is the intrinsic pathway characterized by

depolarization of the mitochondrial membrane, leading to caspase 9 activation. Finally, activated caspase 8 and 9 promote caspase 3 cleavage and activation.[13] In our study, activated caspases (3, 8, and 9) and increased Fas expression were detected in the presence of SNPE, implying that SNPE could activate caspase 3 through an amplifying cascade mediated by both the extrinsic and intrinsic pathways (Fig. 7(A)). Moreover, increased Bcl-2 expression is observed in several tumors, highlighting Bcl-2 as a therapeutic target.[30] Several previous reports showed that decreased Bcl-2 protein is detected in the presence of *S. nigrum* L. or its isolated substance.[4,5] Consistent with other reports, we also found that SNPE significantly reduces Bcl-2 protein levels (Fig. 7(B)). Recent studies showed that mitogen-activated protein kinase (MAPK) activation is required for cancer cell apoptosis induced by anticancer agents.[30] To understand the detailed molecular mechanism of SNPE-mediated apoptosis, we examined the status of MAPK activity by the specific phosphorylated antibody. The result indicated that the p38, extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) activities were not altered by SNPE, suggesting $HepG_2$ cell death initiated by SNPE is not triggered through the MAPK signaling pathway (data not shown). Taken together, our evidence suggests that SNPE could reactivate apoptosis machinery in hepatocarcinoma cells. Based on the present *in vitro* study of $HepG_2$ cells, it was anticipated that SNPE action against HCC growth might also involve cell cycle and apoptosis regulatory mechanisms. The potential molecular mechanisms of SNPE efficacy needed to be further established in a mouse HCC model.

Polyphenolic compounds, mainly flavonoids and steroids, have been demonstrated to exert excellent antioxidative effects. Several studies have shown that *S. nigrum* L. can scavenge free-radical-mediated DNA damage.[31,32] Also, it has been suggested that glycoprotein isolated from *S. nigrum* L. induces apoptosis in hydroxyl-radical stimulated HT-29 cells through inhibiting DNA-binding activity of NF-κB.[33] We have found that SNPE clearly triggered $HepG_2$ cell apoptosis by sub-G_1 fraction accumulation (Fig. 6) as well as DNA fragmentation (data not shown). Whether SNPE induces DNA damage by altering NF-κB activation needs to be further evaluated.

To date, there have been no significant new developments in treating HCC. Surgical resection remains the most effective treatment of HCC. However, patients often have a high rate of relapse. In this study, we that demonstrated SNPE could suppress hepatocarcinoma cell growth and this effect is due to its ability to induce G_2/M phase arrest and apoptosis. Therefore, our finding suggests that SNPE could be a promising agent in treating HCC and needs to be further explored.

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