Apoptotic death in curcumin-treated NPC-TW 076 human nasopharyngeal carcinoma cells is mediated through the ROS, mitochondrial depolarization and caspase-3-dependent signaling responses

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Abstract. Curcumin, a potent candidate anticancer agent, is a dietary pigment (phenolic compound) derived from the food flavoring spice turmeric (*Curcuma longa*), and it has been shown to have inhibitory effects on tumor cells through anti-proliferative and proapoptotic activities. However, there is no report showing curcumin-induced apoptotic cell death in human nasopharyngeal carcinoma cells *in vitro*. Thus, this study was performed to elucidate whether mitochondria and caspase cascades are involved in the modulation of apoptosis and cell cycle arrest in curcumin-treated NPC-TW 076 human nasopharyngeal carcinoma cells. The effects of curcumin on cell cycle arrest and apoptosis were measured by flow cytometry, and caspase-3 activity, apoptosis-associated protein levels and its regulated molecules were studied by flow cytometric assay and immunoblots. The results indicated that curcumin-induced G2/M phase arrest was associated with a marked decrease in the protein expression of cyclin A, cyclin B and cyclin-dependent kinase 1 (Cdk1). Curcumin-induced apoptosis was accompanied with upregulation of the protein expression of Bax and downregulation of the protein levels of Bcl-2, resulting in dysfunction of mitochondria and subsequently led to cytochrome *c* release and sequential activation of caspase-9 and caspase-3 in NPC-TW 076 cells in a time-dependent manner. These findings revealed that mitochondria, AIF caspase-3-dependent pathways play a vital role in curcumin-induced G2/M phase arrest and apoptosis of NPC-TW 076 cells *in vitro*.

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Introduction

Cancer is a one of the major cause of death in human populations. Approximately 90% of all human cancers are carcinomas. Nasopharyngeal carcinoma (NPC) is an endemic disease in southern China and Southeast Asia, and the incidence rates are 15 to 50 per 100,000 (1). In Asian population, especially among Chinese people, NPC exists with high frequency (2,3). NPC has a poor prognosis due to the spread to lymph nodes and metastases to other tissues (4). Currently, the treatments of NPC are still problematic, and thus new therapeutic agents are required. Numerous studies have been tried to identify new naturally occurring chemopreventive or chemotherapy agents for using to inhibit cancer *in vitro* and *in vivo* (5-7).

Based on the epidemic studies, it has been demonstrated that among the potential dietary contributors to cancer incidence is turmeric (*Curcuma longa*), a spice consumed frequently by people from Southeast Asia and India, a continent with low incidence of most cancers. Curcumin (diferuloyl-methane), a yellow-colored polyphenol, is isolated from the plant *curcuma longa*, and it has been shown to have anticancer activities in many types of human cancer cells *in vitro* (8) and *in vivo* (9). Curcumin has been shown to inhibit tumor initiation (10) and promotion (11,12) in animal models.

The anticancer activities of curcumin have been shown to be involved in multiple signaling pathways and on many molecular targets that are related to inhibition of cell proliferation, cell cycle arrest, invasion, metastasis, and angiogenesis of tumor cells (13). Curcumin has been used in clinical trials on cancer patients (9). In a phase I study, the safety of curcumin for oral ingestion of curcumin up to 8,000

mg/day for 3 months did not produce toxicity (14) neither in a phase II trial on pancreatic cancer patients with curcumin daily (15). Curcumin influences structurally unrelated membrane proteins (16), and then inserts deep into the cellular membrane in a transbilayer orientation inducing negative curvature in the bilayer (17). Moreover, the promotion of negative curvature by curcumin may cause a direct effect on apoptosis by increasing the permeabilizing activity of the apoptotic protein tBid (18). Our previous studies have also shown that curcumin induced cell cycle arrest and apoptosis in human colon cancer cells (19), acute promyelocytic leukemia HL-60 cells (20), murine leukemia WEHI-3 cells *in vivo* (21), N18 mouse-rat hybrid retina ganglion cells (22), lung carcinoma A549 cells (23), non-small cell lung cancer NCI-H460 cells *in vivo* (24) and *in vitro* (25). However, there is no available report addressing the curcumin-induced apoptosis in human nasopharyngeal carcinoma cells. Therefore, in the present study, we investigated the effects of curcumin on cell cycle distribution and induction of apoptosis in human nasopharyngeal carcinoma NPC-TW 076 cells. Results indicated that curcumin induced G2/M phase arrest and induced apoptosis through a mitochondria-dependent pathway.

Materials and methods

Chemicals and reagents. Curcumin, RNAse, propidium iodide (PI), dimethyl sulfoxide (DMSO), trypan blue, and Triton X-100 were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA). All primary and secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The fluorescent probes $DiOC_6$ for mitochondrial membrane potential, 2',7'-dichlorofluorescin diacetate (DCFH-DA) for hydrogen peroxide (H_2O_2), and Indo 1/AM for Ca^{2+} staining were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Caspase-3, -8, -9 activity assay kits were obtained from OncoImmunin, Inc (Gaithersburg, MD, USA).

Cell culture. The human nasopharyngeal carcinoma NPC-TW 076 cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 Units/ml penicillin and 100 μg/ml streptomycin in 75 cm^2 tissue culture flasks at 37˚C under a humidified 5% CO_2 and 95% air atmosphere as we have previously reported (26).

Morphological changes and percentage of viable cell examinations in NPC-TW 076 cells. NPC-TW 076 cells at density of $2x10^5$ cells/well were placed on 12-well plates and treated with 0, 1, 5, 10 or 25 μM curcumin, or only with vehicle (DMSO, 1% in culture media) then cells were incubated for 24 and 48 h. Then the cells were photographed under a phase-contrast microscope for morphological changes. Cells were harvested for determination of percentage of viable cells by a PI exclusion method and a flow cytometric assay as previously described (22,27). Briefly, an aliquot of the total cell suspension from each well was mixed with PI (5 μg/ml) dye in PBS and immediately determined by using a FACSCalibur instrument (BD, San Jose, CA, USA) equipped with BD Cell Quest Pro software (19).

Determinations of cell cycle distribution and apoptosis of NPC-TW 076 cells by flow cytometric assay. NPC-TW 076 cells at density of $2x10^5$ cells/well were placed on 12-well plates and exposed to 10 μM curcumin, or only with vehicle (DMSO, 1% in culture media), and then cells were incubated for 6, 12, 24 and 48 h. After incubation, cells from each treatment were individually harvested by brief trypsinization then washed twice, and then PBS containing 40 μg/ml PI, 0.1 mg/ml RNase and 0.1% Triton X-100 was added for 30 min at 37˚C and left in the dark. The PI stained cells were analyzed for quantification, cell cycle distribution and sub-G1 phase (apoptosis) by flow cytometry (28,29). The sub-G1 group was representative of the mean apoptosis.

DAPI staining and Comet assay. About $2x10^5$ NPC-TW 076 cells/well on 12-well plates were treated with 0, 1, 5, or 10 μM curcumin, and then all cells were incubated for 24 h under 5% CO_2 and 95% air at 37˚C. The treated cells were divided into two parts for DAPI staining and Comet staining by 4'-6-diamidino-2-phenylindole (DAPI) (Invitrogen) for DAPI staining and PI for Comet assay, respectively. Then all samples were examined and photographed using fluorescence microscopy as described elsewhere (29,30).

DNA gel electrophoresis for apoptosis. Cells ($2x10^6$/well) on 12-well plates were cultured with 0, 10, 20, 30 and 40 μM curcumin for 24 h. Chromosomal DNA was isolated (Genomic DNA Purification kit, Genemark technology Co., Ltd. Tainan, Taiwan) and ladder formation assays were performed by DNA gel electrophoresis as described previously (27,28).

Flow cytometric assay for mitochondrial membrane potential ($\Delta\Psi_m$), reactive oxygen species (ROS) and cytosolic Ca^{2+} release. About $2x10^5$ NPC-TW 076 cells/well on 12-well plates were incubated with 10 μM curcumin for different time periods to measure the levels of $\Delta\Psi_m$, the productions of ROS and cytosolic Ca^{2+}. After cells were incubated for various time periods, all cells from each treatment were harvested, washed twice by PBS, then re-suspended in 500 μl of $DiOC_6$ (1 μmol/l) for evaluation of the level of $\Delta\Psi_m$, 500 μl of DCFH-DA (10 μM) for ROS and in Indo 1/AM (3 μg/ml) for cytosolic Ca^{2+} production in the dark for 30 min at 37˚C, then all samples were analyzed immediately by flow cytometry as described previously (27,29,31).

Determinations of viability and ROS in NPC-TW 076 cells by pretreatment with N-acetylcysteine (NAC) before curcumin treatment. Approximately $2x10^5$ cells/well on 12-well plates were pretreated with 20 mM NAC for 2 h, and then all samples except the control were treated with 10 μM curcumin for 24 h. The cells were harvested and examined for percentage of viability and the ROS productions as described above then assayed by flow cytometry (22,27).

Determination of caspase-3 activity. NPC-TW 076 cells ($2x10^5$) on 12-well plates were incubated with 10 μM curcumin for 0, 24 and 48 h. Cells from each well were harvested and washed twice with PBS, then substrates were used (PhiPhiLux-G_1D_2 for caspase-3, OncoImmunin, Inc.) and the

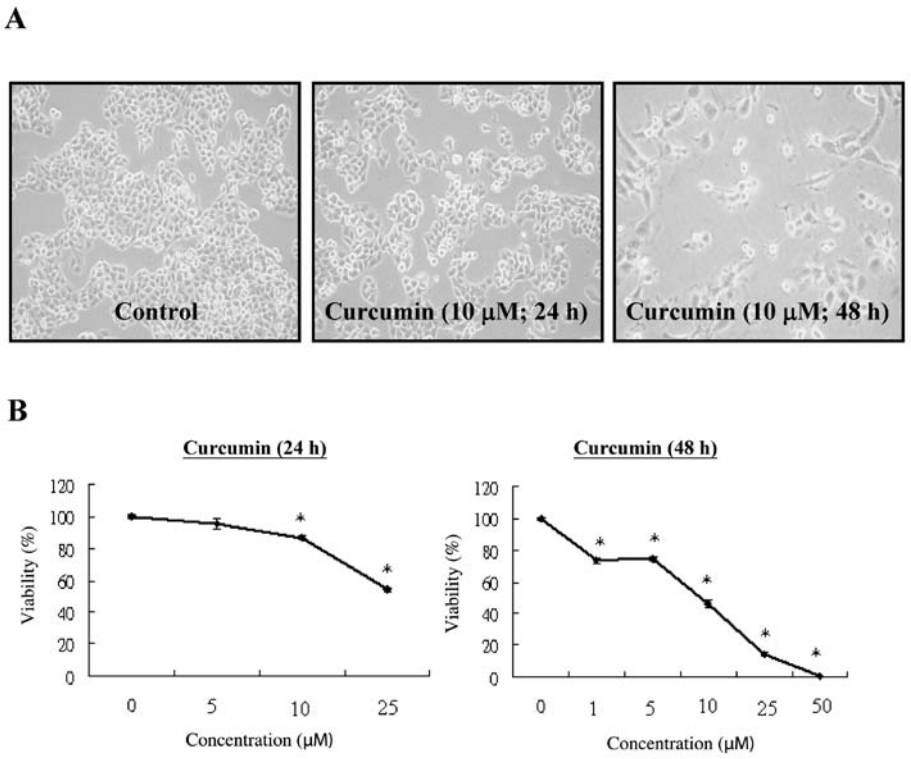

Figure 1. Curcumin induced cell morphological changes and decreased the percentage of viable NPC-TW 076 cells. Cells were treated with 10 μM curcumin for 24 and 48 h. The cell morphological changes were examined and photographed by phase-contrast microscopy (x100) at 24 and 48 h of treatment (A). Cells were treated with 0, 1, 5, 10, 25 or 50 μM curcumin for 24 and 48 h. The percentage of viable NPC-TW 076 cells (B) were determined as described in Materials and methods. Each point is the mean ± SD of three experiments. *p<0.05, significantly different compared with DMSO-treated control and curcumin treatment.

activities of caspase-3 was determinated by flow cytometry as described previously (22,27).

Western blot analysis. Approximately 5x10^6 NPC-TW 076 cells/well on 6-well plates were incubated with 10 μM curcumin for 0, 3, 6, 12, 24, 48 or 72 h. Then cells from each treatment were harvested by centrifugation and washed twice with PBS, then total protein was determined. The protein levels (Cyclin B, CDK1, cdc25c, Bcl-2, cytochrome *c*, pro-caspase-9, PARP, XRCC-1, GADD153, SOD and catalase) associated with cell cycle arrest and apoptosis were determined by Western blotting. Lysates of treated cells from each well or isolated nuclei were prepared by using the PRO-PREP™ protein extraction solution (iNtRON Biotechnology, Seongnam, Gyeonggi-Do, Korea) as described previously (22,27). Each sample was incubated with primary antibody (Santa Cruz Biotechnology Inc.) and then washed twice followed by secondary antibody and then detected with an ECL kit (Millipore, Bedford, MA, USA) and autoradiography using X-ray film (22,27). Each membrane was stripped and anti-β-actin antibody was used to ensure equal protein loading.

Confocal laser scanning microscopy. NPC-TW 076 cells at density of 5x10^4 cells/well were cultured on 4-well chamber slides, and then treated without or with 10 μM curcumin for 24 h. At the end of incubation, cells were fixed directly in 4% formaldehyde in PBS for 15 min then permeabilized with 0.3% Triton X-100 in PBS for 1 h. The fixed cells were stained by using the primary antibody to AIF (1:100 dilution) overnight. Then all cells were washed twice with PBS and were stained with secondary antibody (FITC-conjugated goat anti-mouse IgG at 1:100 dilution) (green fluorescence) followed by DNA staining with DAPI (blue fluorescence) as described previously (22,32). Photomicrography was performed using a Leica TCS SP2 Confocal Spectral Microscope (22,32).

Statistical analysis. The quantitative data are shown as the mean ± SD and results are representative of three independent experiments. The statistical differences between the curcumin-treated and control samples were calculated by Student's *t*-test. A p<0.05 was considered significant.

Results

Curcumin induces morphological changes and decreases percentage of viable NPC-TW 076 cells. Cells were treated with different concentrations of curcumin for 24 and 48 h, and then cell morphological changes were examined and photo-graphed by a phase-contrast microscope. Results are shown in Fig. 1A, indicating that curcumin induced morphological changes of NPC-TW 076 cells. The results revealed that some cancer cells became round, smaller and blunt in size and cells became detached and suspended in the medium, especially for 48 h exposure with 10 μM curcumin. Then all cells from each treatment were harvested by centrifugation for determining the percentage of viable cells by flow cytometric assay. The results shown in Fig. 1B

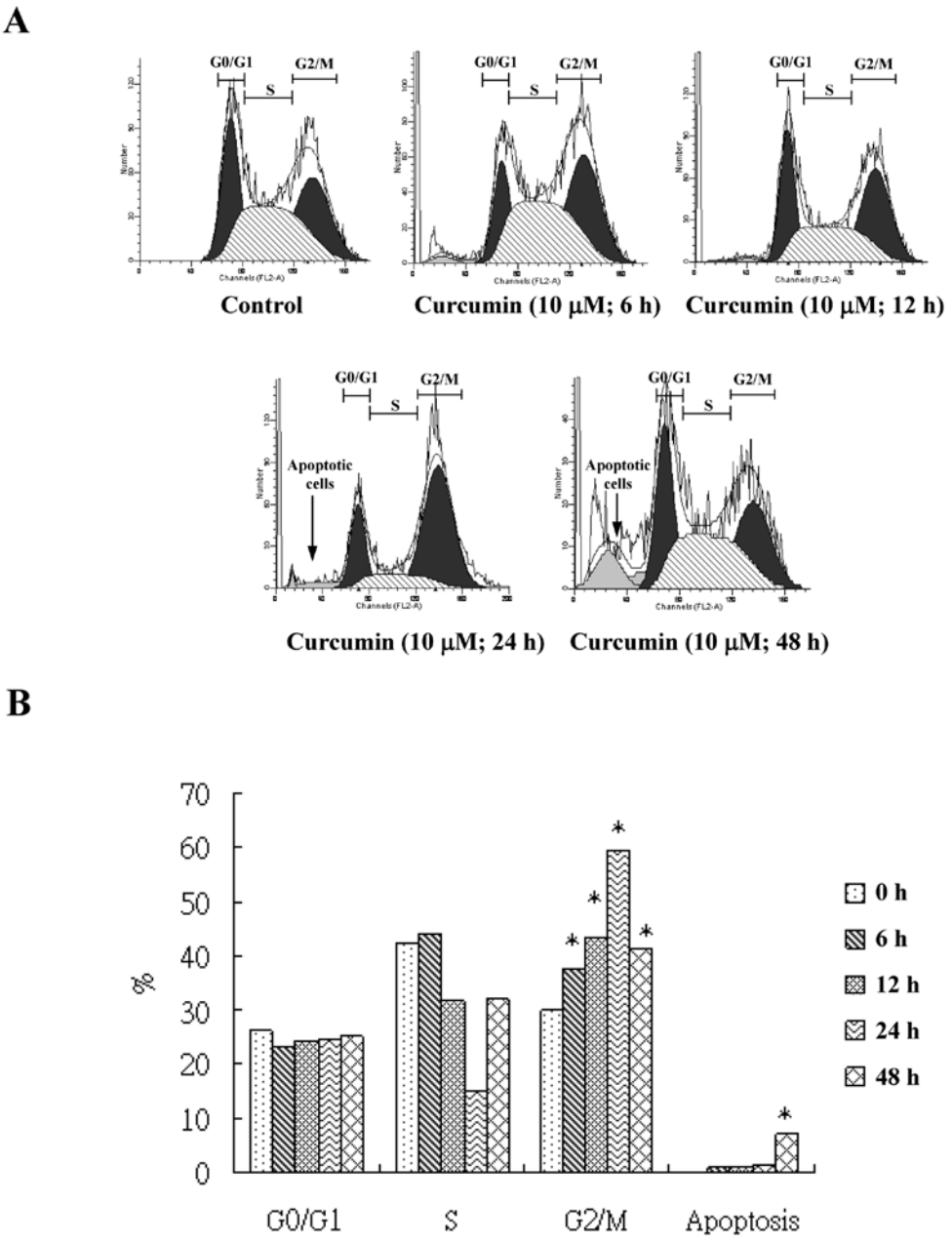

Figure 2. Curcumin induced G2/M phase arrest and apoptosis in NPC-TW 076 cells. Cells were treated with 10 μM curcumin for 0, 6, 12, 24 and 48 h. The cells were harvested and analyzed for cell cycle distribution and apoptosis (A) by flow cytometry. Data from flow cytometic profile were quantified (B) as described in Materials and methods. Each point is the mean ± SD of three experiments. $^{*}p<0.05$, significantly different compared with DMSO-treated control and curcumin-treated groups.

indicate that curcumin decreased the percentage of viable NPC-TW 076 cells in a dose-dependent manner. The percentage of viability decreased by more than 50% in NPC-TW 076 cells after exposure to 10 μM curcumin for 48 h. This concentration of 10 μM curcumin was used in all further experiments in this study.

Curcumin induces cell cycle arrest and sub-G1 (apoptosis) in NPC-TW 076 cells. NPC-TW 076 cells were treated with 10 μM of curcumin for different times then harvested for determining the cell cycle distribution and sub-G1 phase (apoptosis) by using flow cytometric assay. Results are shown in Fig. 2A and B, indicating that the number of cells in each compartment of the cell cycle distribution and the sub-G1 phase (apoptosis) was expressed as a percentage of the total number of cells. The results indicated that curcumin induced G2/M phase arrest and the sub-G1 phase (apoptosis) in a time-dependent manner (Fig. 2B). NPC-TW 076 cells show (Fig. 2A and B) that treatment with 10 μM for 48 h resulted in a higher number of cells in the G2/M phase (65%) compared with the control (28%). This increase was coupled with the decreased percentage of cells in S phase. After 24 h treatment, the percentages of S phase in curcumin-treated cells was 16%, compared to 25% in the control cells. In addition, flow cytometric analysis also revealed the effect of curcumin on the induction of apoptosis. As shown in Fig. 2B, the percentage of

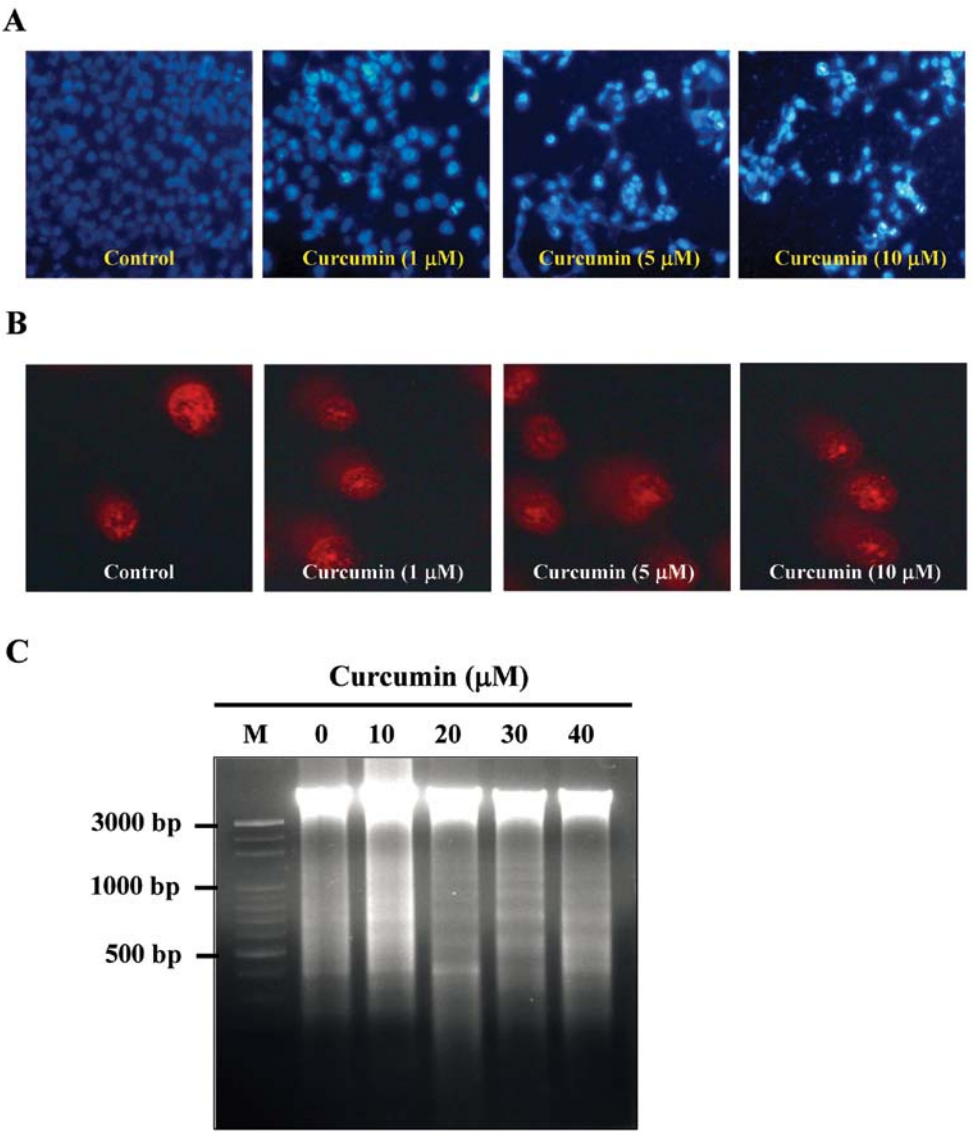

Figure 3. Curcumin induced apoptosis and DNA damage in NPC-TW 076 cells. Cells were treated with various concentrations of curcumin for 24 h. The cells were harvested and were examined for apoptosis by DAPI staining (A), DNA damage by Comet assay (B) and the DNA fragmentation (C) was performed by DNA gel electrophoresis as described in Materials and methods.

the sub-G1 fraction in curcumin-treated cells was increased in a time-dependent manner, indicative of apoptotic cell death.

Curcumin induces apoptosis and DNA damage in NPC-TW 076 cells. Cells were exposed to different concentrations of curcumin for 24 h, and then all samples were harvested for determining apoptosis by DAPI staining and for DNA damage by Comet assay and for DNA fragmentation occurrence by DNA gel electrophoresis. Fig. 3A demonstrates that curcumin induced apoptosis in a dose-dependent manner based on the higher density of white color on the nuclei from a fluorescence microscope examination (DAPI staining). Comet assay demonstrated that curcumin (1, 5 and 10 μM) for 24 h induced DNA damage (Fig. 3B) and DNA gel electrophoresis indicated that curcumin induced DNA fragmentation in a dose-dependent manner (Fig. 3C).

Curcumin changes the levels of mitochondria membrane potential ($\Delta\Psi_m$), reactive oxygen species (ROS) and the production of cytosolic Ca^{2+} in NPC-TW 076 cells. Cells were treated with 10 μM curcumin for different periods of time and then all samples were harvested and the levels of $\Delta\Psi_m$, ROS and cytosolic Ca^{2+} measured by flow cytometric assay. The results are shown in Fig. 4 indicating a significant decrease of $\Delta\Psi_m$ level (Fig. 4A), and the increases of intra-cellular ROS (Fig. 4B) and cytosolic Ca^{2+} level (Fig. 4C) in the curcumin-treated NPC-TW 076 cells. Fig. 4A indicates that curcumin significantly decreased the level of $\Delta\Psi_m$ around 99 to 92% in 1 to 6 h-treatment. Following 0.5 to 3-h treatment of curcumin in NPC-TW 076 cells, it initially significantly increased cytosolic Ca^{2+} level (Fig. 4C). On the contrary, after 3-h treatment, cytosolic Ca^{2+} level did not have significant difference in comparison to that in the control sample (data not shown). However, at the treatment of curcumin at 6 h until 36 h, the

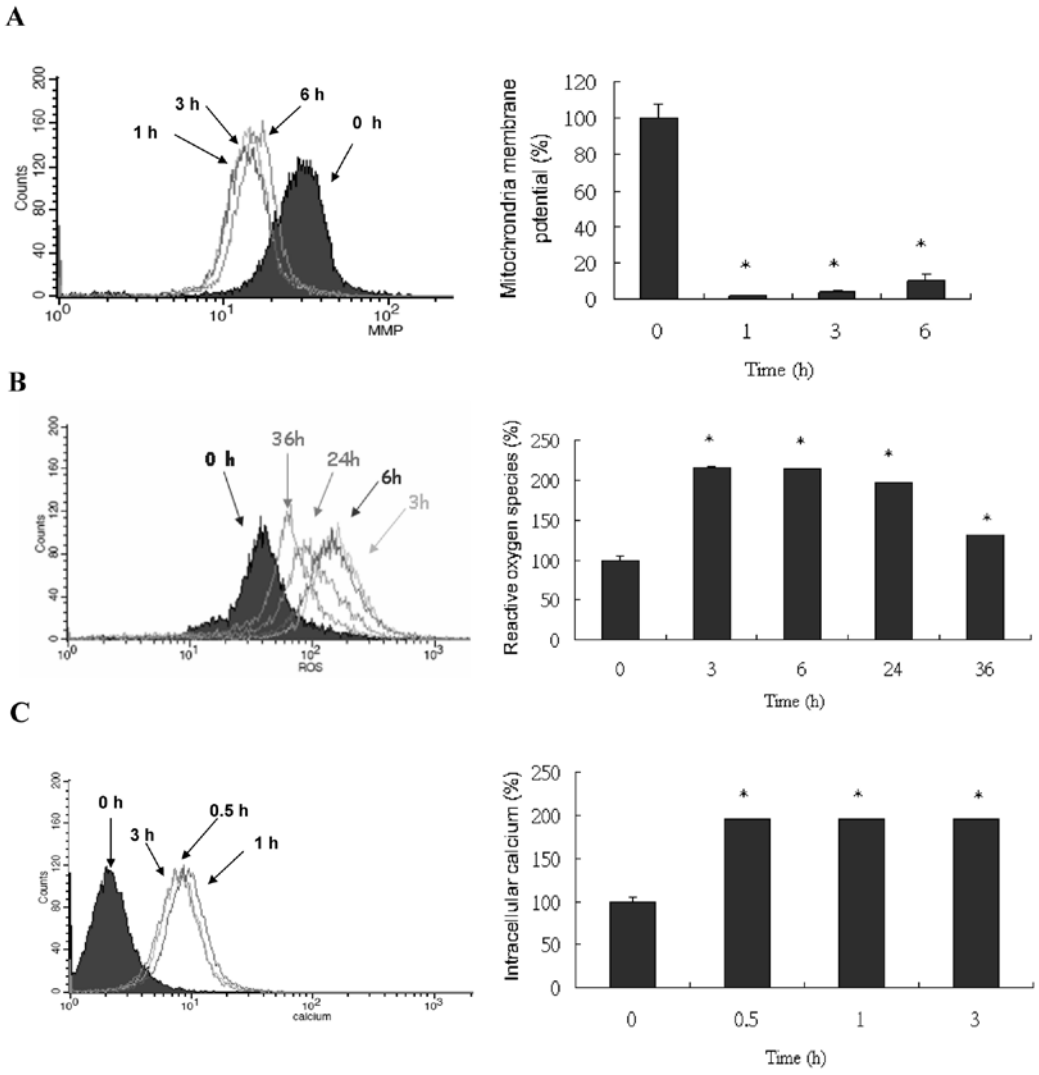

Figure 4. Curcumin altered the levels of mitochondria membrane potential ($\Delta\Psi_m$), reactive oxygen species (ROS) and the production of cytosolic Ca^{2+} in NPC-TW 076 cells. Cells were treated with 10 μM curcumin for 0, 0.5, 1, 3, 6, 24 or 36 h, and then collected, following stain with $DiOC_6$ (1 μmol/l) for the level of $\Delta\Psi_m$ (A), DCFH-DA (10 μM) for ROS (B) and Indo 1/AM (3 μg/ml) for cytosolic Ca^{2+} production (C) as described in Materials and methods. Each experiment was done with triple sets (mean ± SD): $^*p<0.05$, significantly different compared with DMSO-treated control and curcumin-treated groups.

ROS levels were significantly higher than those of the control group (Fig. 4B).

NAC affects curcumin-treated NPC-TW 076 cells on cell viability and ROS productions. Cells were pretreated with 20 mM NAC for 2 h, and then exposed to 10 μM curcumin for 24 h. The cells from each treatment were harvested and examined for percentage of viability and the ROS production. The results shown in Fig. 5, indicate that NAC protected against cell viability of curcumin-treated NPC-TW 076 cells, then led to increase the percentage of viable cells (Fig. 5A) and decreased ROS production (Fig. 5B) in curcumin-treated cells. Based on these results, curcumin-triggered apoptosis is involved in the ROS-dependent pathway.

Curcumin stimulates caspase-3 activity in NPC-TW 076 cells. Cells were treated with or without 10 μM curcumin for 24 and 48 h, and then cells from each treatment were harvested for determination of the caspase-3 activity. The results shown in Fig. 6 indicate that curcumin induced caspase-3 activity in NPC-TW 076 cells. The results suggest that curcumin-induced apoptosis might be mediated through the caspase-3-dependent signal pathway.

Curcumin affects the levels of G2/M phase and apoptosis-associated proteins in NPC-TW 076 cells. Cells were incubated with 10 μM curcumin for 0, 3, 6, 12, 24, 48 and 72 h, and then were harvested for determining the protein levels by Western blotting. The results are shown in Fig. 7, indicating that curcumin decreased Cyclin B, CDK1 and cdd25c (Fig. 7A), Bcl-2, pro-caspase-9 and XRCC1 (Fig. 7B). However, it promoted the levels of cytochrome *c*, PARP, GADD153 (Fig. 7B), SOD and catalase (Fig. 7C). In the nucleic fraction, cyclin B was decreased and AIF was increased (Fig. 7D). The level of anti-apoptosis protein Bcl-2 (Fig. 7B) was decreased. The p53 and p21 were also increased which could contribute to cell cycle arrest and apoptosis.

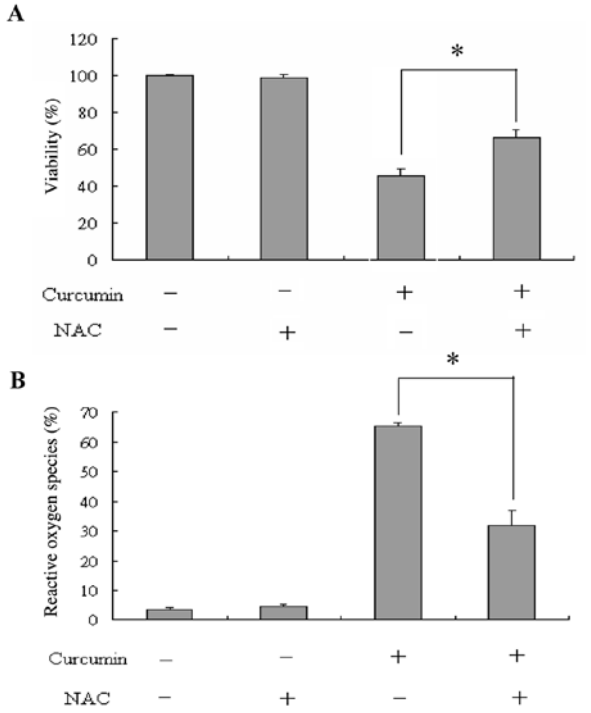

Figure 5. NAC protected the effects of curcumin on the viability and ROS in NPC-TW 076 cells. Cells were pretreated with 20 mM NAC for 2 h before exposure to 10 μM curcumin for 24 h. The cells were harvested and examined for percentage of viability (A) and the productions of ROS (B) were assay by flow cytometric assay as described in Materials and methods. Each point is the mean ± SD of three experiments. *p<0.05, significantly different compared with DMSO-treated control and curcumin-treated groups.

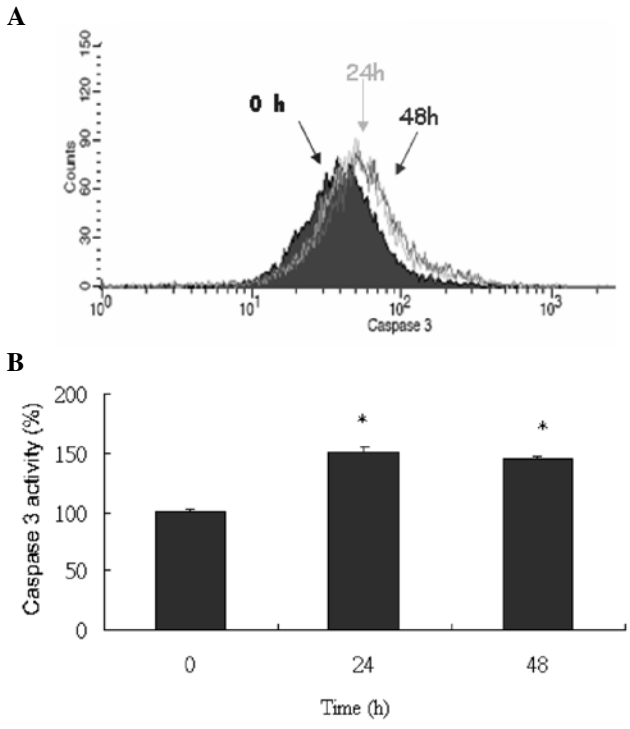

Figure 6. Curcumin stimulated caspase-3 activity in NPC-TW 076 cells. Cells were treated or without 10 μM curcumin for 24 and 48 h. The cells were harvested and washed by using PBS then the caspase-3 activity was measured as described in Materials and methods. (A) Representative profile of caspase-3 activity; (B) quantative data of caspase-3 activity. Each experiment was done in triplicates (mean ± SD): *p<0.05, significantly different compared with DMSO-treated control and curcumin-treated groups.

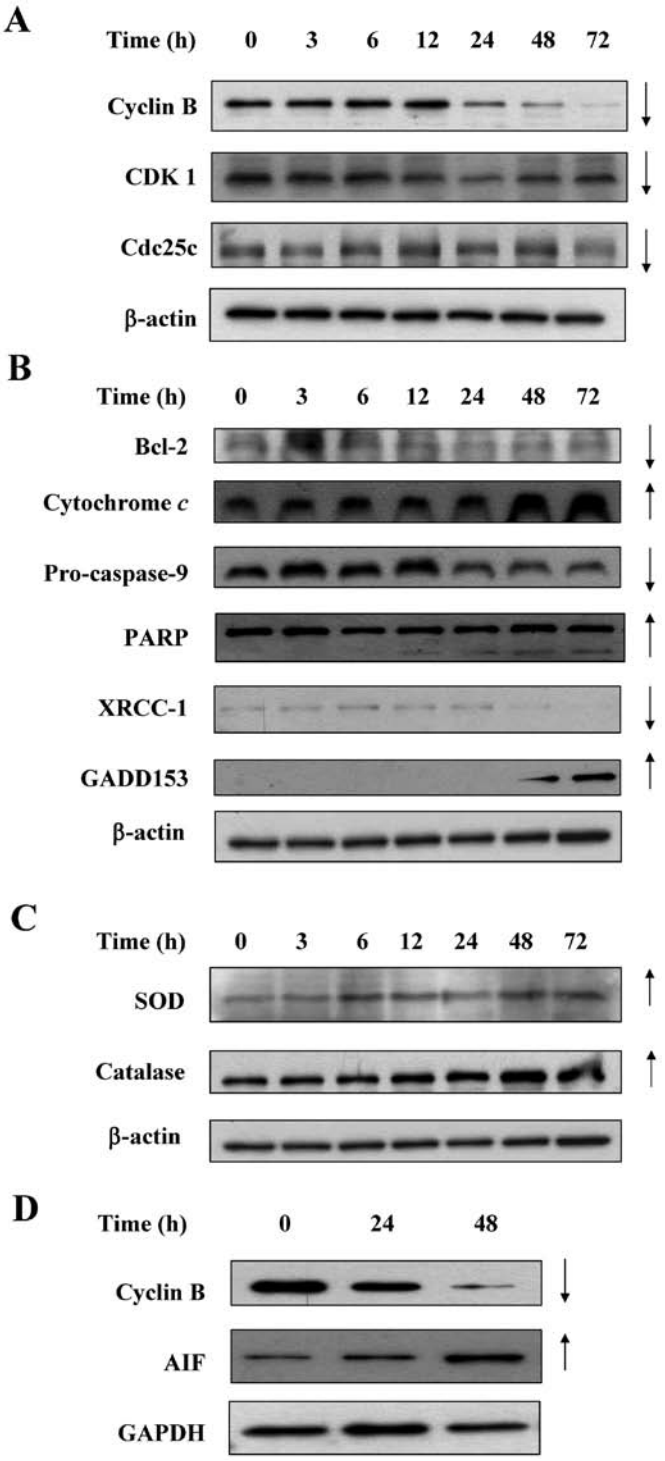

Figure 7. Representative Western blotting showing changes in the levels of apoptosis-associated proteins in NPC-TW 076 cells after exposure to curcumin. Cells were treated with 10 μM curcumin for 0, 3, 6, 12, 24, 48 and 72 h before the total proteins were prepared and determined as described in Materials and methods. The levels of apoptotic relative protein expressions (A) Cyclin B, CDK1, Cdc25c; (B) Bcl-2, cytochrome c, pro-caspase-9, PARP, XRCC-1, GADD153; (C) SOD and Catalase; (D) Cyclin B and AIF were estimated by Western blot analysis, as described in Materials and methods.

Curcumin promotes AIF distribution in NPC-TW 076 cells. Cells were treated with 10 μM curcumin for 24 h, and were harvested for determination of location of AIF. The results

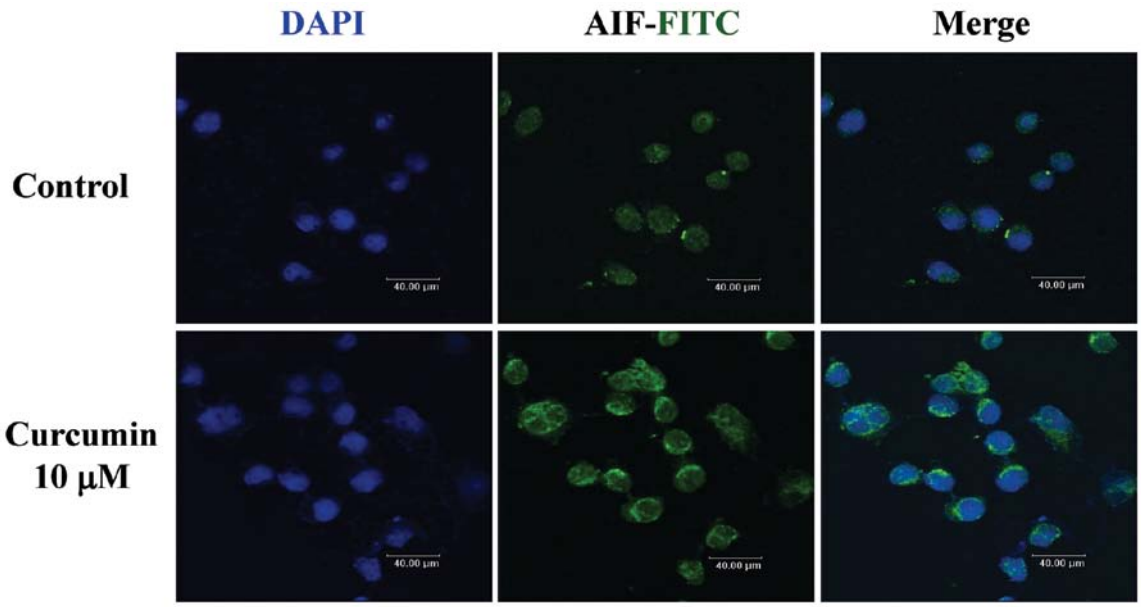

Figure 8. Curcumin translocated AIF distribution in NPC-TW 076 cells. Cells were incubated with 10 μM curcumin for 24 h, and then were fixed and stained with primary antibodies to AIF before FITC-labeled secondary antibodies were used (green fluorescence) and the proteins were detected by a confocal laser microscopy. The nuclei were stained by DAPI (blue fluorescence). Areas of colocalization between AIF expressions, cytoplasm and nuclei in the merged panels are yellow. Scale bar, 40 μm.

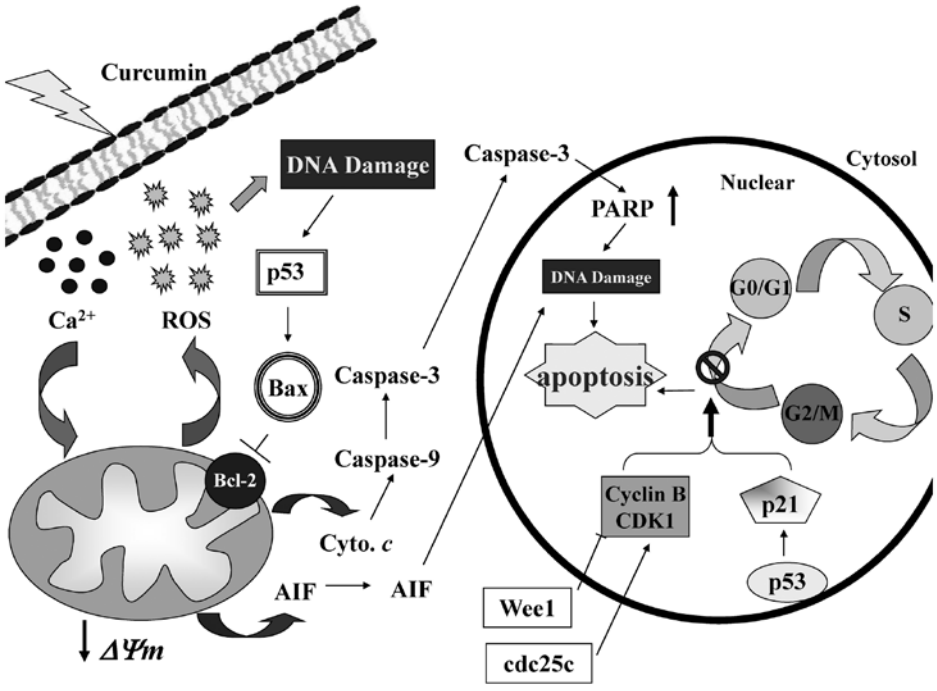

Figure 9. The proposed model of molecular signal pathways from human nasopharyngeal carcinoma NPC-TW 076 cells after treatment with curcumin.

indicated that curcumin promoted the release of AIF from mitochondria to nuclei followed by apoptosis in NPC-TW 076 cells as seen in Fig. 8.

Discussion

The efficacy of treatment with chemotherapeutic agents is largely based on their ability to induce cell death in tumor cells; therefore, the inducer of apoptosis is able to offer a new therapeutic approach design for anticancer agents (33-35). In mammalian cells, the levels of CDKs regulated the cell cycle event (36,37) and among the CDKs such as CDK1 and CDK2 kinases were activated primarily in association with cyclin A and B in the G2/M phase progression (37). In this study, curcumin induced morphological changes and decreased the percentage of viable NPC-TW 076 cells in a dose- and time-

dependent manner. Furthermore, the DNA content indicated that a prominent G2/M phase arrest of NPC-TW 076 cells upon exposure to curcumin (Fig. 2A), and the levels of CDK1, cyclin B and cdc25c proteins were decreased after curcumin treatment in a time-dependent manner (Fig. 7A). Thus, it is plausible that alterations in cell cycle-associated proteins lead to arrest at G2/M phase in curcumin-treated NPC-TW 076 cells.

Our data also demonstrated that curcumin induced apoptosis of NPC-TW 076 cells as verified by condensation of DNA by DAPI staining, sub-G1 phase occurred as shown by flow cytometry. DNA fragmentation from DNA gel electrophoresis indicated a decrease of the level in XRCC1 by Western blotting. It was reported that apoptosis is a tightly regulated process under the control of several different signaling pathways including caspase and mitochondrial pathways (38,39). In the present study, curcumin treatment caused the productions of ROS and Ca^{2+}, but it decreased the level of $\Delta\Psi_m$ in NPC-TW 076 cells (Fig. 4A-C). Furthermore, curcumin promoted the caspase-3 activity in a time-response manner that is consistent with the DNA fragmentation (Fig. 2C).

Herein, we also found that curcumin stimulated the ROS production in NPC-TW 076 cells. However, cells were pretreated with NAC (an antioxidant scavenger) and then treated with curcumin that led to decrease of the level of ROS and increased the percentage of viable NPC-TW 076 cells (Fig. 5A and B). An increase of cytosolic cytochrome *c* was observed in curcumin-treated NPC-TW 076 cells (Fig. 7B). Bcl-2 family proteins including the anti-apoptotic Bcl-2 and the pro-apoptotic Bax proteins have been reported to regulate cytochrome *c* release from mitochondria (39). The proto-oncogene Bcl-2, encodes an inner mitochondrial protein that reportedly antagonizes apoptosis in many tumor cells (40). Interestingly, it was reported that a decreased expression of Bcl-2 might contribute to the drug-mediated lethality (41,42).

Our earlier experiments have revealed that curcumin-induced apoptosis in human non-small cell lung cancer NCI-H460 cells and colon cancer colo 205 cells were associated with decreased Bcl-2 expression (19,32). In the present study, consistent with these observations, down-regulation of Bcl-2, a decrease of the mitochondrial membrane potential ($\Delta\Psi_m$), and the release of cytochrome *c* occurred with curcumin-treated NPC-TW 076 cells. It was reported that the agent distorted mitochondrial function may be *via* an increase in the ratio of Bax and anti-apoptotic (Bcl-2) members leading to an increase in mitochondrial membrane permeability, the release of cytochrome *c*, and activation of caspases (43,44).

In conclusion, curcumin induces cell cycle arrest at the G2/M phase and apoptosis of NPC-TW 076 cells. Curcumin-induced cell cycle arrest was associated with reduction of CDK1, Cyclin B protein expression. Curcumin-induced apoptosis was accompanied with up-regulation of Bax and down-regulation of Bcl-2 leading to cytochrome *c* release, then to activation of caspase-9 and caspase-3, leading to apoptosis of NPC-TW 076 cells.

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References

1. Ho JH: An epidemiologic and clinical study of nasopharyngeal carcinoma. Int J Radiat Oncol Biol Phys 4: 182-198, 1978.
2. Tomatis L: The IARC program on the evaluation of the carcinogenic risk of chemicals to man. Ann NY Acad Sci 271: 396-409, 1976.
3. Liu YH, Du CL, Lin CT, Chan CC, Chen CJ and Wang JD: Increased morbidity from nasopharyngeal carcinoma and chronic pharyngitis or sinusitis among workers at a newspaper printing company. Occup Environ Med 59: 18-22, 2002.
4. Sham JS, Choy D and Choi PH: Nasopharyngeal carcinoma: the significance of neck node involvement in relation to the pattern of distant failure. Br J Radiol 63: 108-113, 1990.
5. Wang LG and Chiao JW: Prostate cancer chemopreventive activity of phenethyl isothiocyanate through epigenetic regulation (review). Int J Oncol 37: 533-539, 2010.
6. Kassie F, Melkamu T, Endalew A, Upadhyaya P, Luo X and Hecht SS: Inhibition of lung carcinogenesis and critical cancer-related signaling pathways by N-acetyl-S-(N-2-phenethyl thiocarbamoyl)-l-cysteine, indole-3-carbinol and myo-inositol, alone and in combination. Carcinogenesis 31: 1634-1641, 2010.
7. Cabrespine-Faugeras A, Bayet-Robert M, Bay JO, Chollet P and Barthomeuf C: Possible benefits of curcumin regimen in combination with taxane chemotherapy for hormone-refractory prostate cancer treatment. Nutr Cancer 62: 148-153, 2010.
8. Anand P, Sundaram C, Jhurani S, Kunnumakkara AB and Aggarwal BB: Curcumin and cancer: an 'Old-age' disease with an 'Age-old' solution. Cancer Lett 267: 133-164, 2008.
9. Hatcher H, Planalp R, Cho J, Torti FM and Torti SV: Curcumin: from ancient medicine to current clinical trials. Cell Mol Life Sci 65: 1631-1652, 2008.
10. Huang MT, Wang ZY, Georgiadis CA, Laskin JD and Conney AH: Inhibitory effects of curcumin on tumor initiation by benzo[a] pyrene and 7,12-dimethylbenz[a]anthracene. Carcinogenesis 13: 2183-2186, 1992.
11. Conney AH, Lysz T, Ferraro T, *et al*: Inhibitory effect of curcumin and some related dietary compounds on tumor promotion and arachidonic acid metabolism in mouse skin. Adv Enzyme Regul 31: 385-396, 1991.
12. Huang MT, Smart RC, Wong CQ and Conney AH: Inhibitory effect of curcumin, chlorogenic acid, caffeic acid, and ferulic acid on tumor promotion in mouse skin by 12-O-tetradecanoylphorbol-13-acetate. Cancer Res 48: 5941-5946, 1988.
13. Goel A, Kunnumakkara AB and Aggarwal BB: Curcumin as 'Curecumin': from kitchen to clinic. Biochem Pharmacol 75: 787-809, 2008.
14. Cheng AL, Hsu CH, Lin JK, *et al*: Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. Anticancer Res 21: 2895-2900, 2001.
15. Dhillon N, Aggarwal BB, Newman RA, *et al*: Phase II trial of curcumin in patients with advanced pancreatic cancer. Clin Cancer Res 14: 4491-4499, 2008.
16. Bilmen JG, Khan SZ, Javed MH and Michelangeli F: Inhibition of the SERCA Ca^{2+} pumps by curcumin. Curcumin putatively stabilizes the interaction between the nucleotide-binding and phosphorylation domains in the absence of ATP. Eur J Biochem 268: 6318-6327, 2001.
17. Barry J, Fritz M, Brender JR, Smith PE, Lee DK and Ramamoorthy A: Determining the effects of lipophilic drugs on membrane structure by solid-state NMR spectroscopy: the case of the antioxidant curcumin. J Am Chem Soc 131: 4490-4498, 2009.
18. Epand RF, Martinou JC, Fornallaz-Mulhauser M, Hughes DW and Epand RM: The apoptotic protein tbid promotes leakage by altering membrane curvature. J Biol Chem 277: 32632-32639, 2002.
19. Su CC, Lin JG, Li TM, *et al*: Curcumin-induced apoptosis of human colon cancer colo 205 cells through the production of Ros, Ca^{2+} and the activation of caspase-3. Anticancer Res 26: 4379-4389, 2006.

20. Tan TW, Tsai HR, Lu HF, *et al*: Curcumin-induced cell cycle arrest and apoptosis in human acute promyelocytic leukemia HL-60 cells via mmp changes and caspase-3 activation. Anticancer Res 26: 4361-4371, 2006.
21. Su CC, Yang JS, Lin SY, *et al*: Curcumin inhibits WEHI-3 leukemia cells in BALB/c mice in vivo. In Vivo 22: 63-68, 2008.
22. Lu HF, Lai KC, Hsu SC, *et al*: Curcumin induces apoptosis through FAS and FADD in caspase-3-dependent and -independent pathways in the N18 mouse-rat hybrid retina ganglion cells. Oncol Rep 22: 97-104, 2009.
23. Lin SS, Huang HP, Yang JS, *et al*: DNA damage and endoplasmic reticulum stress mediated curcumin-induced cell cycle arrest and apoptosis in human lung carcinoma A-549 cells through the activation caspases cascade- and mitochondrial-dependent pathway. Cancer Lett 272: 77-90, 2008.
24. Yang MD, Lai KC, Lai TY, *et al*: Phenethyl isothiocyanate inhibits migration and invasion of human gastric cancer AGS cells through suppressing mapk and NF-kappaB signal pathways. Anticancer Res 30: 2135-2143, 2010.
25. Su CC, Yang JS, Lu CC, *et al*: Curcumin inhibits human lung large cell carcinoma cancer tumour growth in a murine xenograft model. Phytother Res 24: 189-192, 2010.
26. Lin ML, Lu YC, Chung JG, *et al*: Down-regulation of MMP-2 through the p38 MAPK-NF-kappaB-dependent pathway by aloe-emodin leads to inhibition of nasopharyngeal carcinoma cell invasion. Mol Carcinog 49: 783-797, 2010.
27. Lai TY, Yang JS, Wu PP, *et al*: The quinolone derivative CHM-1 inhibits murine WEHI-3 leukemia in BALB/c mice in vivo. Leuk Lymphoma 51: 2098-2102, 2010.
28. Chiang JH, Yang JS, Ma CY, *et al*: Danthron, an anthraquinone derivative, induces DNA damage and caspase cascades-mediated apoptosis in SNU-1 human gastric cancer cells through mitochondrial permeability transition pores and Bax-triggered pathways. Chem Res Toxicol 24: 20-29, 2011.
29. Lu CC, Yang JS, Huang AC, *et al*: Chrysophanol induces necrosis through the production of ROS and alteration of ATP levels in J5 human liver cancer cells. Mol Nutr Food Res 54: 967-976, 2010.
30. Lu HF, Wang HL, Chuang YY, *et al*: Danthron induced apoptosis through mitochondria- and caspase-3-dependent pathways in human brain glioblastoma multiforms GBM 8401 cells. Neurochem Res 35: 390-398, 2010.
31. Wu PP, Kuo SC, Huang WW, *et al*: (-)-Epigallocatechin gallate induced apoptosis in human adrenal cancer NCI-H295 cells through caspase-dependent and caspase-independent pathway. Anticancer Res 29: 1435-1442, 2009.
32. Wu SH, Hang LW, Yang JS, *et al*: Curcumin induces apoptosis in human non-small cell lung cancer NCI-H460 cells through ER stress and caspase cascade- and mitochondria-dependent pathways. Anticancer Res 30: 2125-2133, 2010.
33. Raez LE and Lilenbaum R: Chemotherapy for advanced non-small-cell lung cancer. Clin Adv Hematol Oncol 2: 173-178, 2004.
34. Kelly K: The role of targeted agents in adjuvant therapy for non-small cell lung cancer. Clin Cancer Res 11: S5027-S5029, 2005.
35. Steinke K: Lung tumors. Recent results. Cancer Res 167: 107-122, 2006.
36. Hartwell LH and Weinert TA: Checkpoints: controls that ensure the order of cell cycle events. Science 246: 629-634, 1989.
37. Molinari M: Cell cycle checkpoints and their inactivation in human cancer. Cell Prolif 33: 261-274, 2000.
38. Salvesen GS and Dixit VM: Caspases: intracellular signaling by proteolysis. Cell 91: 443-446, 1997.
39. Gross A, McDonnell JM and Korsmeyer SJ: Bcl-2 family members and the mitochondria in apoptosis. Genes Dev 13: 1899-1911, 1999.
40. Scopa CD, Vagianos C, Kardamakis D, Kourelis TG, Kalofonos HP and Tsamandas AC: Bcl-2/bax ratio as a predictive marker for therapeutic response to radiotherapy in patients with rectal cancer. Appl Immunohistochem Mol Morphol 9: 329-334, 2001.
41. Adams JM and Cory S: The Bcl-2 protein family: arbiters of cell survival. Science 281: 1322-1326, 1998.
42. Antonsson B and Martinou JC: The Bcl-2 protein family. Exp Cell Res 256: 50-57, 2000.
43. Ghatan S, Larner S, Kinoshita Y, *et al*: P38 map kinase mediates bax translocation in nitric oxide-induced apoptosis in neurons. J Cell Biol 150: 335-347, 2000.
44. Mandal C, Dutta A, Mallick A, Chandra S, Misra L and Sangwan RS: Withaferin a induces apoptosis by activating p38 mitogen-activated protein kinase signaling cascade in leukemic cells of lymphoid and myeloid origin through mitochondrial death cascade. Apoptosis 13: 1450-1464, 2008.