

# Curcumin causes DNA damage and affects associated protein expression in HeLa human cervical cancer cells

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**Abstract.** Cervical cancer is one of the most common cancers in women worldwide and it is a prominent cause of cancer mortality. Curcumin is one of the major compounds from Turmeric and has been shown to induce cytotoxic cell death in human cervical cancer cells. However, there is no study to show curcumin induced DNA damage action via the effect on the DNA damage and repair protein in cervical cancer cells in detail. In this study, we investigated whether or not curcumin induced cell death via DNA damage, chromatin condensation in human cervical cancer HeLa cells by using comet assay and DAPI staining, respectively, we found that curcumin induced cell death through the induction of DNA damage, and chromatin condensation. Western blotting and confocal laser microscopy examination were used to examine the effects of curcumin on protein expression associated with DNA damage, repair and translocation of proteins. We found that curcumin

at 13  $\mu$ M increased the protein levels associated with DNA damage and repair, such as O<sup>6</sup>-methylguanine-DNA methyltransferase, early-onset breast cancer 1 (BRCA1), mediator of DNA damage checkpoint 1, p-p53 and p-H2A.X<sup>Ser140</sup> in HeLa cells. Results from confocal laser systems microscopy indicated that curcumin increased the translocation of p-p53 and p-H2A.X<sup>Ser140</sup> from cytosol to nuclei in HeLa cells. In conclusion, curcumin induced cell death in HeLa cells via induction of DNA damage, and chromatin condensation *in vitro*.

## Introduction

Cervical cancer is the seventh most common cancer overall in human populations and the second most common type of cancer in women in many developing countries (1-3). In Taiwan, cervical cancer is the tenth most common cause of cancer associated death with rates of 5.5/100,000 persons/year based on the 2012 report from the Department of Health, Executive Yuan, Taiwan. The treatments for cervical cancer include surgery, radiotherapy, chemotherapy or combination radiotherapy with chemotherapy. However, the reduction in quality of patients' life caused by the high toxic effects of chemotherapeutic drugs makes it unsatisfactory (4,5). Numerous studies have been undertaken to find new therapies, mechanisms or compounds for cervical cancer treatment. Natural products have been used to treat cancer patients and those compounds can be used as complementary and/or alternative therapies in psychiatric medicine (6).

Curcumin is one of the plant pigments and was obtained from turmeric (*Curcuma longa* L.), which has been demonstrated to have wound healing function in diabetic animals (7), and anti-inflammatory (8), antibacterial (9) and antioxidant (10) properties. Furthermore, substantial evidence has shown that curcumin could be used as anti-carcinogenic substance through the inhibition of cell proliferation, inductions

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of cancer cell apoptosis, inhibition of angiogenesis and tumor metastasis (11-13). Curcumin has been shown to involve downregulation of nuclear factor- $\kappa$ B and the serine/threonine kinase Akt and is independent of tubulin polymerization (14). Curcumin is safe to human in phase I clinical trials for up to 12 g/day orally and causes histologic improvement of precancerous lesions in some patients; thus it suggested that these doses of curcumin is biologically active (15,16).

Several studies have shown that curcumin-mediated cell cycle arrest involved DNA damage signaling in colorectal carcinoma HCT116 cells (17), human bladder cancer T24 cells (18) and primary adult T-cell leukemia cells (19). It was reported that curcumin induced reactive oxygen species (ROS) production in human colon cancer HCT116 cells (20), nuclear DNA damage in human gastric mucosa cells, lymphocytes (21) and in human hepatoma HepG2 cells (22). Furthermore, there is no available information to show curcumin induced DNA damage in human cervical cancer cells and the exact nature of the trigger and the mechanisms involved are unclear.

Curcumin has been demonstrated to present antitumor activity against many types of human cancers (23-26). However, the inhibitory effects and possible mechanisms of curcumin induced DNA damage in human cervical cancer cells remain to be determined. Therefore, in this study, we used human cervical cancer HeLa cells to further characterize curcumin-induced DNA damage *in vitro*. Curcumin treatment generally resulted in decreased viable cells and DNA damage *in vitro*. We further investigated DNA damage associated protein expression and we found that curcumin inhibited DNA damage and repair associated proteins in HeLa cells.

## Materials and methods

**Chemicals and reagents.** Curcumin, dimethyl sulfoxide (DMSO), propidium iodide (PI), trypsin-EDTA, penicillin-streptomycin, anti-O<sup>6</sup>-methylguanine-DNA methyltransferase (MGMT) (cat. no. M3068), anti-PARP (cat. no. P248), anti-p-ATMSer1981 (cat. no. SBA4300100) and anti- $\beta$ -actin (cat. no. A5316) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Anti-DNA-PK (cat. no. PC127) was purchased from Calbiochem (San Diego, CA, USA). Anti-p-H2A.X (cat. no. GTX80694), anti-breast cancer 1 (BRCA1) (cat. no. GTX70111) and anti-MDM2 (cat. no. GTX110608) were purchased from GeneTex, Inc. (Irvine, CA, USA). Anti-mediator of DNA damage checkpoint 1 (MDC1) (cat. no. 05-1572) and anti-p53 (cat. no. 04-241) were purchased from Millipore Corp. (Billerica, MA, USA). Anti-p-ATR<sup>Ser428</sup> (cat. no. 2853) was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Dulbecco's modified Eagle's medium (DMEM) medium and fetal bovine serum (FBS) were purchased from Gibco®, Invitrogen Life Technologies (Carlsbad, CA, USA). Curcumin was first dissolved in DMSO at 1 mM.

**Cell culture.** The human cervical cancer HeLa cell line was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were placed into 75 cm<sup>2</sup> tissue culture flasks in DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin (100 U/ml penicillin and 100  $\mu$ g/ml streptomycin). Then cells were grown at 37°C

in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> as previously described (27,28).

**Cell morphology examination and cellular viability assay.** HeLa cells (1x10<sup>5</sup> cells/well) were kept on the 12-well cell culture cluster overnight and then were treated with 0, 12, 13 and 14  $\mu$ M curcumin for 48 h. After incubation, cells were examined by contrast-phase microscopy for cell morphological changes. Trypsin was added to the cells, harvested and rinsed three times with in phosphate-buffered saline (PBS). All cells were stained with PI (5  $\mu$ g/ml) in PBS and the total percentage of cell viability was measured by flow cytometry (Becton-Dickinson, San Jose, CA, USA) as previously described (27).

**Comet assay (single cell gel electrophoresis).** HeLa cells (1x10<sup>5</sup> cells/well) were kept on 12-well cell culture plate for 24 h and then treated with 13  $\mu$ M of curcumin or 0.5% H<sub>2</sub>O<sub>2</sub> (positive control) for 0, 6, 24 and 48 h. At the end of incubation, aliquots of 10<sup>5</sup> cells from each treatment were collected and cast into miniature LMA gels on microscope slides as previously described (27), followed by lysing *in situ* to relax the compacted DNA in nuclei of cells. Cells were electrophoresed and DNA was visualized and photographed by EB staining under fluorescence microscopy. Comets (DNA damage) of cells on slides were quantitated for comet tail lengths by the CometScore™ Freeware analysis (TriTek Corp., Sumerduck, VA, USA) (27).

**4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) staining.** HeLa cells (1.5x10<sup>5</sup> cells/well) were maintained on 6-well cell culture plate for 24 h and then were incubated with 13  $\mu$ M curcumin for 0, 6, 24 and 48 h. At the end of incubation, cells were fixed with 4% formaldehyde in PBS for 10 min, followed by washing with PBS and then were stained by DAPI for 1 h at 37°C. Cells from each treatment were examined and photographed by using a fluorescence microscope at x200 as previously described (27).

**Apo-BrdU (bromolated deoxyuridine triphosphate nucleotides) TUNEL assays.** To evaluate HeLa cell DNA fragmentation, the HeLa cells (1.5x10<sup>5</sup> cells/well) were maintained on 6-well plate and treated with 0 and 13  $\mu$ M of curcumin for 0, 12, 24 and 48 h. Cells were fixed in 4% formaldehyde in PBS for 15 min, and then washed twice with ice-cold PBS, and incubated in the dark for 60 min at 37°C in 50  $\mu$ l of DNA Labeling Solution each assay. TUNEL assays were performed by fluorescence microscope in triplicate on three independent experiments. TUNEL staining was performed according to the manufacturer's instructions (Apo-BrdU *in situ* DNA fragmentation assay kit; BioVision, Inc., Milpitas, CA, USA) (27).

**Western blotting.** HeLa cells (1.5x10<sup>6</sup> cells/dish) were maintained on a 10-cm dish with DMEM medium containing 10% FBS for 24 h and were incubated with 13  $\mu$ M of curcumin for 0, 6, 24 and 48 h. After treatment, cells were collected and suspended in sodium dodecyl sulphate (SDS) buffer followed by sonication and boiling for 10 min as described previously (27). The total protein in each sample was quantitated. A total 30  $\mu$ g from each sample was electrophoresed by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE),

transferred to PVDF membrane, and were immunoblotted as previously described (27). Membranes were transferred, and then were followed by staining with primary antibodies such as anti-p-ATM, anti-p-ATR, anti-p53, anti-MDM2, and anti-p-p53, anti-BRCA1, anti-DNA-PK, anti-MDC1, anti-p-H2A.X, anti-PARP and anti-MGMT at 4°C (overnight), each membrane was then washed. After washing, all membranes were stained by secondary antibody, washed and were visualized with a chemiluminescent detection system and the protein expressions were quantitated as described by the manufacturer (27,29).

**Confocal laser microscopy.** HeLa cells ( $1.5 \times 10^5$  cells/well) were maintained on 6-well plate and treated with 0 and  $13 \mu\text{M}$  of curcumin for 48 h. Cells were rinsed and fixed in 4% formaldehyde in PBS for 15 min as previously described (30). Cells were washed and permeabilized with 0.1% Triton X-100 in PBS followed by washing and blocking with 5% BSA in PBS for 60 min and then were washed with PBS. The primary anti-p-H2A.X, anti-p53 and anti-p-p53 (green fluorescence) were used to stain cells and then followed by secondary antibody (FITC-conjugated goat anti-mouse IgG) staining. The PI (red fluorescence) used for nuclei staining and were photomicrographed under a Leica TCS SP2 confocal spectral microscope as previously described (30).

**Statistical analysis.** All quantitative data were presented as the mean  $\pm$  standard deviation (SD) from three independent experiments. Student's t-test was used to compare means between the control and curcumin treated groups. The  $P < 0.05$  was considered as the significant level.

## Results

**Curcumin induces cell morphological changes and decreases the total percentage of HeLa viable cells.** HeLa cells were incubated with 0, 12, 13 and  $14 \mu\text{M}$  curcumin for 48 h and then were examined for cell morphology and were collected for percentage of viable cell determinations and results are shown in Fig. 1A and B. The results from Fig. 1A indicated that curcumin induced cell morphological changes and the total viable cells were decreased when compared to control (without curcumin treatment).

**Curcumin induces DNA damage of HeLa cells.** Cells were incubated with  $13 \mu\text{M}$  curcumin for 0, 6, 24 and 48 h and then collected for comet assay. Results from Fig. 2A and B demonstrated that  $13 \mu\text{M}$  curcumin induced comet tail (DNA damage) production from single cell electrophoresis when compared with control in HeLa cells (Fig. 2A). The longer the incubation time, the longer the comet tail length which indicated that DNA damage is time-dependent caused by curcumin in HeLa cells (Fig. 2B).

**Curcumin induces chromatin condensation of HeLa cells.** For further confirming whether or not curcumin decreased the total viable cells through apoptosis of HeLa cells, cells were incubated with  $13 \mu\text{M}$  of curcumin for 0, 6, 24 and 48 h, and were stained by DAPI to investigate the formation of chromatin condensation which were characterized by nuclear

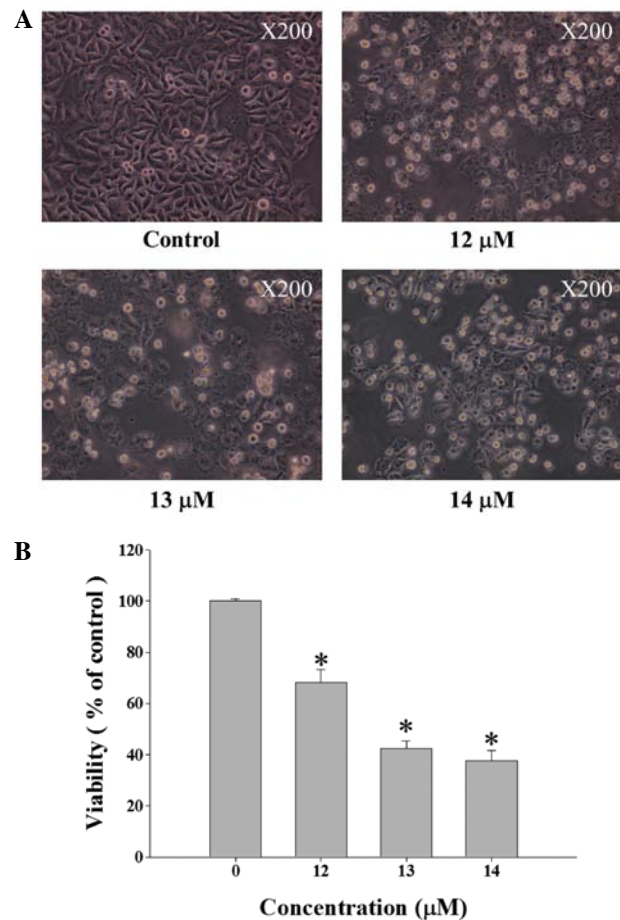


Figure 1. Curcumin induces cell morphological changes and decreases the percentage of viable human cervical cancer HeLa cells. Cells ( $1 \times 10^5$  cells/well) were placed in 12-well plates and were incubated with 0, 12, 13 and  $14 \mu\text{M}$  curcumin for 48 h. (A) All samples were examined for cell morphology by contrast-phase microscopy at x200 magnification and (B) were stained with propidium iodide (PI) ( $5 \mu\text{g/ml}$ ) to analyze the cell viability by flow cytometry as previously described. \* $P < 0.05$  was considered significant.

fluorescence (white color). Results from Fig. 3A and B indicated that curcumin induced chromatin condensation in a time-dependent manner. The chromatin condensation was based on the higher fluorescence (DAPI staining) compared to control under fluorescence microscope examination in HeLa cells.

**Curcumin induces DNA fragmentation of HeLa cells.** HeLa cells were incubated with  $13 \mu\text{M}$  curcumin for 0, 6, 24 and 48 h, and then collected for Apo-BrdU TUNEL assays. In order to investigate the expression of the BrdU in HeLa cells. Cells were exposed to  $13 \mu\text{M}$  of curcumin for 0, 6, 24 and 48 h, and then were examined by confocal microscopy and results are shown in Fig. 4. Comparing with control group, the BrdU-FITC in cells treated with curcumin was found to increase the incorporation with DNA strand breaks and were visualized in the nucleus as shown in the Fig. 4 (merge image). The result demonstrated that curcumin induced DNA fragmentation that may be through the overexpression of BrdU in nuclei in HeLa cells.

**Curcumin affects DNA damage associated proteins of HeLa cells.** In order to further investigate curcumin induced DNA

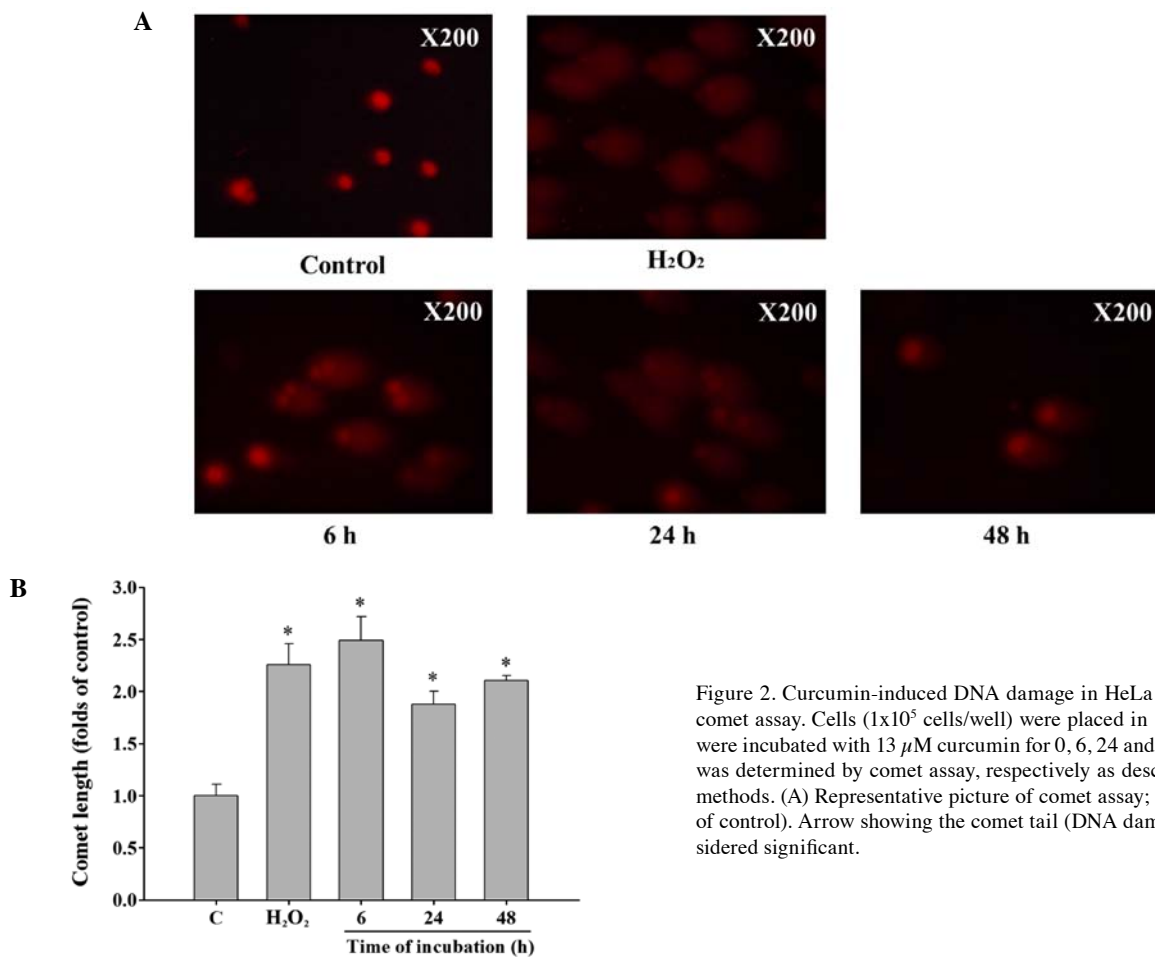


Figure 2. Curcumin-induced DNA damage in HeLa cells was measured by comet assay. Cells ( $1 \times 10^5$  cells/well) were placed in 12-well plates and then were incubated with  $13 \mu\text{M}$  curcumin for 0, 6, 24 and 48 h and DNA damage was determined by comet assay, respectively as described in Materials and methods. (A) Representative picture of comet assay; (B) comet length (folds of control). Arrow showing the comet tail (DNA damage). \* $P < 0.05$  was considered significant.

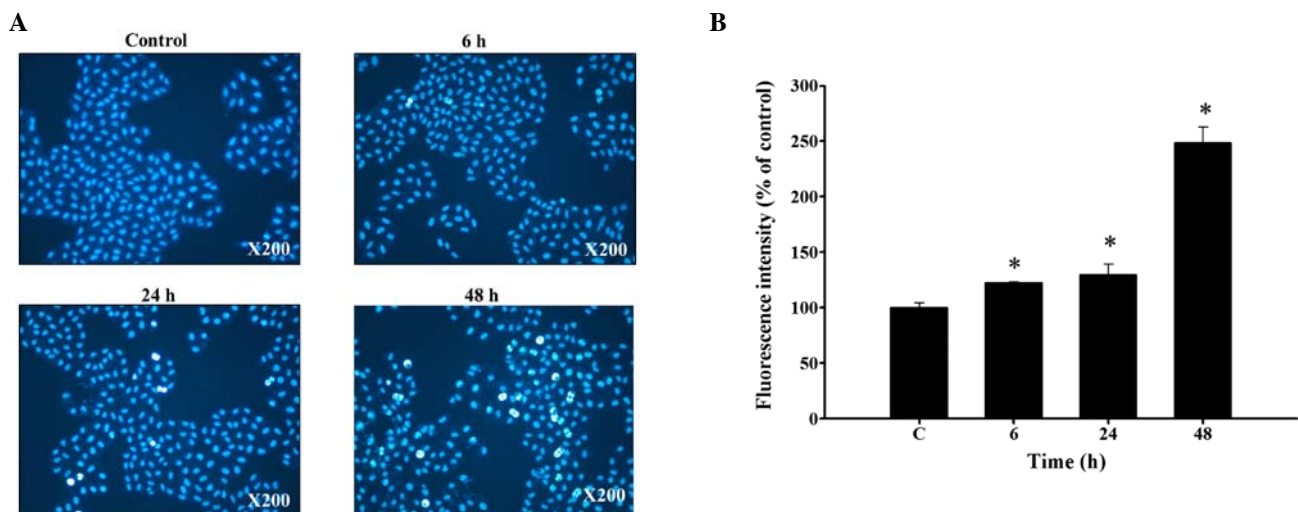


Figure 3. Curcumin-induced chromatin condensation in HeLa cells was examined by 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining. Cells ( $1.5 \times 10^5$  cells/well) were placed in 6-well plates and were treated with  $13 \mu\text{M}$  curcumin for 0, 6, 24 and 48 h. Then cells in each treatment were DAPI stained as described in Materials and methods. (A) Cells were examined and photographed using a fluorescence microscope at x200 magnification. (B) Fluorescence intensity was calculated from each treatment. \* $P < 0.05$  was considered significant.

damage via affect DNA damage and repair associated protein expression in HeLa cells, cells were treated with  $13 \mu\text{M}$  of curcumin for 0, 6, 24 and 48 h and then total protein from cells were quantitated and DNA damage associated proteins were examined by western blotting and results are shown

in Fig. 5A-C. These results demonstrated that curcumin significantly increased the amounts of proteins such as p-ATM, p-ATR, p53 and MDM2 (Fig. 5A), BRCA1, DNA-PK, MDC1 and p-H2A.X (Fig. 5B), PARP and MGMT (Fig. 5C) in HeLa cells.

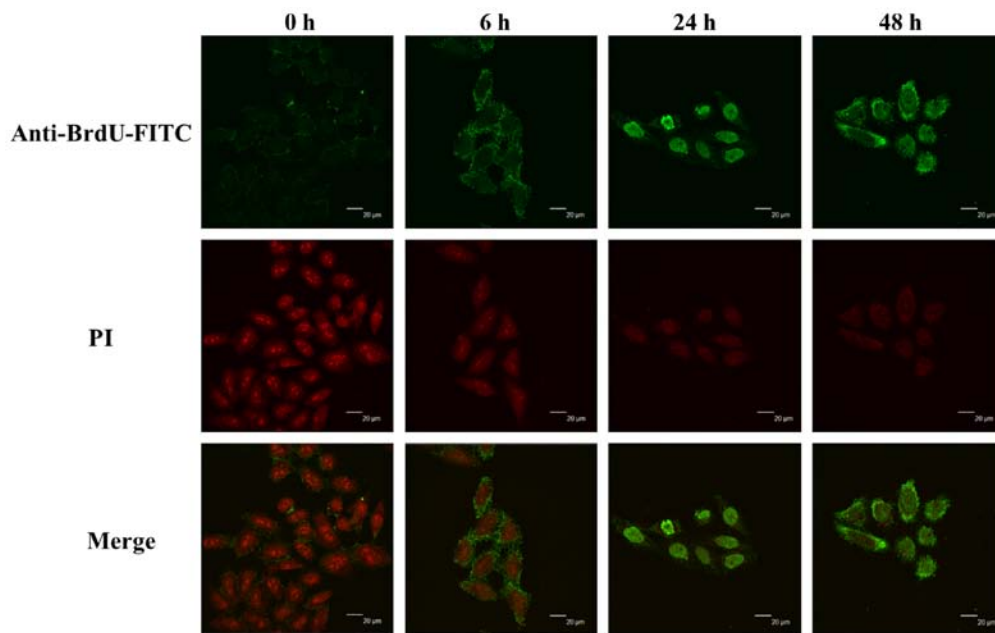


Figure 4. Curcumin affected the BrdU-FITC expression associated with DNA fragmentation in HeLa cells. Cells ( $5 \times 10^4$  cells/well) were placed on a 6-well plate, incubated with  $13 \mu\text{M}$  curcumin for 0, 6, 24 and 48 h and then fixed in 4% formaldehyde in PBS for 15 min. After washing, cells were incubated with 0.1% Triton X-100 in PBS for 1 h and immunostaining by BrdU-FITC was performed as described in Materials and methods. Samples were examined and photo-micrographed under a Leica TCS SP2 confocal spectral microscope.

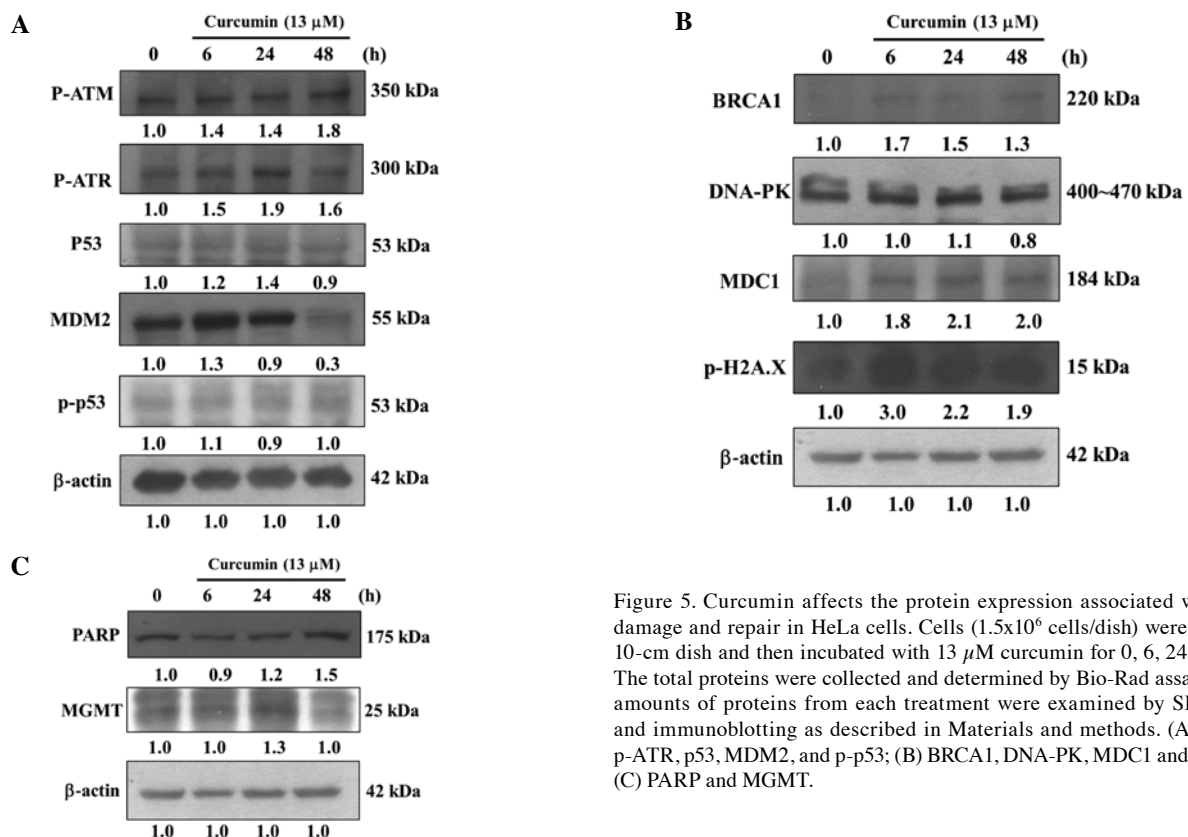


Figure 5. Curcumin affects the protein expression associated with DNA damage and repair in HeLa cells. Cells ( $1.5 \times 10^6$  cells/dish) were placed in 10-cm dish and then incubated with  $13 \mu\text{M}$  curcumin for 0, 6, 24 and 48 h. The total proteins were collected and determined by Bio-Rad assay kit. The amounts of proteins from each treatment were examined by SDS-PAGE and immunoblotting as described in Materials and methods. (A) p-ATM, p-ATR, p53, MDM2, and p-p53; (B) BRCA1, DNA-PK, MDC1 and p-H2A.X; (C) PARP and MGMT.

Curcumin affects the translocation of p53, p-p53 and p-H2A.X in HeLa cells. Based on the results from western blotting (Fig. 5A and B) indicated that curcumin increased the amounts of p53, p-p53 and p-H2A.X in HeLa cells, we investigated the translocation and increase of the proteins in HeLa cells. Cells were exposed to  $13 \mu\text{M}$  of curcumin for 48 h

and then examined by confocal microscopy and results are shown in Fig. 6. Contrasting to the control, the p53 (Fig. 6A), p-p53 (Fig. 6B) and p-H2A.X (Fig. 6C) in cells treated with curcumin was found to increase the cytosol, and more labelled p53, p-p53 and p-H2A.X were visualized in the nucleus as shown in the Fig. 6 (Merge image). These observations indi-



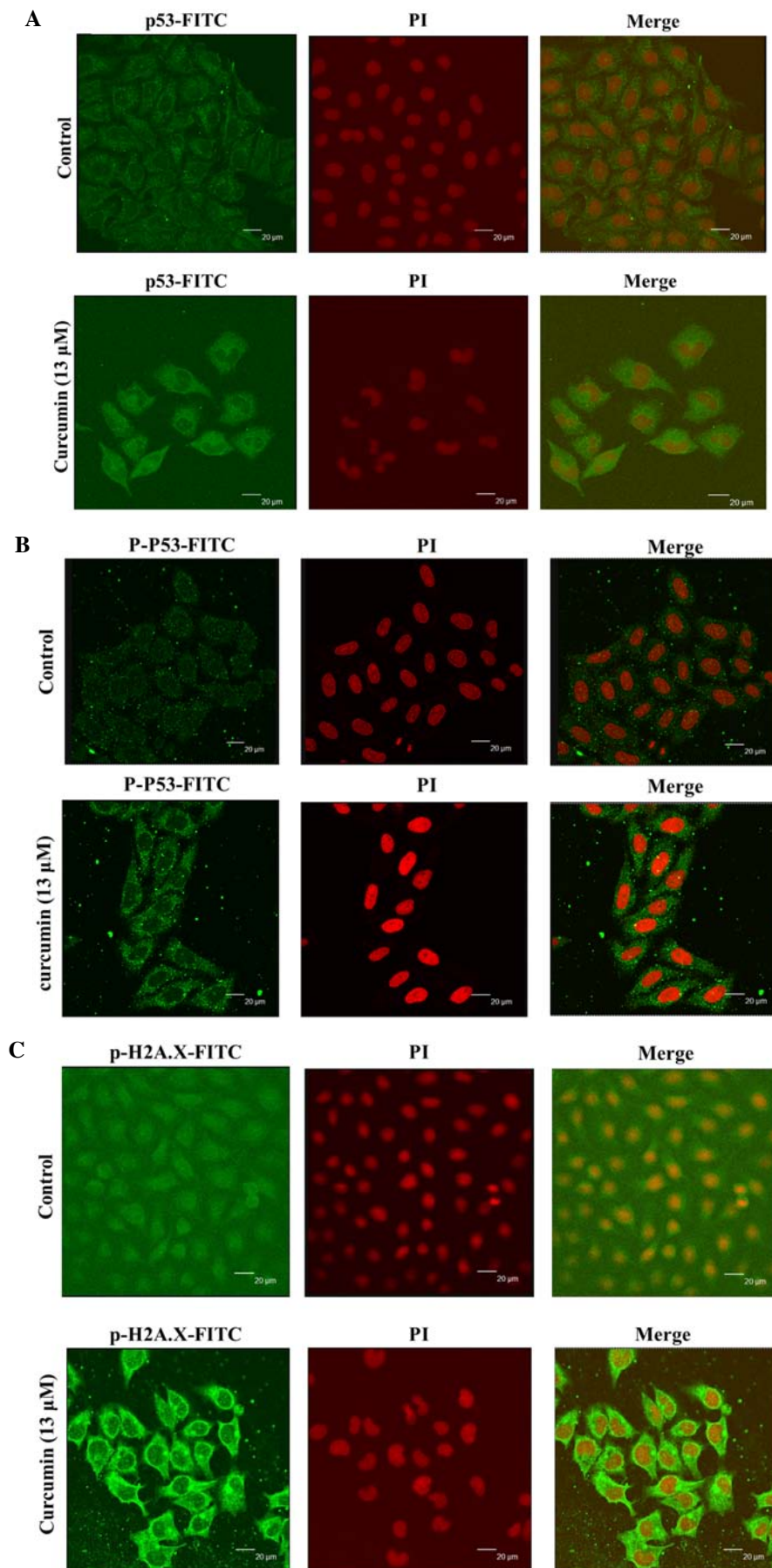


Figure 6. Curcumin affected the protein translocation in HeLa cells. Cells ( $1.5 \times 10^4$  cells/well) were placed on a 6-well plate, incubated with  $13 \mu\text{M}$  curcumin for 48 h and then fixed in 4% formaldehyde in PBS for 15 min. After washing, cells were incubated with 0.1% Triton X-100 in PBS for 1 h and immunostaining was performed as described in Materials and methods. (A) p53, (B) p-p53 and (C) p-H2A.X.. Both samples were examined and photo-micrographed under a Leica TCS SP2 confocal spectral microscope.

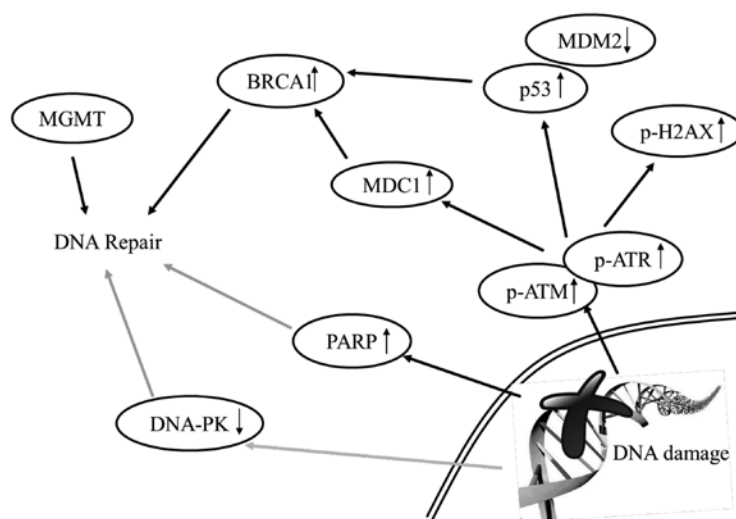


Figure 7. The proposed flow chart for curcumin-induced DNA damage and inhibition of DNA repair associated protein expression in human cervical cancer HeLa cells.

cated that curcumin induced DNA damage and repair that may be via the translocation of p53, p-p53 and p-H2A.X from cytoplasm into nuclei in HeLa cells.

## Discussion

It is well documented that curcumin induced cell death may be via cell cycle arrest and induction of apoptosis in many human cancer cell lines, furthermore, animal studies revealed that oral administration of curcumin inhibited the incidence of cancers and it is under clinical trials to various cancers and related diseases (9). It was also reported that curcumin induced DNA damage and repair in several human cancer cells including human peripheral blood mononuclear cells (PBMCs) which was measured by the comet assay (21,31). Recently, it was reported that curcumin can induce apoptosis of normal resting human T cells that is not connected with DNA damage (17). However, there is no available information to show curcumin induced DNA damage and affect DNA damage and repair associated protein expression in human cervical cancer cells. Therefore, we investigated whether or not curcumin induced DNA damage in HeLa cells and we found that: i) curcumin decreased the percentage of viable cells (Fig. 1); ii) a time-dependent increase in DNA damage was measured by comet assay (Fig. 2); iii) curcumin induced chromatin condensation time-dependently which was examined by DAPI staining (Fig. 3); iv) curcumin induced DNA fragmentation which was examined by TUNEL assay (Fig. 4); v) curcumin significantly increased p-ATM, p-ATR, p53 and MDM2 (Fig. 5A), BRCA1, MDC1 and p-H2A.X (Fig. 5B), PARP and MGMT (Fig. 5C) in HeLa cells; and vi) curcumin induced DNA damage and repair that may also involve p53, p-p53 and p-H2A.X (Fig. 6) translocation from cytoplasm into nuclei that were measured by confocal laser microscopy in HeLa cells.

Several studies have shown that curcumin induced DNA damage in cancer cells (32). Herein, we also showed that curcumin induced DNA damage (Fig. 3) and chromatin condensation (Fig. 2) in HeLa cells. Both were assayed by

single cell electrophoresis (comet assay) and DAPI staining, respectively. Both assays are well known for measuring DNA damage (32,33) and chromatin condensation (34,35), respectively. There are studies that cells can repair DNA damage from agent induction and also can use DNA repair systems to eliminate these damaged DNA even to repair DNA base for cell maintaining survival (36,37). In addition, previous studies showed that DNA fragmentation was closely related with apoptosis (27,38). Our result showed that curcumin induced DNA fragmentation in Fig. 4 as well. The above descriptions indicated that curcumin could inhibit HeLa cell proliferation by induced DNA damage.

Results from western blotting indicated that curcumin increased DNA damage and repair associated protein expression such as p-ATM, p-ATR, p53 and MDM2 (Fig. 5A), BRCA1, MDC1 and p-H2A.X (Fig. 5B), PARP and MGMT (Fig. 5C), however, the protein levels of p-p53 (Fig. 5A) and DNA-PK (Fig. 5B) were no significantly affected. It was reported that MGMT in human cervical cancer (39,40) and polymorphism in MGMT increases the susceptibility of women to cervical carcinoma (40), herein; we found that curcumin inhibited the expression of MGMT in HeLa cells (Fig. 5). It was reported that inhibiting MGMT could increase tumor susceptibility (41). MGMT can repair the pre-mutagenic, pre-carcinogenic and pre-toxic DNA damage O<sup>6</sup>-methylguanine (42) and MGMT has been recognized as an important change that takes place in cervical cancer (43).

BRCA1, is a tumor-suppressor gene, whose mutation has been correlated with the appearance of breast and/or ovarian cancer. Herein, we found that curcumin increased the expression of BRCA1 in HeLa cells (Fig. 5B). The BRCA1 gene products have been demonstrated to be associated with DNA damage repair, transcriptional control, cell growth, and apoptosis (44,45). NSCLC patients with reduced *BRCA1* mRNA expression levels have greater overall survival benefit (46). Numerous studies have reported that MDC1 plays important roles in DNA damage response pathway (47) and have been shown also in human cervical cancer (48). Our findings showed that curcumin increased MDC1 expression in HeLa

cells (Fig. 5B). Recently, it was reported that MDC1 seems to be related to the oncogenic potential of cervical cancer, furthermore, the suppression of its expression can inhibit cancer cell growth (48) and MDC1 could be a potential therapeutic target in human cervical cancer.

Fig. 5 shows that curcumin increased p53, p-p53 and p-H2A.X, respectively, in HeLa cells. We also used confocal laser microscopy to measure the translocation of p53 (Fig. 6A), p-p53 (Fig. 6B) and p-H2A.X (Fig. 6C) from cytoplasm to nuclei. It is well known that after DNA damage is present in cells, the protein level of p53 (a transcription factor) will increase. The p53 have been shown to mediate G<sub>1</sub> arrest for cells in response to genotoxic stress and allowing time for DNA repair (49). Herein, we found p53 was increased but p-p53 was not significantly increased. The phosphorylation of p53 has been shown to be the one type of upstream signal for triggering the p53 regulatory functions (50). The phospho-H2A.X, is a reliable marker of DNA double-strand breaks (DSBs) (51) and the H2A.X deficient mice will have higher radiosensitivity (52). In the present studies, we found that curcumin increased the expression of p-H2A.X (Fig. 6C) that was also confirmed by confocal laser microscopic examination (Fig. 6).

In conclusion, the results of the present study indicate that: i) curcumin induced cytotoxic effects (cell death) in HeLa cells; ii) curcumin induced DNA damage and cell death which were measured by comet assay and DAPI staining, respectively, in HeLa cells; and iii) curcumin increased the proteins levels of p-ATM, p-ATR, MGMT, BRCA1, MDC1, p53, p-p53 and p-H2A.X that may lead to cell death in HeLa cells as summarized in Fig. 7.

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