Curcumin Triggers DNA Damage and Inhibits Expression of DNA Repair Proteins in Human Lung Cancer Cells

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Abstract. *The study goal was to evaluate the effects of curcumin on DNA damage and expression of DNA-repair proteins in human lung cancer. Thus, NCI-H460 cells were used to study the effects of curcumin on DNA damage and repair in vitro. We investigated curcumin induces DNA damage by comet the assay and 4',6-diamidino-2-phenylindole (DAPI) staining. The DNA damage/repair-related protein levels were examined and monitored by western blotting and confocal microscopy. Curcumin significantly increased the length of comet tails and DNA condensation in NCI-H460 cells. Curcumin reduced expression of DNA-repair proteins such as 14-3-3 protein sigma (14-3-3σ), O6-methylguanine-DNA methyltransferase (MGMT), breast cancer susceptibility gene 1 (BRCA1), and mediator of DNA damage checkpoint 1 (MDC1). Curcumin also increased phosphorylation of p53 and Histone H2A.X (S140) in the nuclei of NCI-H460 cells. Taken together, our findings indicated that curcumin triggered DNA damage and inhibited expression of DNA-repair-associated proteins in NCI-H460 cells.*

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Most chemotherapeutic agents can directly or indirectly induce damage DNA, causing cell-cycle arrest and cell apoptosis in many different types of human cancer cells (1). Unfortunately, chemotherapeutic agents on induced DNA lesions can be recognized and repaired by the activation of various DNA-repair mechanisms in target cells, resulting in treatment failure (2). The activating mechanisms of DNA repair have been shown to mediate acquired resistance to chemotherapeutic agents in tumor cells and the inhibition of DNA-repair mechanisms sensitizes tumor cells to these agents (3).

Lung cancer is the most common malignancy and is also a major cause of cancer-related mortality worldwide (4). Chemotherapeutic agents are used in the treatment of lung cancer and the therapeutic efficacy of these agents is limited by chemoresistance which results in poor prognosis (5). Certain clinical studies indicated that overexpression of DNA-repair proteins is often found in lung cancer and is related to chemoresistance during cancer treatment, therefore the development of inhibitors for DNA-repair mechanisms may offer additional treatment benefit to patients with lung cancer (5, 6).

Curcumin [1,7-*bis*(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione), a polyphenol, is extracted from *Curcuma longa* and has been shown to have anti-inflammatory, anti-viral, and anticancer activities (7). Ogiwara *et al*. found that curcumin sensitizes osteosarcoma, fibrosarcoma, cervical and colorectal cancer to poly (ADP-ribose) polymerase (PARP) inhibitors *via* the suppression of DNA damage checkpoint and repair of double-strand breaks (8). However, whether curcumin can influence the expression of DNA-repair proteins in lung cancer is ambiguous.

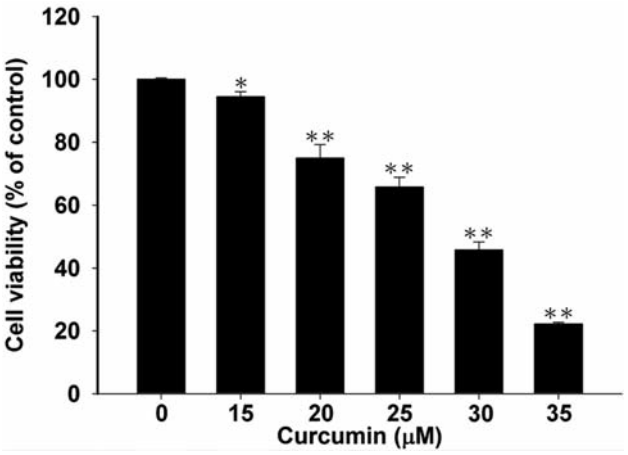

Figure 1. *The effects of curcumin on total cell viability in NCI-H460 cells. Cells were treated with 0, 15, 20, 25, 30 and 35 μM in 0.1% DMSO for 24 h. The cell viability was evaluated with trypan blue exclusion as described in the Materials and Methods. *p<0.05 and **p<0.01, significantly different from the control.*

The aim of the study was to verify whether curcumin can induce DNA damage and inhibit the expression of DNA-repair proteins in lung cancer cells *in vitro*.

Material and Methods

Cell culture. The NCI-H460 human lung cancer cell line was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC). Cells were maintained in PRMI-1640 culture medium with supplemental 10% fetal bovine serum (FBS), 2 mM L-glutamine, and penicillin-streptomycin (100 units/ml and 100 μg/ml, respectively) in 75 cm^2 tissue culture flasks and cultured at 37˚C in a humidified incubator containing 5% CO_2.

Trypan blue exclusions test of cell viability. Approximately 2×10^5 cells/well of NCI-H460 cells in 12-well plates were treated with different concentrations of curcumin (0, 15, 20, 25, 30, and 35 μM in 0.1% Dimethyl sulfoxide (DMSO)) for 24 h. Cell viability was measured by using trypan blue exclusion assay as described previously (9).

Comet assay for evaluation of DNA damage. A total of 2×10^5 cells/well in 12-well plates were kept for 24 h and then treated with different concentrations of curcumin (0, 15, 25, and 35 μM in 0.1% DMSO) for 24 h. The detection of DNA damage was evaluated with comet assay as described previously (10).

4,6-Diamidino-2-phenylindole dihydrochloride (DAPI) staining for changes in nuclear morphology. A total of 3×10^5 cells/well were kept in 12-well plates for 24 h and then were treated with different concentrations of curcumin (0, 15, 20, 25, 30, and 35 μM in 0.1% DMSO) for 24 h. The examination of changes in nuclear morphology was performed with DAPI staining as described previously (11). After DAPI staining, cells were photographed by using a fluorescence microscope at ×400.

Western blotting assay for analysis of levels of proteins associated with DNA repair and damage. A total of 1×10^6 cells were cultured in a 10 cm dish for 24 h and then treated with 35 μM curcumin for different time periods. Lysis buffer (50 mM Tris-HCl pH 8.0, 120 mM NaCl, 0.5% NP-40, 1 mM phenylmethanesulfonyl fluoride) was used for protein extraction from cells at different specific time points. The protein levels of 14-3-3 protein sigma (14-3-3σ), O^6-methylguanine-DNA methyltransferase (MGMT), breast cancer susceptibility gene 1 (BRCA1), and mediator of DNA damage checkpoint 1 (MDC1), p-p53 and p-H2A.X(S140) were investigated with western blotting assay as described previously (12). ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used for the quantification of the protein levels.

Monitoring of phosphorylation of p53 and H2A.X (S140) in the nucleus by using confocal laser microscopy. A total of 3×10^5 cells/well were maintained on 6-well chamber slides and treated with 35 μM curcumin for 24 h. After curcumin treatment, the cells were fixed with 4% formaldehyde in phosphate-buffered saline (PBS) for 15 min and then permeabilized with 0.3% Triton X-100 in blocking buffer (2% bovine serum albumin in PBS) for 1 h. The slides were incubated with green fluorescence anti-p-p53, anti-p-H2A.X (S140) primary antibodies overnight and then exposed to fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (secondary antibody) for 2 h and then stained with propidium iodode (PI) (red fluorescence) for nuclear localization. Imaging of immunofluorescence was obtained by using a Leica TCS SP2 Confocal Spectral Microscope (Leica, Mannheim, Germany) (11).

Statistical analysis. Student's *t*-test was used for the comparison between control and curcumin-treated groups and data are shown as the mean±standard error. Difference were significant when the *p*-value was 0.05 or less.

Results

Curcumin-induced cytotoxicity in NCI-H460 cells. NCI-H460 cells were treated with 0, 15, 20, 25, 30, and 35 μM curcumin for 24 h and then the percentage of viable cells was measured by trypan blue exclusion. Data from Figure 1 indicate that curcumin reduced total cell viability of NCI-H460 cells in a dose-dependent manner, with 5-75% reduction compared to that of the vehicle treatment ($p<0.05$ or $p<0.01$).

Curcumin-induced DNA damage in NCI-H460 cells. For further confirmation that curcumin reduced the percentage of total viable NCI-H460 cells through induction of DNA damage, the comet assay was used to verify DNA damage in NCI-H460 cells and results are shown in Figure 2. Figure 2A demonstrates that curcumin significantly increased the length of comet tails in NCI-H460 cells. After calculating the lengths of comet tails (Figure 2B), it was found that curcumin induced DNA damage dose-dependently.

Curcumin-induced DNA condensation in NCI-H460 cells. After we showed that curcumin induced cell death and DNA damage, we further investigated curcumin-induced DNA

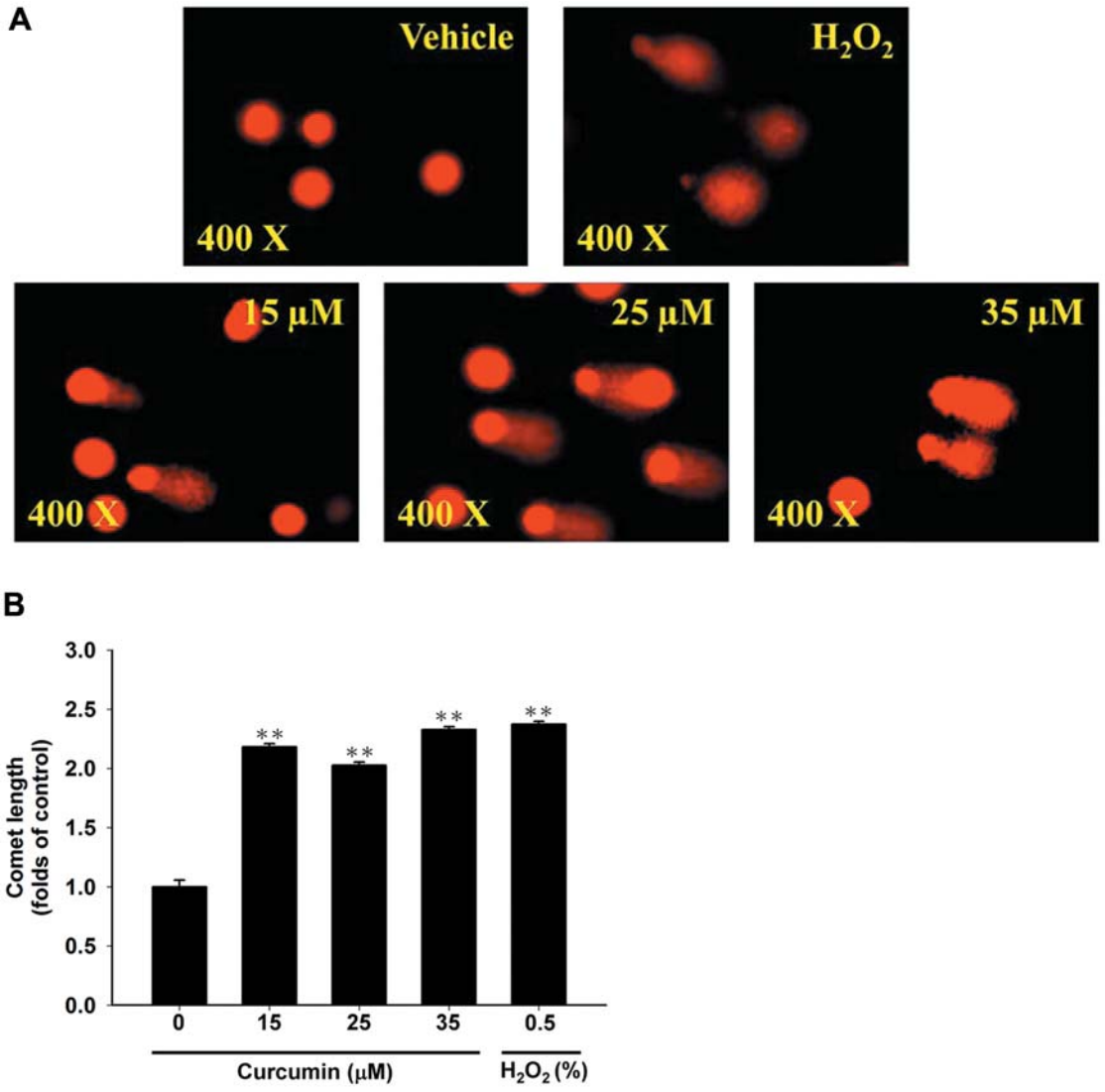

Figure 2. *The effects of curcumin on DNA damage in NCI-H460 cells. Cells were treated with 0, 15, 25 and 35 μM in 0.1% DMSO for 24 h. The DNA damage levels were evaluated with the comet assay as described in the Materials and Methods. A: Representative images of DNA comets in NCI-H460 cells from each treatment. B: Quantification of the comet length. The 0.5% H_2O_2 was used as a positive control. **p<0.01, significantly different from the control.*

condensation (changes in nuclear morphology) in NCI-H460 cells by DAPI staining. Results shown in Figure 3 indicate that curcumin significantly induced DNA condensation in NCI-H460 cells in a dose-dependent manner.

Curcumin influenced levels of proteins associated with DNA repair and damage in NCI-H460 cells. In order to investigate whether curcumin induced cell death, DNA damage and condensation by affecting DNA damage-repair-associated protein expression. Western blotting assay was used to examine the protein expression in NCI-H460 cells after exposure to 35 μM curcumin for 0, 6, 24 and 48 h. The results shown in Figure 4 demonstrate that curcumin not only reduced expression of DNA-repair proteins (14-3-3 σ, MGMT, BRCA1, MDC1) but also increased the expression of DNA-damage proteins (p-p53 and p-H2A.X) in NCI-H460 cells.

Curcumin increased phosphoryaltion of DNA-damage proteins in the nucleus of NCI-H460 cells. Western blotting already showed that curcumin increased expression of p-p53 and p-H2A.X (S140); we further used confocal laser microscopy to measure whether curcumin induces expression

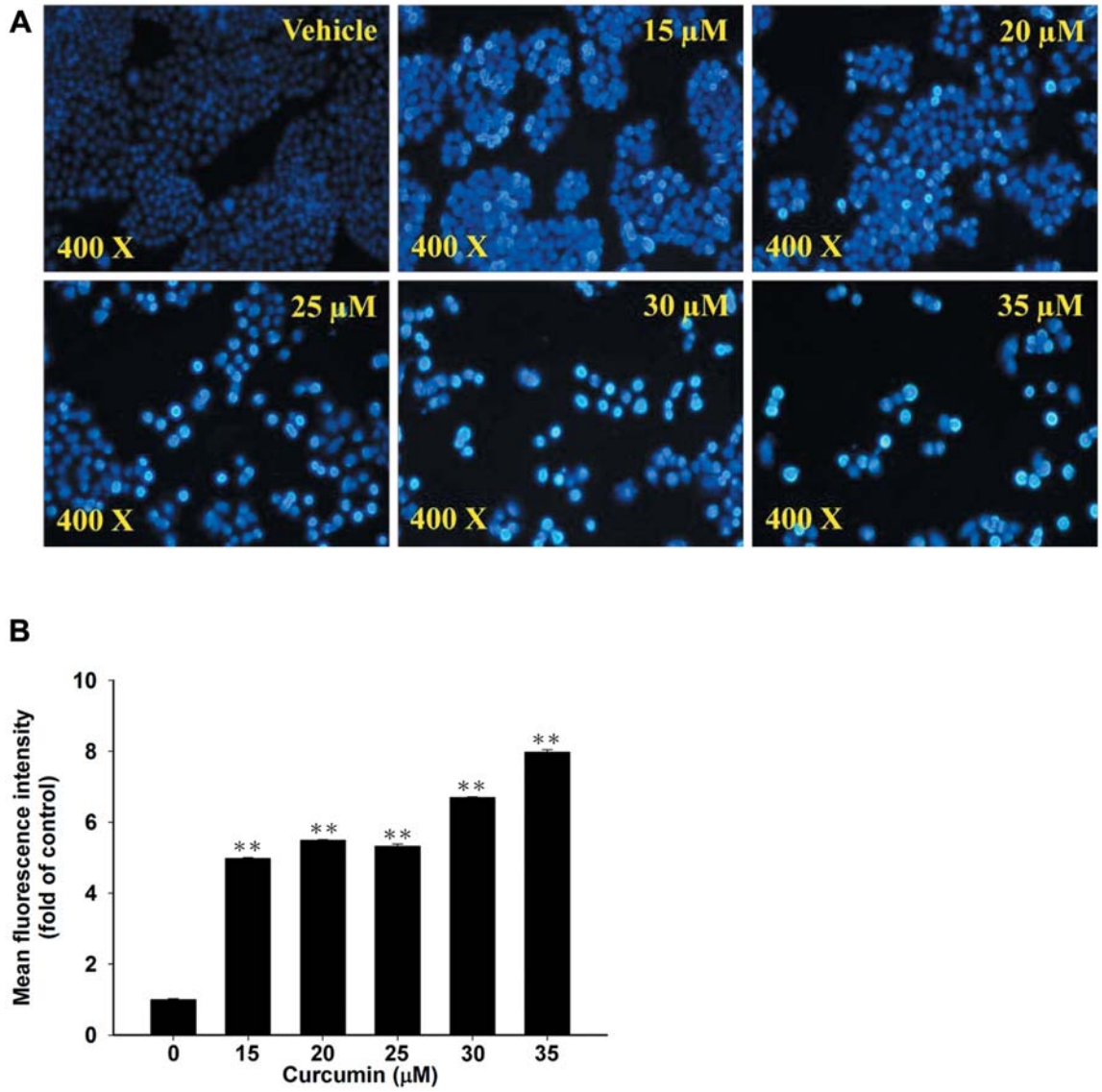

Figure 3. *The effects of curcumin on the nuclear morphology in NCI-H460 cells. Cells were treated with 0, 15, 20, 25, 30 and 35 μM in 0.1% DMSO for 24 h. The change of nuclear morphology was determined with DAPI staining as described in the Materials and Methods. A: Representative images of DAPI staining in NCI-H460 cells from each treatment. B: Quantification of the DNA condensation. **p<0.01, significantly different from the control.*

of p-p53 and p-H2A.X (S140) in the nucleus of NCI-H460 cells. We found that curcumin indeed increases phosphorylation of p53 and H2A.X (S140) in the nucleus of NCI-H460 cells (Figure 5).

Discussion

It is known that curcumin, as multi-target inhibitor, can abolish activation of various molecules which regulate tumorigenesis, resulting in suppression of lung cancer progression *in vitro* and *in vivo* (13). However, whether curcumin can affect expression of DNA-repair proteins in lung cancer is unclear. In the present study, we investigated the effects of curcumin on DNA-damage and repair-associated protein levels in NCI-H460 cells. We firstly found curcumin reduced the percentage of total viable cells (Figure 1). Secondly, we found that curcumin induced DNA damage (Figure 2) and condensation (Figure 3) in dose-dependent manners. Thirdly, we found curcumin reduced expression of DNA-repair proteins (14-3-3 σ, MGMT, BRCA1, MDC1) and increased expression of DNA-damage proteins (p-p53 and p-H2A.X) in NCI-H460 cells (Figure

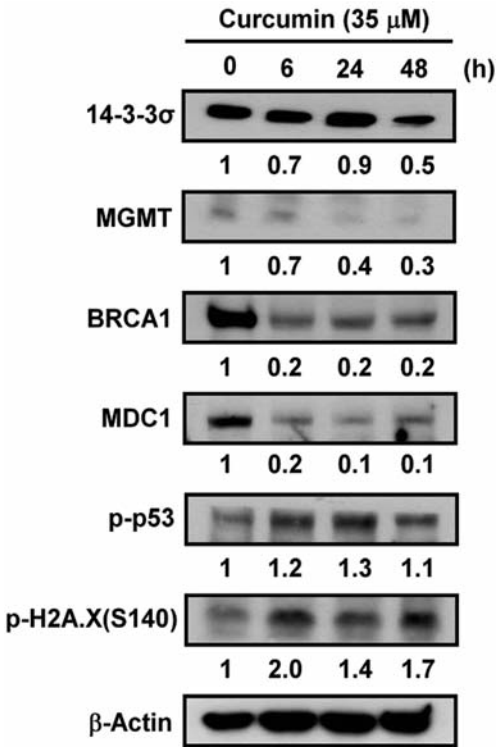

Figure 4. *The effects of curcumin on protein expression associated with DNA repair and damage in NCI-H460 cells. Cells were treated with 35 μM curcumin for 0, 6, 24, 48 h. Cells were collected for examining the change of protein levels using western blotting assay as described in the Materials and Methods Section.*

4). Finally, we found that curcumin increases phosphorylation of p53 and H2A.X (S140) in the nucleus of NCI-H460 cells (Figure 5A) that were assayed by confocal laser microscopic examination.

It is well-documented that there are several major DNA-repair pathways that can identify and repair chemotherapeutic agent-triggered DNA lesions in order for cancer cells to resist chemotherapy (14). Numerous studies have also demonstrated that in human lung cancer, excision repair cross-complementing 1 (ERCC1), BRCA1, 14-3-3σ, MGMT, and MDC1 are overexpressed and contribute to the development of chemoresistance (5, 6, 15-17).

Cisplatin-based regimens are widely used in the treatment of lung cancer but ovrexpression of BRCA1 and ERCC1 can diminish the anticancer efficacy of these agents in patients with lung cancer (5-6). Furthermore, DNA-damage and repair-associated proteins such as 14-3-3 σ, MGMT and MDC1 have been proposed to be predictive biomarkers for survival in patients with lung cancer (15-17). Thus, targeting DNA-repair proteins has emerged as a potential approach in treating human lung cancer. Herein, our results from western blotting showed that curcumin inhibited the protein expressions of 14-3-3 σ, MGMT, BRCA1 and MDC1 in NCI-H460 cells (Figure 4). Other investigators have shown that curcumin down-regulates the expression of ERCC1 *via* inhibition of extracellular signal-regulated kinases phosphorylation (18).

It was reported that the transcription factor p53 can induce the expression of genes which modulate cell-cycle arrest, apoptosis and DNA repair when cell DNA is damaged by chemotherapeutic agents (19). Sharma *et al*. demonstrated that histone H2A.X phosphorylation is a marker of DNA damage (20). Furthermore, numerous studies have shown that increased phosphorylation of p53 and H2A.X represents nuclear DNA damage (19-21). Results from western blotting also indicated that curcumin increased the phosphorylation of p53 and H2A.X in NCI-H460 cells (Figure 4). We also confirm these finding by using confocal laser microscopic examination which indicated that curcumin increases phosphorylation of p53 and H2A.X in nucleus of NCI-H460 cells (Figure 5).

In conclusion, based on these observations of curcumin triggering DNA damage and inhibiting expression of DNA-repair proteins14-3-3 σ, MGMT, BRCA1 and MDC1 in NCI-H460 cells, we suggest the possible molecular signal pathways in Figure 6. Curcumin induced cell death through induction of DNA damage and inhibition of DNA damage-associated proteins.

Acknowledgements

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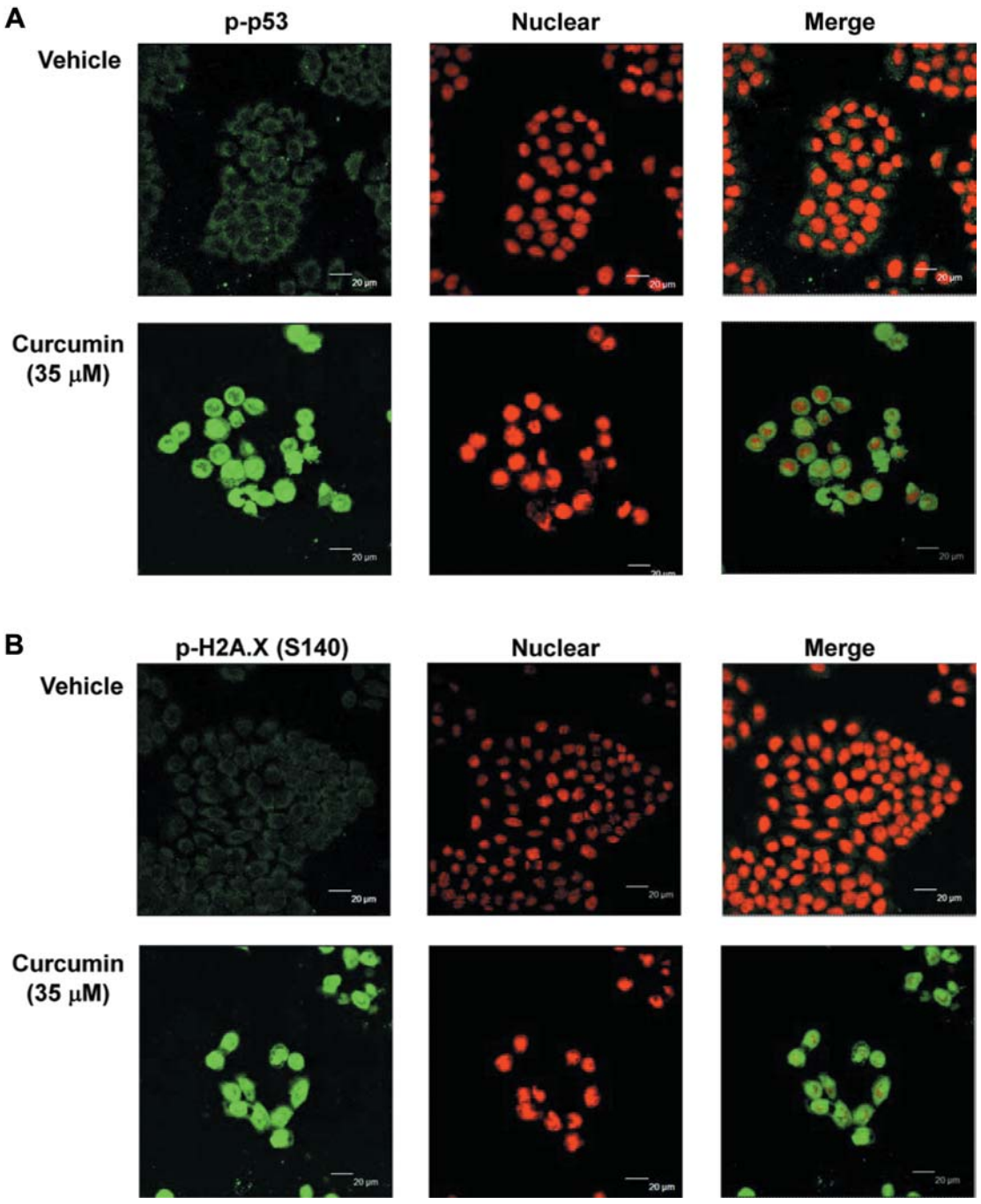

Figure 5. *The effects of curcumin on phosphorylation of p53 and H2A.X (S140) in nucleus of NCI-H460 cells. Cells were treated with 35 μM curcumin for 24 h. The expression of p-p53 and p-H2A.X (S140) in nucleus of NCI-H460 cells was monitored by using confocal laser microscopy. A: p-p53. B: p-H2A.X (S140). (H2A.X: Histone H2A.X).*

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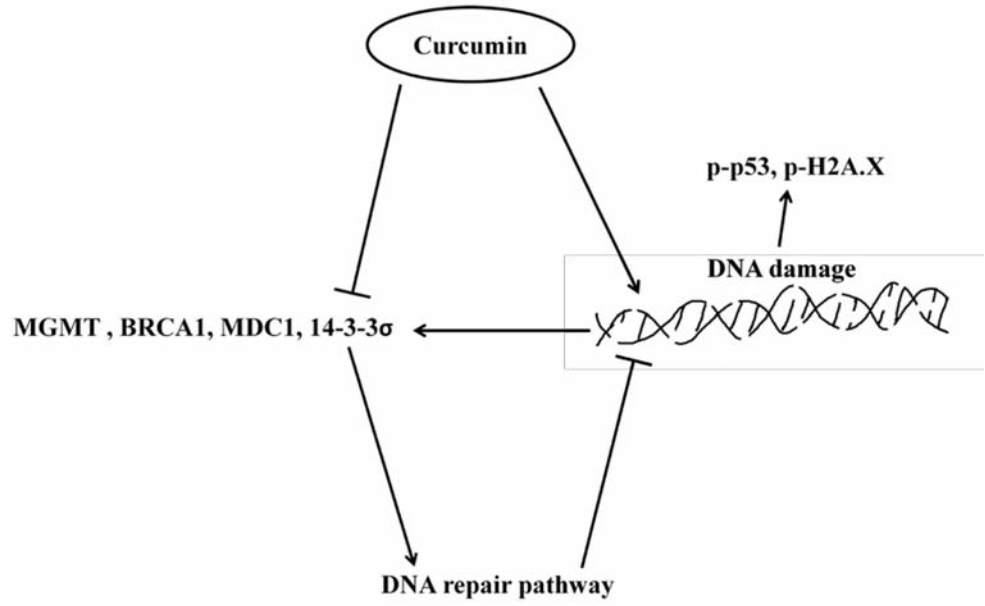

Figure 6. *The proposed pathway of curcumin-induced cell death via induction of DNA damage and inhibition of expression of DNA-damage and repair-associated proteins in NCI-H460 cells.*

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