

Anti-proliferative and pro-apoptotic effects of 3,3'-diindolylmethane in human cervical cancer cells

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Abstract. The antitumor effects of Indo-3-carbinol (I3C) have been proven in many human carcinoma cells. However, the roles of 3,3'-diindolylmethane (DIM), an important polymer converted from I3C under pH 5.0-7.0, on the growth and proliferation of cervical cancer HeLa and SiHa cells still remain unrevealed. In the present study, we investigated the potential anti-proliferative and pro-apoptotic effects of DIM on HeLa and SiHa cells. Cell proliferation was detected by Cell Counting kit-8 and apoptosis was analyzed by flow cytometry. In addition, morphological changes accompanying cell apoptosis were observed using an inverted microscope after Hoechst 33258 staining. In addition, expression changes of proteins involved in the MAPK and PI3K pathways were determined by western blotting. DIM treatment inhibited the proliferation and induced apoptosis of HeLa and SiHa cells significantly in a time- and dose-dependent manner. Moreover, SiHa cells were more sensitive to DIM treatment than HeLa cells ($P < 0.05$). In addition, the expression of ERK, p38 and p-p38, which are involved in the MAPK pathway, was downregulated by DIM treatment. Another protein involved in the MAPK pathway, JNK, was upregulated. Furthermore, DIM treatment significantly suppressed the expression of Akt, p-Akt, PI3K p110 α , PI3K p110 β , PI3K class III, GSK3- β , p-PDK1 and p-c-Raf which are involved in the PI3K pathway. These results demonstrate that DIM exerts antitumor effects on HeLa and SiHa cells through its anti-proliferative and pro-apoptotic roles, especially for SiHa cells. The molecular mechanism for these effects may be related to its regulatory effects on MAPK and PI3K pathway and apoptosis proteins. DIM may be a preventive and therapeutic agent against cervical cancer.

Introduction

Cervical carcinoma is the second most common malignancy of female reproductive tract and ranks second among female deaths

caused by cancers. More than 50 million people worldwide suffer from this disease each year. Although early diagnosis of cervical cancer has made great progress, the incidence and mortality of this disease still remains high, and the onset age is becoming younger, seriously threatening women's health and lives. The treatments mainly depend on surgery and radiotherapy, supplemented with chemotherapy. Five-year survival rate of terminally-ill patients is only 50% although therapeutic techniques have been improved greatly in the past years (1). It was reported that the poor effects of chemotherapy on advanced stage of cervical cancer may be directly due to obstacles in apoptosis of tumor cells (2).

Indole-3-carbinol (I3C), a type of compound derived from cruciferous vegetables (3), was demonstrated to exhibit significant anticancer efficacy both *in vivo* and *in vitro* by inducing cell apoptosis and cell cycle arrest in G1 phase (4,5), affecting DNA damage repair (6-8), as well as resisting angiogenesis, metastasizing and invasion of cancer cells (9,10). 3,3'-diindolylmethane (DIM), an important polymer converted from I3C under pH 5.0-7.0 (11), was characterized with anti-proliferative and pro-apoptotic activities in cancer cells (12,13). Chinnakannu *et al* (13) reported that a formulated DIM (B-DIM) could inactivate NF- κ B signaling and induce apoptosis through inhibiting proteasome activity in S phase of LNCaP and C4-2B cells, two types of prostate cancer cells, suggesting that B-DIM could be a potent agent for prevention and/or treatment of both hormone-sensitive and hormone-refractory prostate cancers. I3C-6, a derivative of I3C, was proven to significantly inhibit the growth and migration of HeLa cells (adenocarcinoma type) (14). However, there are no reports on anti-tumor effects of DIM in cervical carcinoma cells, or comparisons of its effects on two different types of cervical cancer cells, including HeLa cells (adenocarcinoma type) and SiHa cells (squamous carcinoma). The present study aimed to investigate the potential anticancer effects of DIM in cervical carcinoma cells *in vitro* and compare its different influence on HeLa cells and SiHa cells, thus providing useful information for clinical therapies of cervical carcinoma.

Materials and methods

Reagents. Cell Counting kit-8 (CCK-8) was purchased from Dongji (Japan). All antibodies were purchased from Cell Signaling Technology (USA), including p-p38, t-p38, p-ERK, t-ERK, p-JNK, t-JNK, PI3K p85, PI3K p110 α , PI3K p110 β ,

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p-Akt, t-Akt, p-PDK1, p-c-Raf and GAPDH. Annexin V FITC/PI Apoptosis Detection kit was from Lianke (China).

Cell cultures. The HeLa cells and SiHa cells, kindly provided by the Type Culture Collection of Chinese Academy of Sciences, were maintained in RPMI-1640 medium (Sigma, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, USA) and 20 $\mu\text{g}/\text{ml}$ antibiotics (ampicillin and kanamycin). The cultivations were performed in a humidified atmosphere of 5% CO_2 at 37°C. The medium was changed at 48-h intervals and culture transfers were performed every 4 days when the culture almost reached confluence.

Measurement of cell proliferation. Cells were harvested by trypsinization at their logarithmic growth phases and plated in 96-well microwell plates in 100 μl RPMI-1640 medium with 10% FBS at a density of 1×10^3 cells/well. Cells were treated with DIM at different concentrations (25, 50 and 100 μM) after 24 h of incubation and continuously cultured for 0, 24, 48 and 72 h before proliferation analysis. Cell proliferation assays were performed using the WST-8 based Colorimetric Assay Cell Counting kit-8 (CCK-8). In addition, proliferation was also measured in parallel negative groups without DIM treatment and the blank groups without cells in the wells. Six repeats were prepared for each group. Quantification of viable cells was performed by measuring the absorbance at 450 nm (A450) using a microplate reader (Beckman, USA). The following formula was used to calculate the inhibitory rate of DIM on cell proliferation: inhibitory rate (%) = [A450 (negative group) - A450 (treated group)] / [A450 (negative group) - A450 (blank group)] \times 100%.

Apoptosis analysis. After DIM treatment for 48 h, HeLa and SiHa cells were harvested by trypsinization ($\geq 1 \times 10^4$) and washed twice with cold phosphate-buffered saline (PBS). The cells resuspended in 200 μl annexin-V binding buffer were incubated in 5 μl of annexin-V fluorescein isothiocyanate (FITC) solution and 10 μl of propidium iodide (PI) solution for 15 min in the dark at room temperature. The fluorescences of each group were analyzed on a FACSCanto™ II spectrometer (BD Biosciences, San Jose, CA, USA) within 1 h and the cells stained with FITC/PI were counted as apoptotic cells.

Western blot analysis. Proteins were extracted from the cultured cells and loaded onto a polyacrylamide gel (10%). Then electrophoresis was performed at room temperature at 50 V for 30 min and then 200 V for 1.5 h, followed by transferring to nitrocellulose membranes. The membranes were incubated with blocking solution for 2 h at room temperature, and then incubated with different primary antibodies including p-p38, t-p38, p-ERK, t-ERK, p-JNK, t-JNK, PI3K p85, PI3K p110 α , PI3K p110 β , p-Akt, t-Akt, p-PDK1, p-c-Raf and GAPDH (Cell Signaling) for 12 h at 4°C. After washing for 1 h with 20 mM Tris-HCl and 0.5 M NaCl (TBS) with 0.05% Tween-20 (TTBS), membranes were incubated with the secondary antibody conjugated to horseradish peroxidase for 30 min at room temperature and washed again for 1 h. GAPDH was used as an internal reference. Protein bands were finally colored with enhanced chemiluminescence (ECL) reagent (Thermo, USA) and exposed to X-ray.

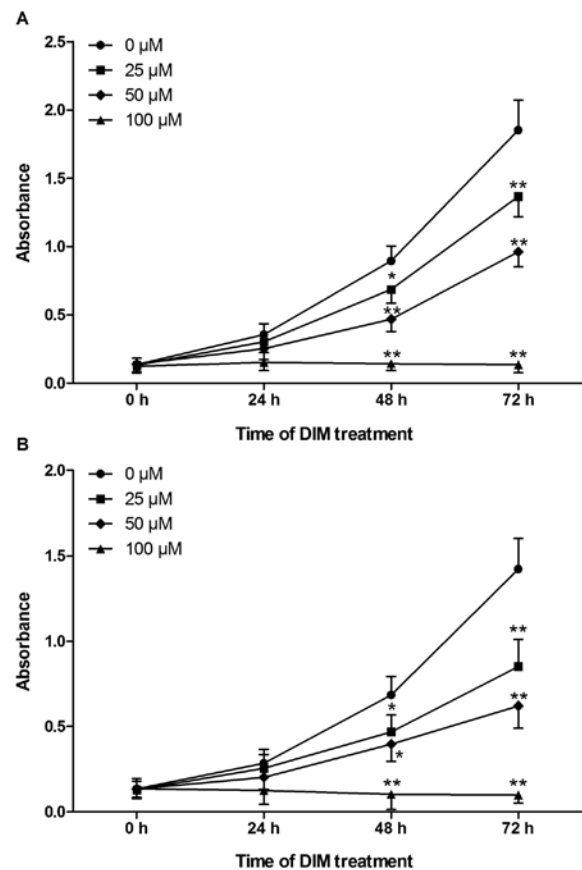


Figure 1. Effects of DIM treatment on the proliferation of HeLa cells (A) and SiHa cells (B). Cells were treated with various concentrations (0-100 μM) of DIM for 0-72 h. Cell proliferation was assessed by the CCK-8 method and represented by absorbance value at 450 nm. Data are the mean \pm SD values obtained from five culture wells per group. * $P < 0.05$ and ** $P < 0.01$ compared with the control group.

Statistical analysis. Data are expressed as mean \pm SD. Difference in the effects of DIM on HeLa and SiHa cells were compared with one-way ANOVA (SPSS statistical software, SPSS Inc., Chicago, IL, USA). $P < 0.05$ (two-tailed) was considered statistically significant.

Results

Effects of DIM on the proliferations of HeLa and SiHa cells. HeLa and SiHa cells were treated with various concentrations (0, 25, 50 and 100 μM) of DIM for 0-72 h. Cell proliferation was assessed by the CCK-8 method and represented by absorbance value at 450 nm. DIM showed a dose- and time-dependent anti-proliferative effect on the growth of HeLa and SiHa cells (Fig. 1).

DIM showed a profound dose-dependent inhibition of the growth of HeLa and SiHa cells with the inhibitory rates ranging from 14.61 to 93.03 (Table I). It was noticeable that the inhibitory effects of DIM on SiHa cells were much stronger than that on HeLa cells after 48 h of treatment in each concentration group (except for 50 μM group after 48 h of treatment). In addition, after DIM treatment for 48 h, the calculated IC_{50} of DIM for HeLa and SiHa cells were 46.8 and 44.44 μM respectively, suggesting a much more sensitive response of SiHa cells to DIM than that of HeLa cells.

Table I. Inhibitory rates of DIM on the proliferation of HeLa and SiHa cells.

DIM (μM)	24 h		48 h		72 h	
	HeLa	SiHa	HeLa	SiHa	HeLa	SiHa
25	14.61	10.18	23.38	31.58	26.19	40.04
50	29.21	28.77	47.54	42.11	48.06	56.30
100	57.02	56.14	84.00	84.94	92.66	93.03

Cells were treated with different concentrations (25-100 μM) of DIM for 0-72 h. Cell proliferation was assessed by the CCK-8 method and represented by absorbance value at 450 nm (A450). Inhibitory rate (%) = [A450 (negative group) - A450 (treated group)] / [A450 (negative group) - A450 (blank group)] x 100%. DIM, 3,3'- diindolylmethane.

Effects of DIM on the apoptosis of HeLa and SiHa cells. Flow cytometry was performed to detect the apoptosis of HeLa cells and SiHa cells treated with various concentrations of DIM for 48 h. As DIM concentrations increased from 0 μM (negative control group) to 100 μM , both HeLa cells and SiHa cells showed the following tendencies (Fig. 2): i) The cells distributed in the 3rd region (normal zone) gradually decreased, indicating that the number of living cancer cells were reduced by DIM treatment. ii) The cells distributed in the 4th region gradually increased, suggesting the number of cells at early stage of apoptosis were increased after DIM treatment. iii) The number of cells with broken nuclei, which would be distributed in the 2nd region (advanced apoptosis cells), also increased with the increment of DIM concentrations.

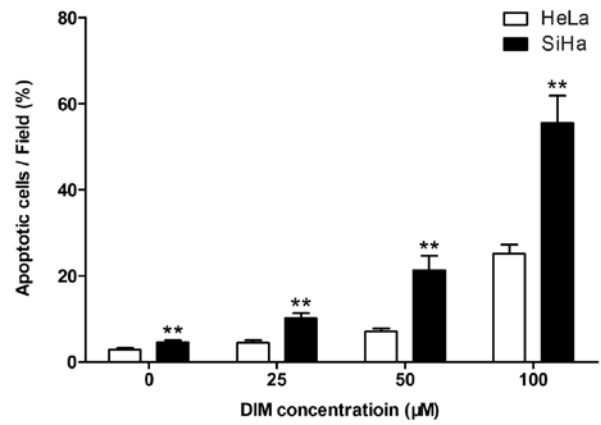


Figure 3. Comparisons of pro-apoptotic effects of DIM treatment on HeLa cells and SiHa cells. Cells were treated with various concentrations (0-100 μM) of DIM for 48 h. Flow cytometry was performed for apoptosis analysis and apoptotic ratio was expressed as the percentage of apoptotic cells over total cells in each field. **P<0.01 compared with HeLa cells.

DIM showed a significantly stronger pro-apoptotic effect on SiHa cells compared with HeLa cells (Fig. 3). Apoptosis was induced in (4.45 \pm 0.56), (7.11 \pm 0.67) and (25.16 \pm 2.12)% of HeLa cells at 25, 50 and 100 μM DIM respectively, while the apoptotic ratio in SiHa cells were (10.09 \pm 1.32), (21.11 \pm 3.36) and (55.46 \pm 6.33)% at each concentration (all P<0.01).

Effects of DIM on the expressions of proteins involved in apoptosis. Among many apoptosis pathways, the activation of mitogen-activated protein kinases (MAPK) and phosphoinositide 3-kinases (PI3K) signaling pathways drive a significant

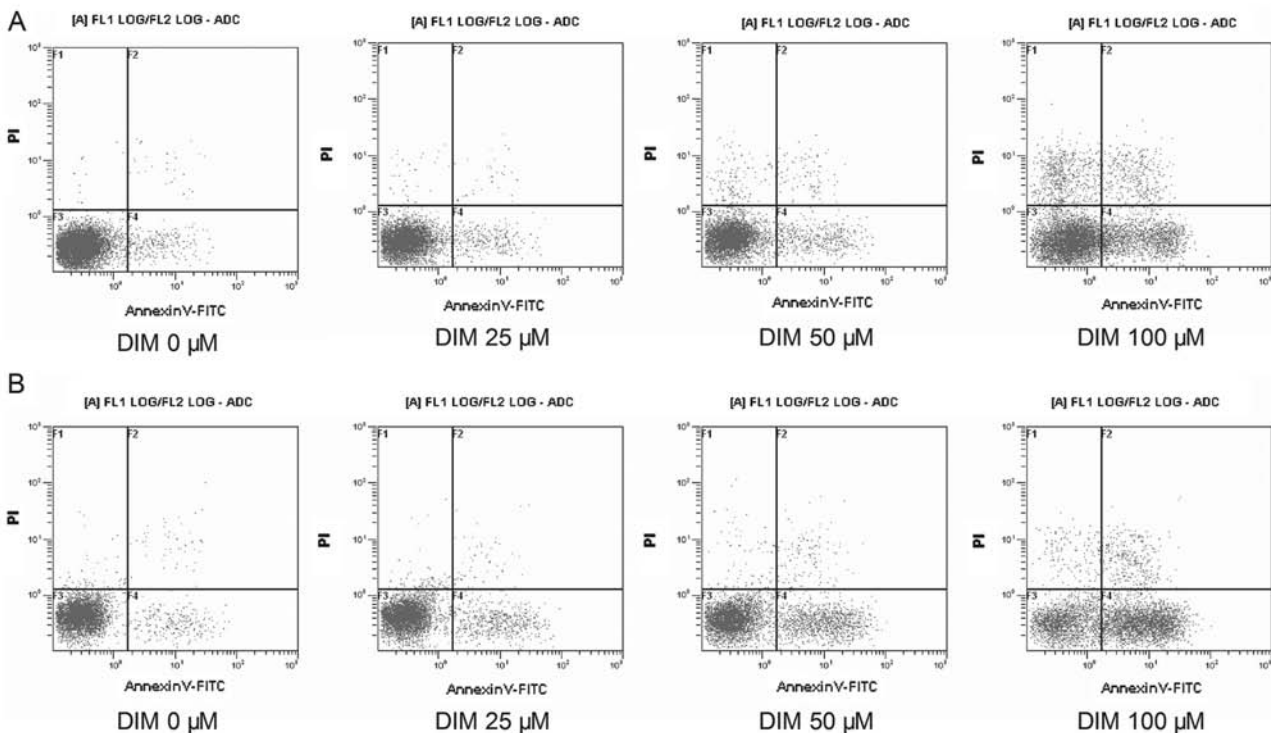


Figure 2. Detection of apoptosis in HeLa cells (A) and SiHa cells (B) after DIM treatment. Flow cytometry was performed for apoptosis analysis of HeLa cells and SiHa cells treated with various concentrations (0-100 μM) of DIM for 48 h.

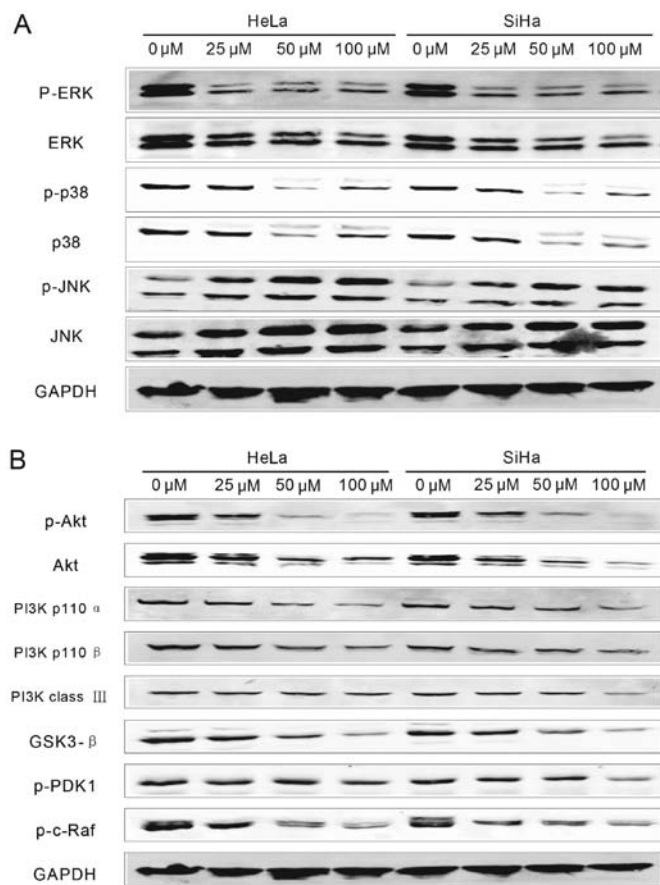


Figure 4. Expression changes of proteins involved in MAPK and PI3K pathways after DIM treatment. Cells were treated with various concentrations (0-100 μM) of DIM for 48 h.

percentage of human cancer and serve as the targets for drug development. Western blotting was performed to determine the expression changes of proteins involved in these two pathways. The results showed that expression levels of different proteins involved were more or less influenced by DIM treatment. As shown in Fig. 4, GAPDH, the internal reference, remained stable in the two types of cells before and after DIM treatment. For MAPK pathway (Fig. 4A), the expression levels of extracellular signal regulated kinase (ERK), as well as the levels of phosphorylated ERK (p-ERK), were all decreased in HeLa and SiHa cells in accordance with the increase of DIM concentration. p38 and p-p38, also proteins involved in MAPK pathway, were downregulated with relatively lower DIM concentrations (25 and 50 μM), but then upregulated when the concentration reached 100 μM . Moreover, the expression levels of p38 and p-p38 in SiHa cells treated with 100 μM DIM were lower than those in HeLa cells. Another protein involved in MAPK pathway, c-Jun N-terminal kinase (JNK), was upregulated by DIM treatment in a dose-dependent manner, accompanied with the increase of phosphorylated JNK (p-JNK) and there were no evident differences between HeLa and SiHa cells.

For proteins involved in PI3K pathway (p110 α , p110 β , class III, Akt, GSK3- β , p-PDK1 and p-c-Raf) (Fig. 4B), their expressions and phosphorylation levels were all downregulated in both HeLa and SiHa cells by DIM treatment with decreasing the drug concentration. In addition, the expression levels of

Akt, PI3K class III, GSK3- β , and p-PDK1 of SiHa cells treated with 100 μM DIM were significantly lower than those of HeLa cells.

Discussion

There is well documented evidence that I3C, a natural anticancer compound extracted from cruciferous vegetables, exerted anti-proliferative effects on many cancers *in vitro*, including breast, colon and prostate cancers (12,13). While DIM, the polymer converted from I3C under acidic conditions, was suggested to be the real actor during anticancer process induced by I3C (15). In the present study we investigated the effects of DIM on the proliferation and apoptosis of HeLa and SiHa cells, as they are two different typical types of cervical cancer cells.

In the present study it was demonstrated that DIM inhibited proliferation and induced apoptosis in HeLa and SiHa cells in a time- and dose-dependent manner, consistent with other reports concerning the anti-proliferative and pro-apoptotic abilities of DIM (12,13). Interestingly, SiHa cells, a type of squamous carcinoma cells, seemed to be more sensitive to DIM treatment than the adenocarcinoma cells (HeLa cells), contrary to the effects of many other anti-cancer compounds. According to Zhou *et al* (16), artesunate inhibited the growth of HeLa cells more efficiently than SiHa cells, and it also increased the radio-sensitivity of HeLa cells but not SiHa cells. Cisplatin, a widely-used drug in cancer chemotherapy, was demonstrated to be more effective in HeLa cells compared with SiHa cells (17). However, Han *et al* (18) found that extracts from shiitake (*Lentinula edodes*) mycelial culture broth, by an organic solvent ethyl acetate, inhibited the proliferation of cultured tumor cells, including Caski, SiHa, HeLa, HP-1 and A375 cells. Among them, HeLa cells showed lower sensitivity to the extracts than SiHa cells, not only in cell growth, but also in fragmentation of DNA, as well as the activity of caspase-3 in cancer cell extracts. In the present study a similar phenomenon for DIM treatment was observed.

Mitogen-activated protein kinases (MAPK), including ERK, JNK, and p38 MAPK (19), are ubiquitous serine/threonine protein kinases that have been implicated in many cellular processes such as proliferation, differentiation, and apoptosis (20,21). Human MAPK pathway was often deregulated in cancer cells (22) and thus considered as potential therapeutic targets (23). Elevated ERK activity was frequently observed in human tumors and suggested to be a good indicator of tumor progression and evaluation of the efficacy of therapeutic agents (24-26). Studies have shown that targeting ERK1/2 using siRNAs effectively reduced lung metastasis and sensitized tumor cells to chemotherapeutic agents such as cisplatin (25,27). The activation of p38 was also reported to be elevated in human non-small cell lung cancer (28) and the inhibition and reduction of p38 by inhibitor or RNAi could restrain the invasion and movement of cancer cells (29-31), suggesting the important roles of p38 in oncogenesis, tumor development, tumor invasion and metastasis. Several reports also provided evidence on JNK's function as proapoptotic kinase in response to various stimuli (32-37). The inhibitory effect of DIM on the proliferation of cervical cancer cells, as well as the induction of apoptosis in the present study, were demonstrated to be accompanied by downregulation of ERK, P38, and upregula-

tion of JNK, indicating that MAPK pathway was involved in the pro-apoptotic effects of DIM treatment on HeLa and SiHa cells, which was consistent to reports on other cancer cells. However, it remained unclear why expression of p38 and p-p38 was reduced at relatively lower concentration of DIM treatment, but then increased at the concentration of 100 μ M. This might be explained by a compensatory mechanism triggered at the advanced apoptosis stage since the main function of p38 was to protect cells from apoptosis (38).

PI3K, a major signaling hub downstream of HER2 (human epidermal growth factor receptor 2) and other receptor tyrosine kinases (RTKs), activates Akt, SGK, PDK1, mTOR, and several other molecules involved in cell cycle progression and survival (39). Three classes of PI3K enzymes have been defined and class I is the most intensely studied which includes p110 α , β , γ , and δ catalytic isoforms (40). As a direct downstream target of PI3K, Akt is also a key oncogenic survival factor and can phosphorylate and inactivate a panel of critical proapoptotic molecules, including Bad, caspase 9, GSK3- β , cell cycle inhibitors p21 and p27, and tumor suppressor TSC2 (41-45). The PI3K pathway is an important regulator in cell survival, proliferation, and apoptosis. Moreover, it is one of the most frequently altered networks in cancer (46). Overexpression of PI3K/AKT was observed recently in various malignant tumors, such as cervical carcinoma (47), prostate cancer (48), as well as breast cancer (49). Furthermore, PI3K p110 expression was detected to be positive in all stages of cervical lesions except normal cervical tissue (50). The inhibitors of PI3K or Akt, curcumin (51), celecoxib (52), perifosine (53-55) and CMEP (56), were all reported to attenuate the growth and proliferation of cancer cells and induce cell apoptosis. In the present study, downregulation of proteins associated with PI3K/Akt signaling pathway in HeLa and SiHa cells suggested that this pathway was also involved in the apoptosis of cervical cancer cells due to DIM treatment. In addition, different expression changes of p38, p-p38, Akt, PI3K classIII, GSK3- β , and p-PDK1 after DIM treatment between SiHa cells and HeLa cells might explain their different sensitivities to DIM treatment.

In conclusion, according to our results in the present study, DIM showed evident anti-proliferative and pro-apoptotic effects on cervical carcinoma cells in time and dose-dependent manner, and SiHa cells presented a stronger sensitivity than HeLa cells. MAPK and PI3K signaling pathways were proven to be involved in the pro-apoptotic effects of DIM on cervical cancer cells. Given that DIM exerted the above anti-tumor effects, especially on SiHa cells, it might be helpful for the development of novel therapeutic compounds for cervical cancers. Specific therapies for different types of cervical carcinoma are necessary since they react differently to different drugs.

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