

Pro-apoptotic and anti-proliferative effects of 3,3'-diindolylmethane in nasopharyngeal carcinoma cells via downregulation of telomerase activity

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Abstract. The pro-apoptotic and anti-proliferative effects of 3,3'-diindolylmethane (DIM) in various tumor cell types have been widely investigated. The underlying mechanisms were suggested to include cell cycle arrest, cell signaling inhibition and downregulation of the androgen receptor. The present study demonstrated that DIM induced apoptosis and inhibited proliferation in nasopharyngeal carcinoma cells by downregulating the activity of telomerase. The nasopharyngeal carcinoma cell line 5-8F was selected for this purpose. A cell counting kit-8 assay and flow cytometry were performed to detect apoptosis and proliferation of 5-8F cells, respectively, which revealed the pro-apoptotic and anti-proliferative effects of DIM. Telomerase activity was detected using a telomeric repeat amplification protocol assay, which revealed that the telomerase activity was inhibited by DIM in a dose-dependent manner. Reverse transcription polymerase chain reaction was used to detect the mRNA expression levels of human telomerase reverse transcriptase (hTERT) and human telomerase RNA (hTR), and western blot analysis was used to detect the protein expression of hTERT. The results showed that the mRNA and protein expression of hTERT were downregulated in 5-8F cells following treatment with DIM; however, the mRNA expression of hTR remained unchanged, suggesting that hTERT was the target of DIM. To further identify the target, the length of telomeres was continually measured using a telomere length detection

kit, revealing that the telomeres were shortened by DIM in an concentration-dependent manner. The present study confirmed that DIM had pro-apoptotic and anti-proliferative effects in nasopharyngeal carcinoma cells by regulating telomerase.

Introduction

Cruciferous vegetables are plants exerting antitumor activity. 3,3' Diindolylmethane (DIM), which extracted from cruciferous vegetables, induces antiproliferative and proapoptotic effects in a variety of tumor cell types, including nasopharyngeal carcinoma (NPC) cells (1,2). Several underlying mechanisms of the anti tumor effects of DIM have been reported (3-6), however, the DIM regulation of telomerase activity, which is important in NPC, and the associated mechanisms remain to be elucidated. Telomeres are located on the ends of eukaryotic chromosomes, are comprised of G- and C-rich hexanucleotide repeats and protect chromosome ends from recombination, fusion and degradation (7). Cell division is accompanied by a gradual reduction in telomere length. A short telomere triggers the apoptotic program, which, however, can be avoided by the activation of telomerase (7,8). Telomerase is composed of human telomerase RNA (hTR), telomerase-associated protein 1 (TP1) and human telomerase reverse transcriptase (hTERT). As the rate-limiting component of telomerase, hTERT induces the immortalization of a number of cell types in culture (8).

However, whether the effects of DIM may be associated with telomerase activity has remained to be elucidated. Previous studies have investigated the underlying mechanisms of the anti-proliferative and pro-apoptotic effects of DIM and have revealed that cell cycle arrest (9), cell signaling inhibition (10-12) and downregulation of the androgen receptor (13) are involved. The inhibition of telomerase was reported to induce anti-proliferative effects (14,15). The present study hypothesized that telomerase may be important in the anti-tumor mechanism of DIM. Therefore, the effects of DIM on the proliferation and apoptotic rate of NPC cells were assessed. Furthermore, telomerase activity, levels of hTERT and hTR, as well as telomere length were assessed in nasopharyngeal cells treated with DIM.

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Materials and methods

Cells and culture. Human nasopharyngeal carcinoma (NPC) 5-8F cells were purchased from the Cell Bank of the Chinese Academy of Science (Shanghai, China). The cell line was cultured in the medium RPMI-1640 (HyClone Corp., Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco Life Technologies, Carlsbad, CA, USA) and cultured in a humidified incubator at 37°C with 5% CO₂.

DIM. DIM (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in dimethylsulfoxide (Sigma-Aldrich) and was diluted to the concentrations of 0, 25, 50, 75 and 100 μM in complete medium (Genom Biotechnology, Hangzhou, China).

Cell proliferation assay. Cells in the logarithmic growth phase were obtained and were seeded into 96-well plates at 2,500 cells/well. Following culturing, cell proliferation was assessed using a cell counting kit-8 (CCK-8; cat. no. C0038; Beyotime Institute of Biotechnology, Shanghai, China) according to manufacturer's instructions. Briefly, 10 μl CCK8 solution was added to the culture medium followed by incubation for 1 h. The absorbance was measured at a wavelength of 450 nm with a reference wavelength of 630 nm.

Flow cytometry. Induction of apoptosis was assessed using flow cytometric analysis. An Annexin V/propidium iodide (PI) apoptosis kit (cat. no. LK-AP101-100; Lianke Biotech Co., Ltd., Hangzhou, China) was used for the detection. The cells were seeded into six-well plates and the next day, the medium was changed to medium containing 0, 25, 50, 75 and 100 μM DIM for up to 24 h, prior to digestion and collection. The samples were stained with Annexin V-fluorescein isothiocyanate (FITC) and PI for 15 min. The cells were analyzed immediately by flow cytometry (FACSCanto II; BD Biosciences, San Jose, CA, USA) using the fluorescence channels FL1 (emission at 525 nm) and FL3 (emission at 670 nm).

Telomeric repeat amplification protocol (TRAP) assay. For the detection of telomerase activity, a TRAP-polymerase chain reaction (PCR) assay was used (Millipore, Billerica, MA, USA). The primers were as follows: TS, 5'-AATCCGTGAGCAGAGTT-3' and CX primer, 5'-CCCTTACCCCTTACCCCTAACCAA-3'. Cells (~1x10⁶) were washed once with ice-cold wash buffer, re-suspended and centrifuged at 3,000 × g for 5 min at 4°C. The precipitate was homogenized with 40 μl cold lysis buffer for 30 min on ice, followed by centrifugation at 13,000 × g for 30 min at 4°C. The supernatants were used for subsequent analyses. The extension reaction was as follows: 5 μl 10X TRAP buffer, 1 μl deoxyribonucleoside triphosphates, 1 μl Taq-DNA polymerase, 1 μl telomere strand primer, 2 μl telomerase extraction, 39 μl diethylpyrocarbonate-treated H₂O and 1 μl CX primer. Prior to PCR, a solution without CX primer was pre-incubated at 23°C for 30 min. The PCR for the TRAP assay was performed as follows: 94°C for 5 min; 35 cycles of 94°C for 30 sec, 50°C for 30 sec and 72°C for 90 sec; followed by 72°C for 10 min. The PCR products were electrophoresed using 10% SDS-PAGE. The gels were stained with ethidium bromide for 15 min, subsequently scanned and images were

captured (Geliance 200 Gel Imaging system; Perkin Elmer, Waltham, MA, USA).

Reverse transcription (RT)PCR. The total RNA was isolated from ~1x10⁶ cells and a 25 μl reaction mixture, containing 8.5 μl 5X RT buffer, 2 μl RT enzyme mix, 2 μl primer mix and 12.5 μl nuclease-free water, which were contained in the RT-PCR kit (Takara, Dalian, China), were incubated at 65°C for 5 min, followed by 42°C for 1 h. The cDNA was amplified using the following specific primers: hTERT forward, 5'-CGGAAGAGTGTCTGGAGCAA-3' and reverse, 5'-GGATGAAGCGGAGTCGGA-3'; hTR forward, 5'-TCTAACCCCTAACTGAGAAGGGCGTAG-3' and reverse, 5'-GTTTGCTCTAGAATGAACGGGAAAG-3'; Actin forward, 5'-CGTACCACTGGCATCGTGAT-3' and reverse, 5'-GTGTTGGCGTACAGGTCTTG-3'; GAPDH forward, 5'-GAAGGTGAAGGTGGAGTCA-3' and reverse, 5'-GGCAGAGATGATGACCCTTT-3'. All primers were designed and synthesized by Sangon Biotechnology, Shanghai, China. The PCR assay was performed as follows: 94°C for 5 min; 35 cycles at 94°C for 30 sec, 60°C for 30 sec and 72°C for 90 sec; followed by 72°C for 10 min. The products were subsequently electrophoresed on a 1.5% agarose gels. The gels were stained with ethidium bromide for 15 min, scanned and images were captured (Geliance 200 Gel Imaging system; Perkin Elmer).

Western blot analysis. The cells were harvested and lysed with lysate buffer (Guge Biotechnology, Wuhan, China), mixed with protease inhibitor cocktail (Roche, Basel, Switzerland) and phosphorylase inhibitor (Roche) on ice for 15 min, and was subsequently quantified using a BCA kit (Thermo Fisher Scientific, Grand Island, NY, USA). The total protein extracts from each group of cells were resolved by 10% SDS-PAGE (Guge Biotechnology) and transferred on polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Following blocking with 5% sealing liquid (Guge Biotechnology) for 2 h at room temperature, the PVDF membranes were washed three times for 15 min with Tris-buffered saline containing Tween-20 (TBST; Guge Biotechnology) at room temperature and incubated with primary antibody (rabbit anti-hTERT; 1:500; cat. no. sc-7212; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. Following extensive washing with TBST three times for 15 min each, the membranes were incubated with the secondary antibody, donkey anti-rabbit immunoglobulin G (cat. no. 926-32213; LI-COR, Lincoln, NE, USA) for 1 h. Following washing three times for 15 min with TBST at room temperature, the membranes were scanned with the Odyssey CLx Infrared Imaging system (LI-COR).

Telomere length detection. A telomere peptide nucleic acid (PNA) fluorescence *in situ* hybridization (FISH) kit FITC (cat. no. K5325; Dako Denmark A/S, Glostrup, Denmark) was used for the detection of telomere length. The nuclei were isolated by re-suspending 3x10⁵ cells in 2% Triton X-100 and 0.1 M citric acid buffer (Guge Biotechnology), vortexed and incubated for 10 min at room temperature. The samples were washed once with phosphate-buffered saline (PBS) and directly subjected to denaturation/hybridization. Denaturation

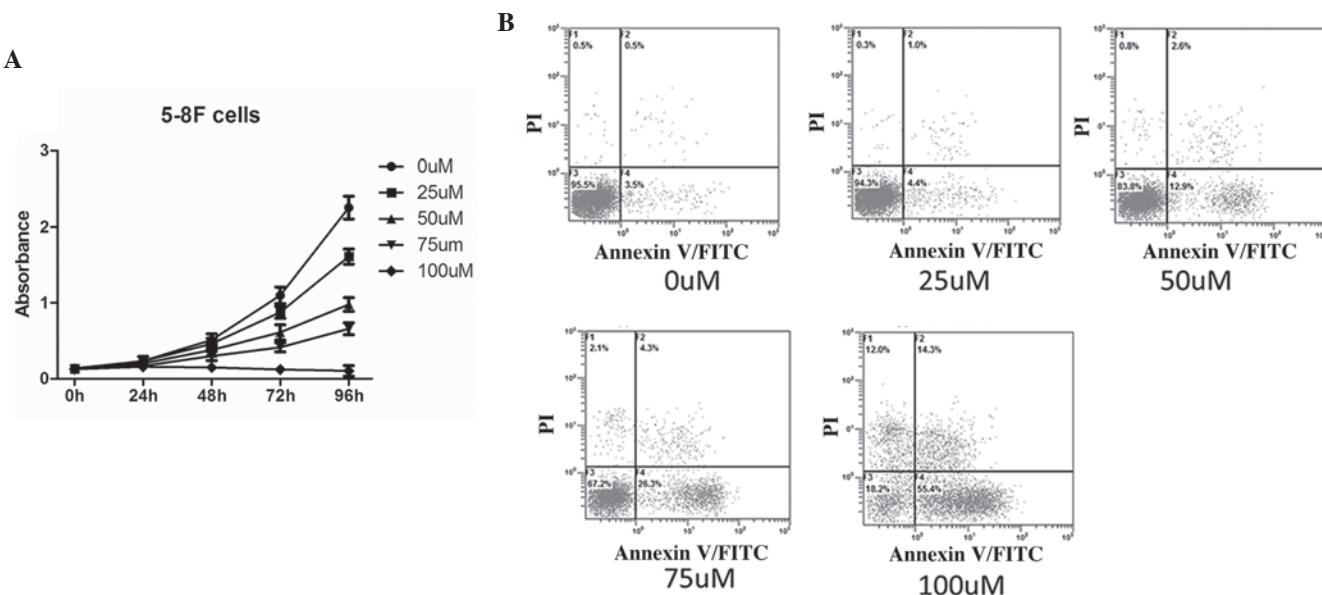


Figure 1. Proapoptotic and antiproliferative effects of DIM in the 5-8F NPC cell line. (A) The proliferation rate of the NPC cells treated with DIM at various concentrations was determined. At the time points of 0, 24, 48, 72 and 96 h, the proliferation ability was detected by CCK-8 assay. As the concentration of DIM increased, the proliferation ability of the 5-8F cells was reduced ($P<0.05$). (B) The apoptotic response of the NPC cells treated with DIM at various concentrations was determined. Following 24 h treatment with DIM, the apoptosis rate of the 5-8F cells was detected by flow cytometry. The apoptotic rates were 4.0 ± 0.4 , 5.4 ± 0.6 , 15.5 ± 0.5 , 30.6 ± 0.5 and $69.7\pm0.6\%$, respectively. As the concentration of DIM increased, there was an increasing trend in the apoptotic response of the NPC cells ($P<0.05$). DIM, 3,3'-diindolylmethane; NPC, nasopharyngeal carcinoma; CCK, cell counting kit; PI, propidium iodide; FITC, fluorescein isothiocyanate.

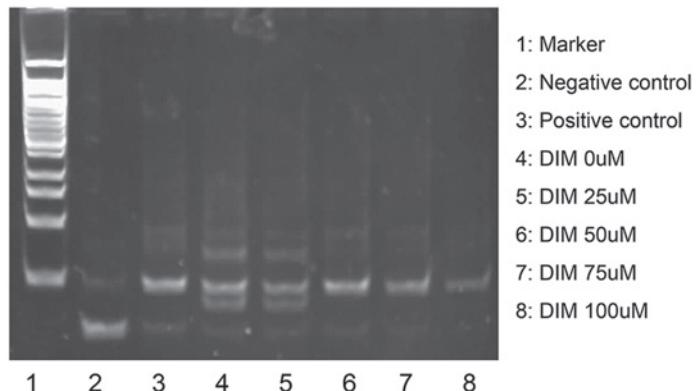


Figure 2. Telomerase activity of 5-8F cells and 5-8F cells treated with DIM. A TRAP assay was performed to detect the telomerase activity. It was demonstrated that the telomerase activity was decreased in a concentration-dependent manner. DIM, 3,3'-diindolylmethane; TRAP, telomeric repeat amplification protocol.

was performed on a thermo block at 80°C for 10 min and the samples were allowed to hybridize at room temperature overnight. The samples were washed with PBS, incubated on a heat block at 40°C for 10 min and subsequently centrifuged at 700 x g for 5 min. The samples were re-suspended in 200 ml DNA staining solution. For DNA counterstaining, the cells were re-suspended in 500 μ l staining solution for 2 h prior to acquisition on a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). The mean telomere fluorescence of the cells was analyzed using Cell Quest software (Becton Dickinson).

Statistical analysis. All statistical analyses were performed using SPSS version 20.0 software (IBM SPSS, Chicago, IL, USA). The values are expressed as the mean \pm standard deviation. A t-test was used to determine the significance.

$P<0.05$ was considered to indicate a statistically significant difference.

Results

NPC cell growth is repressed and apoptosis is increased following treatment with DIM. To identify the effects of DIM on NPC cells, the proliferation ability and the apoptotic rate of NPC cells were determined following treatment with DIM. A CCK-8 assay was used to detect the proliferative rate of the NPC cells. The NPC cells were treated with DIM at various concentrations: 0, 25, 50, 75 and 100 μ M, for five durations: 0, 24, 48, 72 and 96 h. As the duration increased, the absorbance was increased. In addition, at each time-point, the absorbance decreased as the concentration of DIM increased. Of note, the absorbance at each time-point revealed no change at a

concentration of 100 μ M, indicating that cells failed to grow substantially (Fig. 1A).

The apoptotic response of NPC cells was detected by flow cytometry. As the concentration of DIM increased, there was an increasing trend in the apoptotic rate of the NPC cells (Fig. 1B).

Telomerase deactivation is involved in the anti-cancer effect of DIM. To identify whether telomerase activity was changed following treatment with DIM, telomerase activity was detected. A TRAP assay was performed to detect the telomerase activity in 5-8F cells and 5-8F cells treated with DIM at a range of concentrations as mentioned above. The results demonstrated that the telomerase activity was reduced in the DIM-treated cells compared with that in the control 5-8F cells, and the decreasing effect occurred in a concentration-dependent manner (Fig. 2).

Telomerase subunit hTERT is the possible target of DIM. To assess which part of telomerase was the target of DIM, the expression levels of the two components of telomerase, hTERT and hTR, were detected. The mRNA expression levels of hTERT and hTR were detected by RT-PCR, and the protein expression of hTERT was detected by western blot analysis. The results demonstrated that the mRNA and protein expression levels of hTERT were downregulated in the 5-8F cells treated with DIM compared with those in the control 5-8F cells (Fig. 3). The mRNA expression of hTR remained unchanged (Fig. 3), indicating that hTERT was the possible target of DIM in the regulation of the proliferation and apoptosis of NPC cells.

Telomeres are shortened in the NPC cells treated with DIM. To further confirm the functions of DIM with hTERT, the length of telomeres was detected. A telomere length detection kit was used for the detection and the results demonstrated that the telomeres in the 5-8F cells treated with DIM were shortened compared with those in the control 5-8F cells in a concentration-dependent manner (Fig. 4). Combined with the finding of apoptosis being induced by DIM in NPC cells, the present study hypothesized that telomere shortening leads to cell death, as indicated by the increased apoptotic rate.

Discussion

DIM is a tumor-preventive agent and is a natural product present in cruciferous plants. The anti-tumor effect of DIM has been vastly investigated (2-6,16,17). The underlying mechanisms of the anti-tumor effects were reported in several previous studies which demonstrated that DIM induces apoptosis of tumor cells (1-4), inhibits proliferation (2,5,6), regulates several signaling pathways (2-4), including phosphoinositide 3-kinase, mitogen-activated protein kinase, Akt and nuclear factor- κ B, regulates the cell cycle (5), inhibits tumor angiogenesis (16,17), downregulates the androgen receptor (7) and exerts a preventive and curative role in the development and progression of certain types of tumor (2,5).

Proliferation and apoptosis are important hallmark properties of cancer cells (18). Based on this, the present study aimed to investigate the effects of DIM on the proliferation

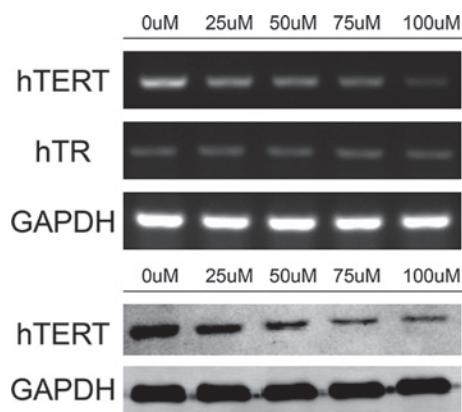


Figure 3. Expression of the telomerase sub-unit of 5-8F cells and 5-8F cells treated with DIM. The cells were treated with 0, 25, 50, 75 and 100 μ M DIM for 24 h, and the cells were harvested to detect the protein and mRNA expression levels. The mRNA expression levels of hTERT and hTR were detected by reverse transcription polymerase chain reaction and the protein expression of hTERT was detected by western blot analysis. It was demonstrated that the mRNA and protein expression levels of hTERT were downregulated in the cells treated with DIM compared with those in the untreated 5-8F cells ($P<0.05$), while the mRNA expression levels of hTR remained unchanged ($P>0.05$). DIM, 3,3'-diindolylmethane; hTERT, human telomerase reverse transcriptase; hTR, human telomerase RNA.

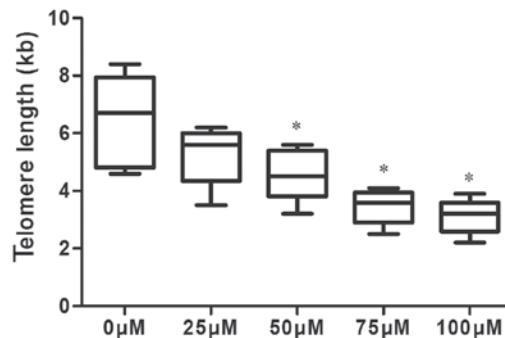


Figure 4. Telomere length in 5-8F cells and 5-8F cells treated with DIM. The cells were treated with 0, 25, 50, 75 and 100 μ M DIM for 24 h, and a telomere length detection kit was used to detect telomere length. The telomere length was graphed as boxes and error bars indicate the mean \pm standard deviation of three replicates. The results were 6.44 ± 1.6 , 5.26 ± 1.0 , 4.58 ± 0.92 , 3.46 ± 0.61 and 3.12 ± 0.61 , respectively, and these results indicated that telomeres in cells treated with DIM were shortened compared with those in untreated 5-8F cells. * $P<0.05$, compared with the 0 μ M DIM group. DIM, 3,3'-diindolylmethane.

and apoptosis of NPC cells, to identify its effects on telomerase and elucidate the possible underlying mechanism. The proliferation ability of cells with and without DIM treatment was detected using a CCK-8 assay, and the apoptosis of the cells was detected by flow cytometry. The results demonstrated that DIM inhibited proliferation and induced apoptosis of the NPC cells.

Telomerase was first characterized by its ability to elongate and maintain telomere length; however, previous studies on mice and cells have revealed that it also functions in other processes (19-22). The novel functions of telomerase were assessed following the elimination of telomerase activity and several functions were reported, including induction of apoptosis, participation in DNA repair, association with DNA replication protein primase, regulation of DNA damage

responses, modulation of several signaling pathways and regulation of gene expression.

Telomerase activity was hypothesized to affect changes in proliferation and apoptosis of NPC cells (23); therefore, the present study used the TRAP-PCR assay for the detection of telomerase activity. The results demonstrated that telomerase activity was inhibited following treatment with DIM. Based on these results, the present study aimed to determine which sub-unit of telomerase is a target of DIM. RT-PCR and western blot analyses were performed to detect the mRNA and protein expression levels of hTERT, respectively, and the mRNA expression of hTR. The results demonstrated that the mRNA and protein expression levels of hTERT were downregulated in the DIM-treated cells; however, the mRNA expression of hTR remained unchanged. This suggested that DIM decreased telomerase activity by downregulating the mRNA and protein expression of hTERT.

The expression of hTERT was downregulated in the cells treated with DIM, which confirmed that hTERT was the target of DIM. Inhibition of hTERT by DIM resulted in the inhibition of the telomerase activity. The present study next aimed to determine whether the function of telomerase in this process was associated with telomere length. A FISH assay was used to assess whether the telomere length was affected by DIM, and the results revealed that telomere length was shortened in the DIM-treated cells, suggesting that telomerase inhibition led to decreases in telomere length, inhibited proliferation and directly induced the apoptosis of NPC cells.

Several previous studies have reported that telomerase has a role in the effect of the anti-proliferative and anti-tumor effects of drugs (24-26). Zhao *et al* (24) revealed that telomerase was inhibited by harmine and the proliferation of MCF-7 cells was decreased. Long-term effects of hTERT inhibition were demonstrated by Qian *et al* (25), who revealed that SGC-7901 cell proliferation was inhibited by the inhibition of telomerase activity. The present study demonstrated that telomerase activity was inhibited by DIM through the inhibition of hTERT.

Telomerase was identified to be associated with the sensitivity of NPC cells to radiotherapy and chemotherapy (23,27,28). It was hypothesized that DIM may also be involved in the sensitivity to radiotherapy and chemotherapy, and several previous studies confirmed this. Ahmad *et al* (29) reported that DIM increased the chemotherapeutic sensitivity of prostatic cancer cells, which were multidrug-resistant. Fan *et al* (30) reported that physiological sub-micromolar concentrations of DIM protected cultured cells against radiation. DIM was thoroughly studied for its functions in cancer therapy and prevention, and indole-3-carbinol, the precursor of DIM, was already approved for clinical use in the treatment of respiratory papillomatosis in the USA (31). It is no exaggeration to suggest that there is a wide prospect for DIM in clinical application; however, further study is required.

In conclusion, the present study reported that the proliferation of NPC cells was inhibited and apoptosis was induced by DIM. NPC cells treated with DIM showed obvious decreases in telomerase activity, and it was speculated that the changes in cell proliferation and apoptosis of NPC cells treated by DIM were associated with telomerase. The mRNA and protein expression of hTERT were downregulated following treatment

with DIM, while the expression of hTR mRNA was unaltered, indicating that hTERT was the target of DIM. Furthermore, telomeres were shortened in DIM-treated NPCs, which confirmed that pro-apoptotic and anti-proliferative effects of DIM were mediated through the regulation of telomerase.

Acknowledgements

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