ERK signaling mediates long-term low concentration 3,3'-diindolylmethane inhibited nasopharyngeal carcinoma growth and metastasis: An *in vitro* and *in vivo* study

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Abstract. It is well known that crucifers have antitumor effects and 3,3'-diindolylmethane (DIM) is one of the major bioactive components, and the associated molecular mechanisms in a short-term high-dose manner are widely discussed. However, the antitumor effects of DIM in a long-term low-dose manner in nasopharyngeal carcinoma (NPC) has not been reported yet, as to the potential mechanisms in the human body. In the present study, NPC cells were induced by 20 μmol/l DIM for over a month, and the proliferation, apoptosis, migration and *in vivo* metastasis were investigated. The results showed that DIM significantly reduced the proliferation and migration; however, changes in apoptosis were not observed. *In vivo* study showed the metastasis was significantly reduced. Compared to the short-term high-dose manner, incomplete similar qualities were observed; next we explored the possible signal pathway revolved, the ERK signaling showed similar changes, while the PI3K/Akt, NF-κB, P38, JNK pathways were significantly altered in the short-term high-dose manner (our previous study) showed no obvious change, indicating the ERK signaling may be the main effector of DIM.

Introduction

Epigenetic studies have demonstrated that long-term intake of cruciferous vegetables, such as broccoli, carrots and cauliflower, effectively reduced the risk of cancer, as the variety of natural anticancer compounds contained in the crucifers could be absorbed in human body. 3,3'-Diindolylmethane (DIM) is one of the most important natural compounds with anticancer properties extracted from crucifers (1). Presently, the natural compounds with anticancer effects causing only slight side-effects are of great interests to medical researchers. Several studies have reported a series of natural compounds, which were extracted from natural materials, with the anticancer effects of inhibiting tumor cell growth and division (2-4), or with anticancer effects of inhibiting tumor metastasis in animal experiments (5,6). Besides, several natural compounds were applied in combination with radiotherapy or chemotherapy in tumor treatment, and enhanced effects of treatments were observed (5,7-9).

DIM was reported as an effective cell proliferation inhibitor and apoptosis inducer in a variety of tumors, such as breast (3), prostate (10,11), pancreatic (8), ovarian (12) and thyroid cancers (13). However, the majority of the studies focused on the short-term killing effects of DIM on tumor cells. Similarly to most of the researches, in our previous study, short-term high-dose manner was designed, and we found that DIM effectively induced cell death in nasopharyngeal carcinoma (NPC) cells both *in vitro* and *in vivo*, and no obviously toxic effects were observed on the normal tissues and organs (14). While, whether this type of model could induce similar response in the human body with long-term dietary intake of DIM was unclear, more likely the different key effectors activated in the long-term low-dose manner, thus, in the present study, a long-term low concentration of DIM was chosen to treat the NPC cells, the selection criteria of the concentration referred to several published results (15,16), the detectable concentration of DIM in serum or tissue of human or animal was reported to be 20 μmol/l, whereas, no significant inhibitory effects on NPC cell proliferation were observed with 20 μmol/l DIM in the short-term response in our previous study (14).

We further explored the antitumor effects of DIM in long-term low-dose manner on NPC cells, as well as the potential targets or effectors playing important roles in the long-term low-dose manner of DIM, which would provide reliable technical indications for clinical use of DIM in long-term low-dose manner.

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Materials and methods

Reagents. DIM was purchased from Sigma-Aldrich, dissolved in dimethylsulfoxide (both from Sigma-Aldrich St. Louis, MO, USA) and was diluted to 20 μM in complete medium (HyClone Corp., Logan, UT, USA). Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Tokyo, Japan), the antibodies used in the present study were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA).

Cell culture. Human NPC cell lines CNE-2, and 5-8F were maintained in our laboratory, and were stored in liquid nitrogen. The cells were cultured in RPMI-1640 (HyClone Corp.), supplemented with 10% fetal bovine serum (FBS; Gibco Life Technologies, Carlsbad, CA, USA) and 20 μg/ml antibiotics (ampicilin and kanamycin; Genome Biotechnology, Hangzhou, China), at 37˚C, 5% CO_2. Cells in logarithmic growth phase were used in the experiment. The NPC cells CNE-2, and 5-8F were treated with 20 μM DIM for over a month then renamed as CNE-2/DIM^{LL} and 5-8F/DIM^{LL}.

Cell proliferation assay. Cells were seeded into 96-well plates with 1x10^3 cells/well for normal culture. Six double wells were set. After 24, 48, 72 and 96 h culture, 10 μl of CCK-8 was added in each well, and were incubated at 37˚C for 1 h, and absorbance value was measured at 450 nm.

Transwell assay. Transwell assay was performed using polycarbonate membrane Transwell (Corning Inc., Corning, NY, USA). The bottom chamber included medium (0.5 ml) containing 5% FBS. The cell density seeded was 3.0x10^5/ml in the upper chamber, incubated 24 h at 37˚C, 5% CO_2. Membranes were then washed, fixed and stained by Methyl violet (Guge Biotechnology, Wuhan, China). The invasion ability of the cells was determined by counting the cells that had pass through to the lower side of the filter with a microscope.

Flow cytometry. Annexin V/PI apoptosis kit (cat. no. LK-AP101-100; Lianke Biotech Co., Ltd., Hangzhou, China) was used for the detection. Cells were harvested and washed twice with cold phosphate-buffered saline (Genom Biotechnology). The cells were resuspended in Annexin V binding buffer, and were then stained with 5 μl of Annexin V-FITC solution and 10 μl of propidium iodide (PI) solution for 15 min in the dark. Fluorescence was analyzed on a FACSCanto™ II spectrometer (BD Biosciences, San Jose, CA, USA).

Animal feeding and grouping. The animal experiment was approved by the Ethics Committee of Renmin Hospital of Wuhan University. Forty-eight female BALB/c nude mice (4-6 weeks old) were purchased from Beijing HuaFukang Biological Technology Co. Ltd. (HFK Bioscience, Beijing, China), and underwent adaptive feeding one week before the experiment. The NPC cells at logarithmic phase were collected, resuspended in serum-free medium, then the cell concentration was adjusted to 1x10^7/ml. Syringe (1 ml) was used for injection of 200 μl cell suspension for each animal (14). After the inoculation, the animals were raised for 8 weeks. The short and long diameter of transplanted tumors at 1-8 weeks after the inoculation were measured, 8 weeks after inoculation, animals were sacrificed.

Hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC). Specimens of transplanted tumors were fixed, embedded and sliced. The H&E staining was performed following the protocol of the H&E staining kit (Guge Biotechnology). Then neutral balsam was used for mounting and the section was observed and photographed under a microscope. The IHC was performed with the method of SABC, following the protocol of the IHC kit (Guge Biotechnology).

Western blotting. Cells were harvested and lysed in buffer containing 1% Nonidet-P40 supplemented with complete protease inhibitor 'cocktail' (Roche) and 2 mM dithiothreitol. Lysates were resolved by 10% SDS-PAGE, transferred to polyvinylidene defluoride (PVDF) (Immobilon-FL; Millipore, Billerica, MA, USA) membranes and immuno-blotted with primary antibodies. After immunoblotting with the secondary antibody, donkey anti-rabbit immunoglobulin G (cat. no. 926-3221), the membranes were scanned with Odyssey CLx Infrared Imaging System (both from LI-COR, Lincoln, NE, USA).

Statistical analysis. The values are expressed as the mean ± SD. Statistical analyses were carried out by one-way ANOVA performed using the SPSS statistical software (SPSS, Inc., Chicago, IL, USA). Probability values of (P-value) <0.05 were considered as statistically significant.

Results

DIM in the long-term low-dose manner significantly reduces the proliferation and migration without influence of apoptosis. To evaluate the effects of long-term low-dose DIM on the proliferation of NPC cells, the proliferation ability was detected with CCK-8 assay. As shown in Fig. 1B, compared to 5-8F group, the proliferation rate of 5-8F treated with long-term low concentration DIM (5-8F/DIM^{LL}) group decreased by 35% (P<0.05), while the proliferation rate of CNE-2 treated with long-term low concentration DIM (CNE-2/DIM^{LL}) group had no significant difference from that of the CNE-2 cell group (P>0.05). To further explore the effects of long-term low-dose DIM on apoptosis of NPC cells, the apoptotic rates were detected with flow cytometric assay. The results in Fig. 1C and D show that the apoptotic rates of 5-8F, 5-8F/DIM^{LL}, CNE-2 and CNE-2/DIM^{LL} were (2.7±0.5%), (2.9±0.4%), (1.0±0.4%) and (1.0±0.6%), respectively. There was no statistically significant difference between the treatments (P>0.05). In addition, the migration ability was explored to evaluate the effects of long-term low-dose DIM on NPC cells. Transwell assay for migration was performed, as shown in Fig. 1E and F, after 36 h incubation in the Transwell chamber, the numbers of the membrane-penetrating cells in the four cell groups were 67.3±8.9, 52.4±10.2, 24.8±6.3 and 21.2±7.1, respectively. Apparently, the number of the membrane-penetrating cells was significantly reduced in the CNE-2/DIM^{LL} and 5-8F/DIM^{LL} cell groups as compared to those of the CNE-2 and 5-8F cell groups (P<0.01).

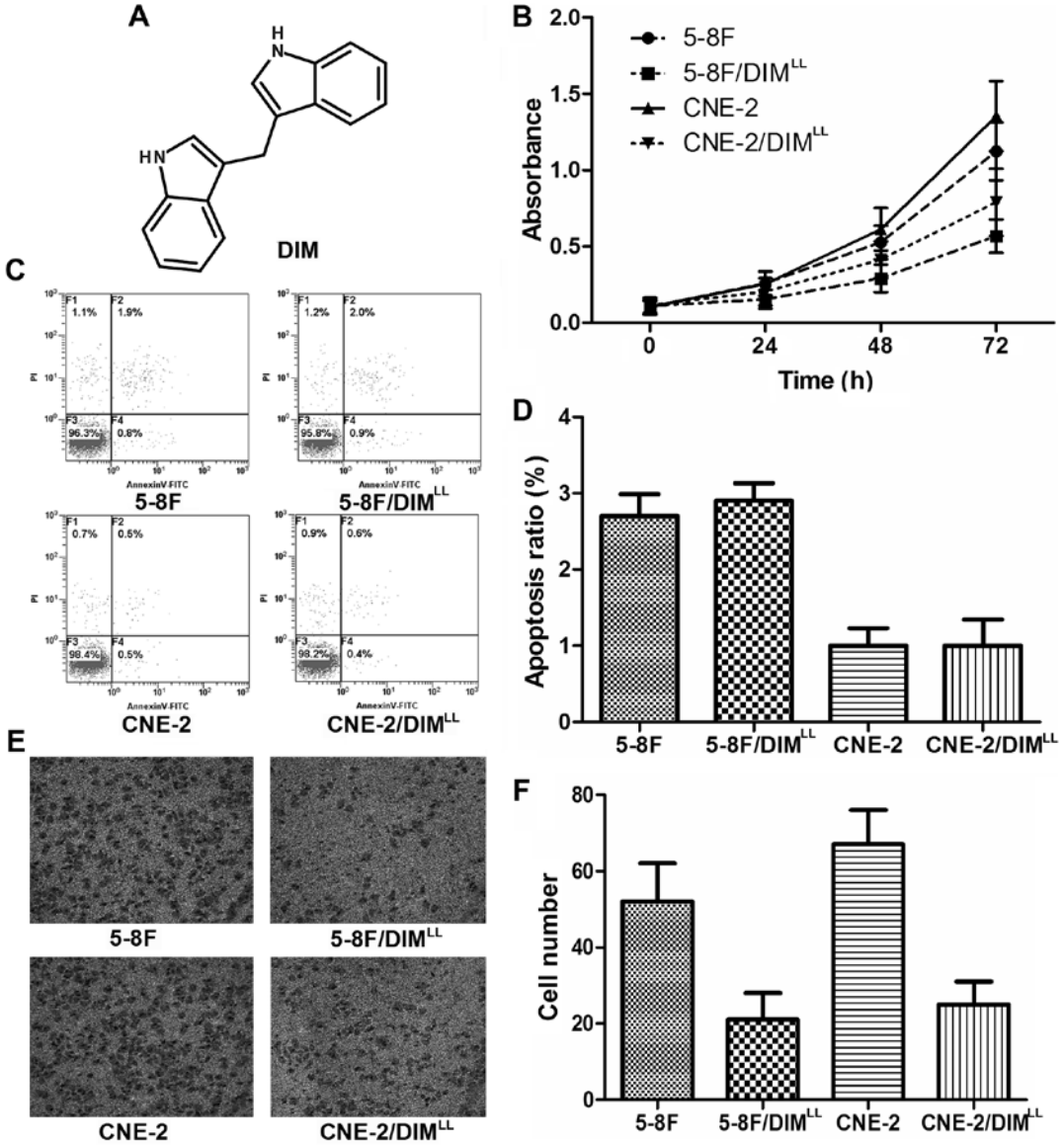

Figure 1. Long-term low-dose of DIM results in the change of proliferative, apoptosis and migration in NPC cells. (A) Molecular formula of DIM; the proliferative capacity was detected by CCK-8 assay (B), compared to 5-8F, 5-8F/DIM^{LL} group was decreased, while the proliferation rate of CNE-2/DIM^{LL} group had no apparently difference when compared to CNE-2. The apoptosis rates of 5-8F, 5-8F/DIM^{LL}, CNE-2 and CNE-2/DIM^{LL} (C and D) were detected by a flow cytometric assay, and there was no statistically significant difference between 5-8F and 5-8F/DIM^{LL}, similar result was observed between group CNE-2 and CNE-2/DIM^{LL} (C and D). Transwell assay was chosen for the detection of the migration ability, the numbers of the membrane-penetrating cells of 5-8F/DIM^{LL}, 5-8F, CNE-2/DIM^{LL}, CNE-2 are shown (E). The histogram of the statistically significant difference between the four groups is shown (F).

DIM in the long-term low-dose manner significantly reduces metastasis in vivo. The *in vitro* experiments demonstrated that the treatment with long-term low concentration DIM significantly decreased the proliferation and migration in NPC cells. Therefore, an animal experiment was conducted by establishing a subcutaneous xenograft tumor model in nude mice with 5-8F, CNE-2, 5-8F/DIM^{LL} and CNE-2/DIM^{LL} cells. Eight weeks after the NPC cell inoculations, the average volume of the xenograft tumor in the 5-8F/DIM^{LL} and CNE-2/DIM^{LL} animal groups were 857.8±126.1 and 1034.4±147.1 mm^3, as shown in Fig. 2, respectively. In comparison, the average volume of the xenograft tumor in the 5-8F and CNE-2 animal groups were significantly larger, 2032.1±223.3 and 2769.1±241.3 mm^3, respectively (P<0.05). Eight weeks after the NPC cells inoculation, the tumor formation rates of the xenografts in the 5-8F and CNE-2

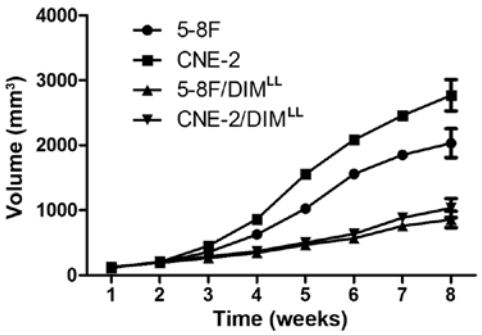

Figure 2. Long-term low-dose of DIM results in the change of proliferative and metastasis *in vivo*. The average volume of the xenograft tumor from the first week to the 8 week after the NPC cell inoculation of the four groups, the average volume of the xenograft tumor in the 5-8F and CNE-2 animal groups were significantly larger than the 5-8F/DIM^{LL} and CNE-2/DIM^{LL} animal groups.

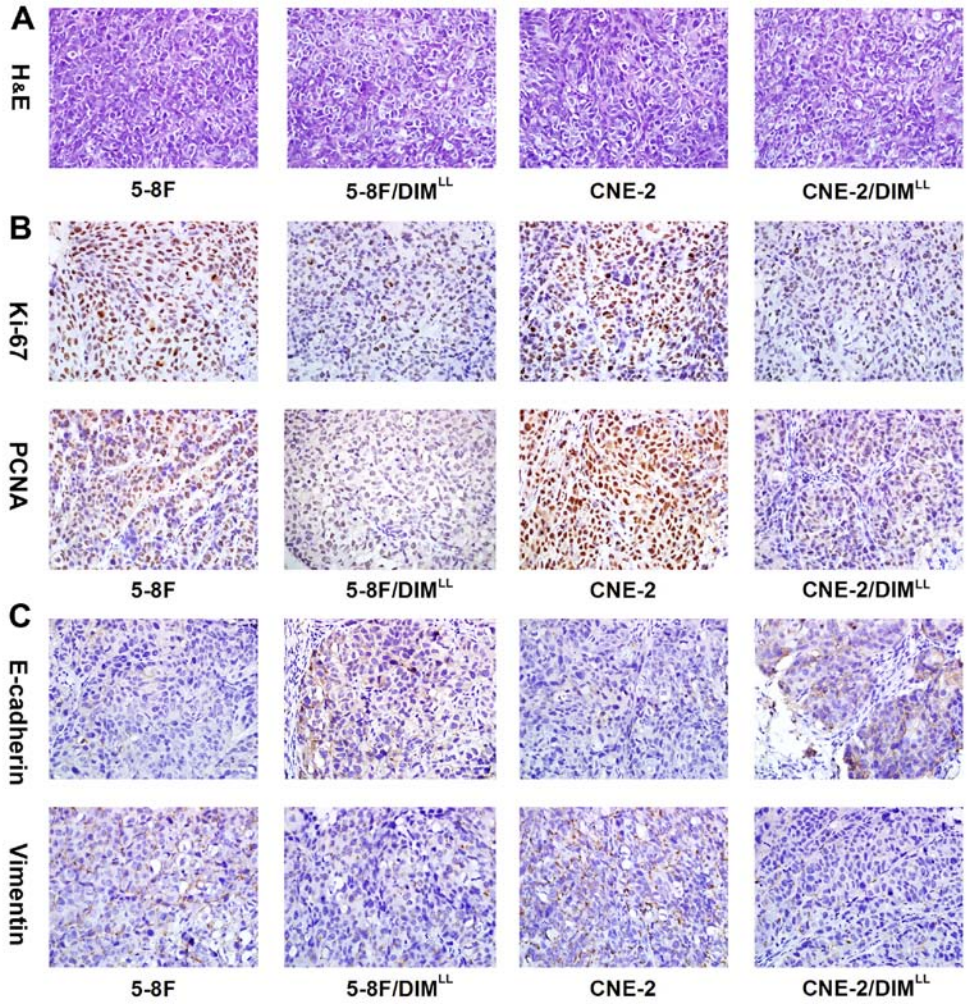

Figure 3. Long-term low-dose of DIM results in the change of proliferation and metastasis-related protein expression *in vivo*. The xenograft tumors of the four groups were made into tissue paraffin blocks, H&E staining of tumor tissues are shown (A), IHC assay was chosen for the detection of the expression of proliferation-related molecules Ki-67 and PCNA (B), and the metastasis-related molecules E-cadherin and vimentin (C), the relative expression intensities of Ki-67, PCNA and vimentin were significantly lower (P<0.01), while the E-cadherin was significantly higher (P<0.01) in the 5-8F/DIM^{LL} and CNE-2/DIM^{LL} groups than the 5-8F and CNE-2 groups.

Table I. Long-term low-dose of DIM resulted in the change of proliferative and metastasis *in vivo*.

Group	No. of mice	Xenograft tumor	Lymph node metastasis
5-8F	12	12/12 (100%)	11/12 (91.7%)
CNE-2	12	12/12 (100%)	10/12 (83.3%)
5-8F/DIM	12	9/12 (75%)	2/9 (22.2%)
CNE-2/DIM	12	10/12 (83.3%)	3/10 (30%)

DIM, 3,3'-diindolylmethane.

animal groups were 12/12 and 12/12, respectively. The lymph node metastasis rates were 10/12 and 11/12, respectively. In comparison, the 5-8F/DIM^{LL} and CNE-2/DIM^{LL} animal groups had a lower tumor formation rates of the xenografts (9/12 and 10/12), and a significantly decreased lymph node metastatic rates (2/9 and 3/10), respectively (P<0.01), and the results are shown in Table I.

Proliferation and metastasis-related protein are altered in the long-term low-dose DIM manner. In the subcutaneous xenograft tumor model of the nude mice, the decrease of proliferation and metastasis of the 5-8F and CNE-2 treated with long-term low-dose DIM were observed, then, to further identify the related effectors involved in the effects of anti-proliferation and antimetastasis of long-term low-dose DIM, we detected the expression of proliferation related PCNA and Ki-67 as well as metastasis related E-cadherin and vimentin in the xenograft of the nude mice through immunohistochemical assay. As shown in Fig. 3B and C, the relative expression intensities of PCNA, Ki-67 and vimentin were significantly lower (P<0.01), while the E-cadherin was significantly higher (P<0.01) in the 5-8F/DIM^{LL} and CNE-2/DIM^{LL} groups than the 5-8F and CNE-2 groups.

ERK signal pathway is significantly decreased in both the short-term high-dose manner and the long-term low-dose manner of DIM. As indicated above, the NPC cell lines 5-8F and CNE-2 underwent significant changes in proliferation, migration, as well as metastasis in the long-term low-dose

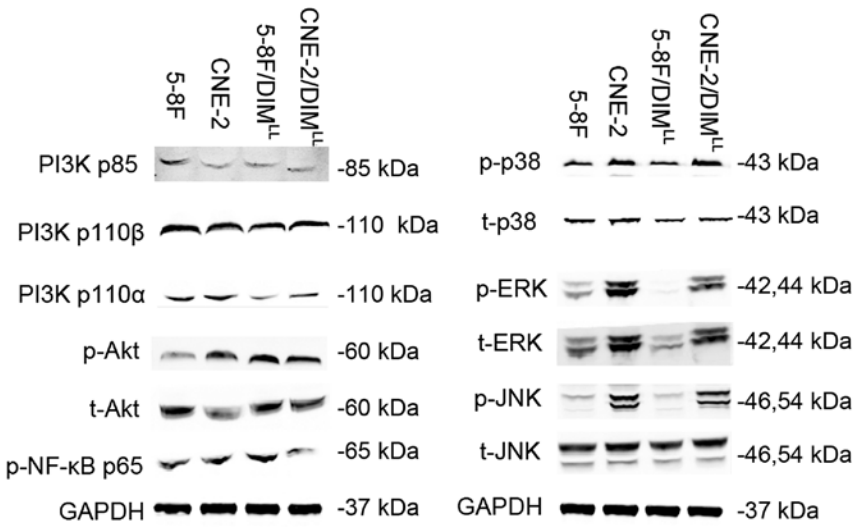

Figure 4. Long-term low-dose of DIM results in the change of proliferation and metastasis-related protein expression in NPC cells. Western blotting was chosen for the detection. The expression of proteins related to the PI3K, NF-κB, MAPK signal pathway were detected, and there were no significant changes between the 5-8F/DIM^{LL}, CNE-2/DIM^{LL} and 5-8F, CNE-2 groups in the PI3K, NF-κB, P38, JNK pathway, however, the ERK pathway showed a significant decreasing trend in the 5-8F/DIM^{LL}, CNE-2/DIM^{LL} groups compared to the 5-8F and CNE-2 group.

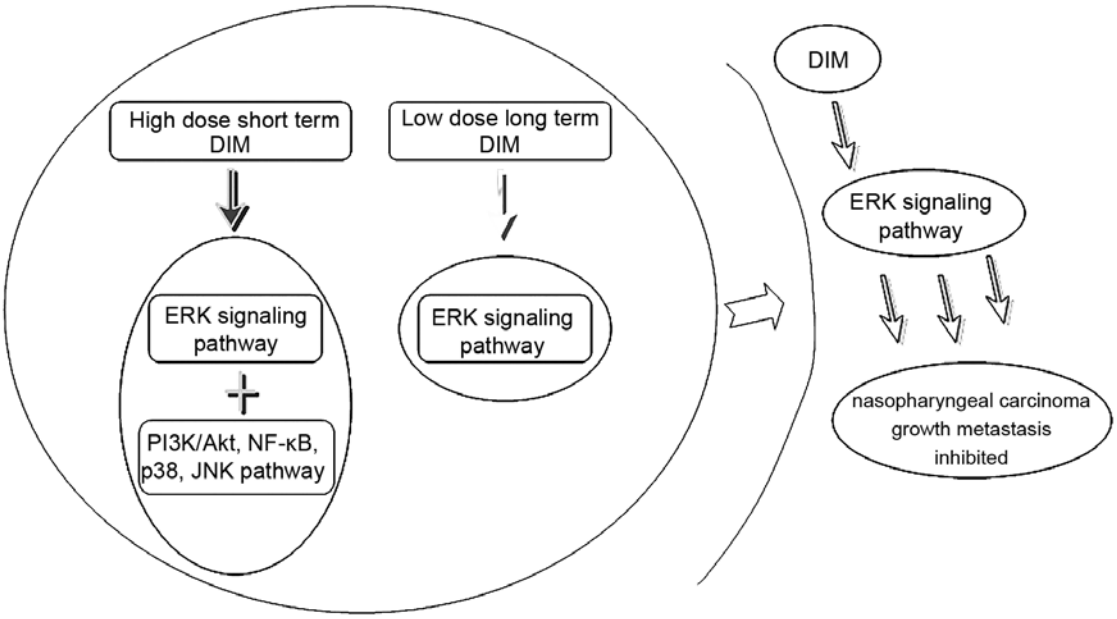

Figure 5. ERK is the major target of DIM in NPC. The ERK signaling pathway was decreased obviously during the long-term low-dose manner of DIM, compared to the short-term high-dose manner of DIM, in which manner that the biological behaviors of NPC cells (CNE-2 and 5-8F) under the treatment of DIM were generally consistent, while the PI3K/Akt, NF-κB, P38 pathway which were significantly reduced, and JNK was significantly increased in the short-term high-dose manner showing no obvious change, thus indicating that the ERK signaling may be the main effector involved in the long-term low-dose manner of DIM treatment.

manner of DIM, therefore, we investigated the key signaling pathways involved in the occurrence and development of NPC, the protein expression in relation to cell proliferation, migration and metastasis was detected by western blotting. Since the short-term high-dose manner of DIM was reported in our previous study (14), the key signaling pathways were detected and compared with the previous results, and the results are shown in Fig. 4, the ERK signaling showed similar changes, while in the short-term high-dose manner (14), the PI3K/Akt, NF-κB, JNK pathways which were significantly reduced, and the P38 pathway, which was significantly increased, showed no obvious change, indicating the ERK signaling may be the main effector involved in the long-term low-dose manner of DIM.

Discussion

DIM is a natural compound extracted from cruciferous vegetables, and widely recognized for its antitumor effects. Several *in vitro* studies had reported the pro-apoptosis effects in tumor

cells in short-term high-dose manner of DIM (2,5,8-10). In our previous study, short-term high concentration of DIM effectively inhibited the proliferation and apoptosis of NPC cells in a dose-dependent manner. Moreover, the results of animal experiments showed that DIM had certain effects on NPC prevention and inhibition, including slowing down the tumor progression and reducing the incidence of tumor metastasis (14). In the present study, we raised the suggestion that, treatment with high-dose of DIM would induce an acute or transient response, as a result, the experiments based on the high-dose DIM may only show an acute or transient response in the body, whereas the continued dietary intake of DIM resulted in a relatively steady-state, the response *in vivo* would be different, therefore the long-term low-dose was designed for the study.

Long-term intake of edible crucifers effectively prevents the occurrence of specific tumors. The possible mechanism is thought to be that, a certain dose of DIM was persistently identified in the blood in the condition of sustaining intake of crucifers, the early mutations which resulted in the proliferation induction or the apoptosis inhibition of the tumor cells were thereby effectively inhibited. Studies have shown that colon cancer spontaneously occurred in heterozygous TRAMP mice, while oral administration of DIM significantly decreased its incidence and alleviated the severity of colon lesions in the transgenic mice. These findings indicated that DIM effectively inhibited the occurrence of colon cancer and prevented its progression (17). In another experiment, Howells *et al* found that DIM was detectable in the blood samples with a concentration up to 20 μmol/l (16,18). Similarly, Moiseeva *et al* (19) found that a continued treatment with low concentration of DIM significantly changed the specific gene expression of the breast cancer MD-MBA-231 cells and affected their biological behavior, such as the doubling time, motility and ability to repair DNA damage. Therefore, in the present study, we conducted *in vivo* and *in vitro* experiments in order to examine the effect of long-term low-dose DIM treatment on the NPC cells; moreover, the related underlying molecular mechanism was further investigated.

Our previous results showed that after 48-72 h of treatment with 20 μmol/l of DIM, no obvious changes were observed in the NPC cells, neither proliferation inhibition nor apoptosis induction (14), however, the continued treatments with 20 μmol/l DIM for over a month resulted in significant changes in proliferation, migration and metastasis. While, there was a slight difference between the two NPC cell lines. For example, after being treated with 20 μmol/l of DIM for over 30 days, the 5-8F cell line had a significant reduction in proliferation ability, migration and metastasis, however, the CNE-2 cell line had no evident decrease in the proliferation ability, but showed a decreased ability for migration and metastasis. Such variations could be related to the differences of gene expression profiles or epigenetic properties between various cancer cell lines.

The signaling pathways PI3K/Akt, MAPK and NF-κB, which played an important role in the occurrence, pathogenesis and metastasis processes of NPC (20-24), were reported in regulation of cell proliferation, migration and metastasis. In addition, they were detected by western blot assay in the study, aimed to explore the possible targets of DIM (20 μmol/l, over a month) in NPC. It turned out that the ERK pathway was significantly decreased during the treatment, while the other primary signal pathways were not obviously changed. Compared to the short-term high-dose manner in our previous study, the expression changes of relevant signaling pathway proteins differed.

ERK signaling pathway was vital *in vivo*, a number of important biological process were under the regulation of ERK signaling pathway, such as proliferation, differentiation, apoptosis, cancerization and other biological reaction. The ERK1/2 was located in the cytosol in an inactivated state, when phosphorylated to activate, translocated from the cytosol to the nucleus, and affected the multiple biological processes through the regulation of the activity of transcription factors. ERK was reported activated in a majority of NPC (25-27). Since we observed that the ERK signaling showed similar changes, while the PI3K/Akt, NF-κB, P38 and JNK pathways which were significantly changed in the short-term high-dose manner (14) showed no obvious change, indicated the ERK signaling may be the main effector involved in the long-term low-dose manner of DIM treatment. Based on the differences of the activated signals (Fig. 5), we speculated that the PI3K/Akt, NF-κB, P38, JNK pathways which were activated/inactivated during the short-term high-dose manner of DIM were only thought to be the drug toxic reaction of the high-dose DIM, and also considered to be the reason that apoptosis increased in the high-dose of DIM, by contrast, apoptosis was not changed in the low-dose of DIM, these results confirmed that ERK may be the real target of DIM in the long-term low-dose treatment of DIM in NPC.

In conclusion, a long-term low-dose DIM treatment (20 μmol/l) inhibited the proliferation, migration, as well as the *in vivo* metastasis in NPC cells, further, the ERK signaling pathway may be the main effector in the long-term low-dose DIM. Our results provide evidence that the use of long-term low-dose DIM suppresses the activation of the ERK pathway, which would add support for the use of DIM in preclinical and clinical settings in the management of NPC patients.

Acknowledgements

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