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# Synergistic antitumor activity of vitamin D3 combined with metformin in human breast carcinoma MDA-MB-231 cells involves m-TOR related signaling pathways

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Metformin is usually used for the treatment of type 2 diabetes. Recently, many studies suggest that metformin and vitamin D have broad-spectrum antitumor activities. Our aim in this research was to study the effects of vitamin D3 combined with metformin on the apoptosis induction and its mechanisms in the human breast cancer cell line MDA-MB-231. Cell proliferation was measured by methylthiazol tetrazolium (MTT) assay. The morphology of cell apoptosis was observed after Hoechst 33342 staining. Here we show that vitamin D3 280 μg/ml or vitamin D3 300 μg/ml or vitamin D3 320 μg/ml seperately combined with metformin 15000 μg/ml exhibited synergistic effects on cell proliferation and apoptosis. The underlying anti-tumor mechanisms may involve m-TOR related pathways, which are related to activating expression of cleaved caspase-3, Bax and p-AMPK, as well as inhibiting expressions of p-Bcl-2, c-Myc, p-IGF-IR, p-mTOR, p-P70S6K, p-S6.

## 1. Introduction

Triple negative breast cancer (TNBC) is a breast cancer subtype that does not express estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). Thus, endocrine therapy and anti-HER2 targeted therapy have no effect on triple negative breast cancer, so chemotherapy plays a vital role in the treatment of TNBC. TNBC has highly invasive, highly malignant, intractable characteristics, which have become a challenging demand (Dent et al. 2007; Jiralerspong et al. 2009).

Metformin is the first-line drug for the treatment of type 2 diabetes. In recent years, many studies suggest that metformin has good anti-tumor effects *in vitro* and *in vivo* (Bost et al. 2012; Xiong et al. 2012; Kisfalvi et al. 2009). Calcitriol is the the most active vitamin D metabolite. Studies show that calcitriol has broad spectrum antitumor activities *in vitro* and *in vivo*, besides, calcitriol can enhance the activity of a variety of chemotherapeutic agents (Yu et al. 2010; Ma et al. 2010; Deeb et al. 2007). This study was devoted to study the effects of metformin combined with vitamin D3 on proliferation and apoptosis in triple negative breast cancer cell line MDA-MB-231 *in vitro* and its molecular mechanism.

## 2. Investigations and results

### *2.1. Effects of vitamin D3 combined with metformin on cell proliferation*

Vitamin D3 (280 μg/ml, 300 μg/ml, 320 μg/ml) combined with 15000 μg/ml metformin acted on cells for 24 h; Results showed a strong inhibitory effect and significant synergistic effect, but, with increasing concentration of vitamin D3 the inhibition rate of drug combination is not obvious, but still a high level (Fig. 1A). Low concentration group: 280 μg/ml vitamin D3, 15000 μg/ml metformin, 280 μg/ml vitamin D3 + 15000 μg/ml metformin, IR was -17.12 ± 1.71%, 29.27 ± 0.9%, 86.13 ± 0.74% respectively; Compared with the negative control group, the inhibition rate were significantly different ($P < 0.01$).

Middle concentration group: 300 μg/ml vitamin D3, 15000 μg/ml metformin, 300 μg/ml vitamin D3 + 15000 μg/ml metformin, IR was 15.75 ± 2.27%, 29.3 ± 0.29%, 90.47 ± 0.32% respectively; Compared with the negative control group, the inhibition rate were significantly different ($P < 0.01$).

High concentration group: 320 μg/ml vitamin D3, 15000 μg/ml metformin, 320 μg/ml vitamin D3 + 15000 μg/ml metformin, IR was 38.74 ± 2.22%, 16.02 ± 1.83%, 92.76 ± 0.29 respectively; Compared with the negative control group, the inhibition rate were significantly different ($P < 0.01$).

### *2.2. Apoptotic morphology of different drug treatment groups observed under a fluorescence microscope*

Hoechst 33342 staining observations showed that, negative control group had normal morphology and clear boundaries, the cytoplasm was full, evenly distributed chromatin, nucleus was well dispersed fluorescence, rare strong fluorescence; Compared with the negative control group, in vitamin D3 or metformin monotherapy group, the apoptotic morphological changes appeared in some cells, such as nuclear shrinkage, the

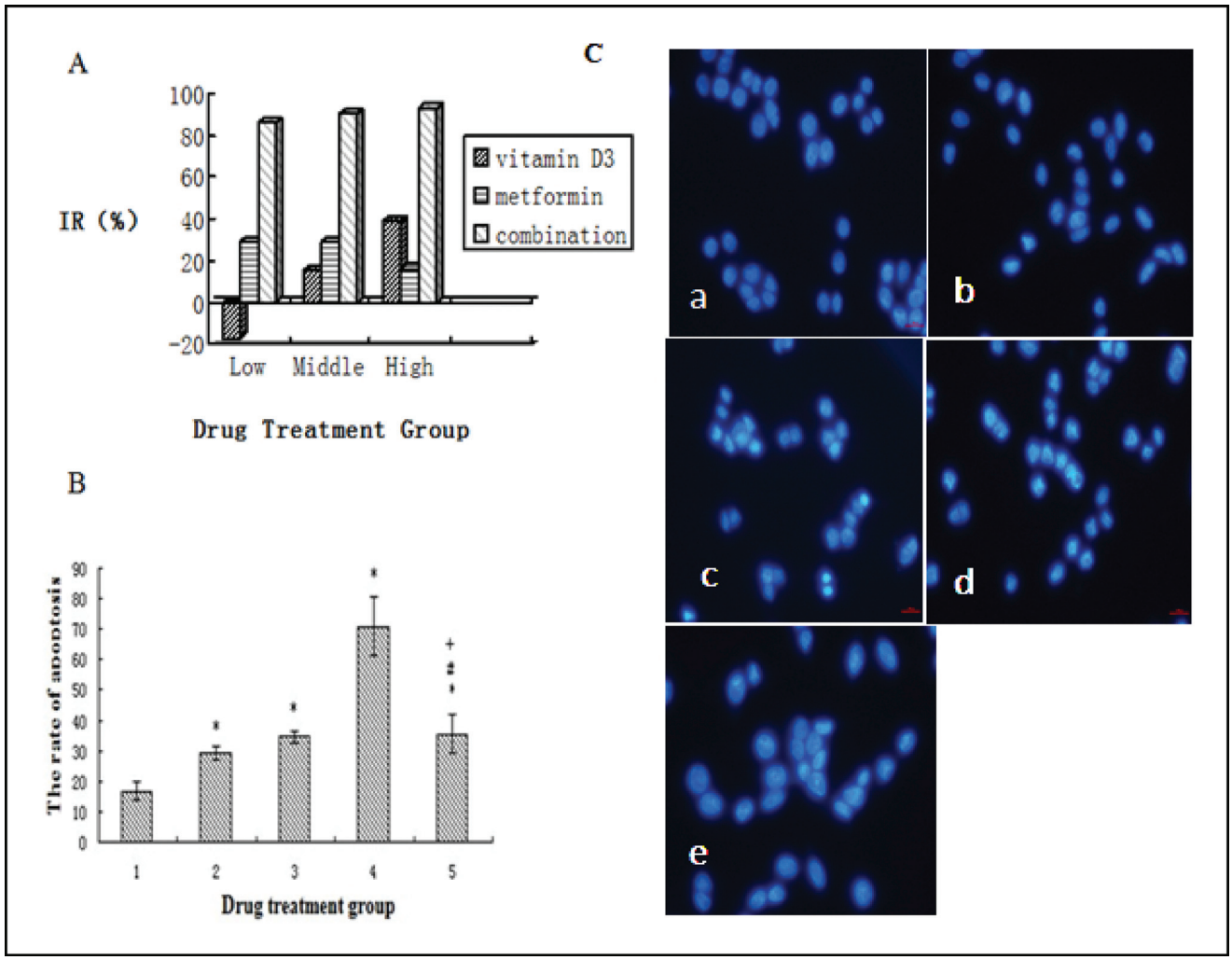


Fig. 1: A. Inhibitory effects of vitamin D3 and metformin monotherapy and combination therapy on MDA-MB-231 breast cancer cells. (24 h, $\bar{x} \pm S$, n = 9), compared with the negative group, $P < 0.05$. B. Apoptosis rate of MDA-MB-231 of different treatment groups (24h, $\bar{x} \pm S$, n = 3) C. Apoptotic morphology of MDA-MB-231 of different drug treatment groups observed by fluorescence microscope. 1, a negative control group; 2, b vitamin D3 treatment group; 3, c metformin treatment group; 4, d combination therapy treatment group; 5, e mitoxantrone treatment group. $^{*}P < 0.01$*vs* control group; $^{\#}P < 0.01$*vs* vitamin D3 group; $^{+}P < 0.01$*vs* metformin group.

nucleus turned into a horseshoe, cell vacuolization of cytoplasm, dense stain, highly aggregated, marginalized, and even cracked into pieces, the formation of apoptotic bodies, and the number of apoptotic cells in the combination group increased significantly with more obvious apoptotic morphology, while the number of cells was significantly reduced (Fig. 1B,C, Table 1).

### *2.3. Expression levels of p-Bcl-2, Bax in the different treatment groups*

Compared with the negative control group, monotherapy treatment groups' protein expression ratio of p-Bcl-2/Bax decreased, while the combination treatment group's decreased more obviously, besides, compared with the negative control group and monotherapy treatment groups, there existed significant differences (Fig. 2A, Table 2).

### *2.4. Expression levels of cleaved caspase-3 in the different treatment groups*

Compared with the negative control group, metformin monotherapy treatment groups' protein expression levels of Cleaved caspase-3 increased, while the combination treatment group's increased obviously, compared with the monotherapy

**Table 1: Apoptosis rate of MDA-MB-231 of different treatment groups (24 h, $\bar{x} \pm S$, n = 3)**

| Groups | Apoptosis rate (%) |
|---|---|
| Negative control group | 16.79 ± 3.2 |
| Vitamin D3 group | 29.37 ± 2.27* |
| Metformin group | 34.51 ± 2.07* |
| Combination group | 70.93 ± 9.52*#+ |
| Positive group | 35.53 ± 6.29* |

$^{*}P < 0.01$ *vs* control group; $^{\#}P < 0.01$*vs* vitamin D3 group; $^{+}P < 0.01$*vs* metformin group.

**Table 2: Ratio of relative IOD value of p-Bcl-2 and Bax in different treatment groups (24 h, $\bar{x} \pm S$, n = 3)**

| Groups | IOD(p-Bcl-2/β-actin)/ IOD(Bax /β-actin) |
|---|---|
| Negative control group | 0.134 ± 0.0016 |
| Vitamin D3 group | 0.07 ± 0.0021* |
| Metformin group | 0.099 ± 0.0061* |
| Combination group | 0.0292 ± 0.0017*#+ |

$^{*}P < 0.01$*vs* control group; $^{\#}P < 0.01$*vs* vitamin D3 group; $^{+}P < 0.01$*vs* metformin group.

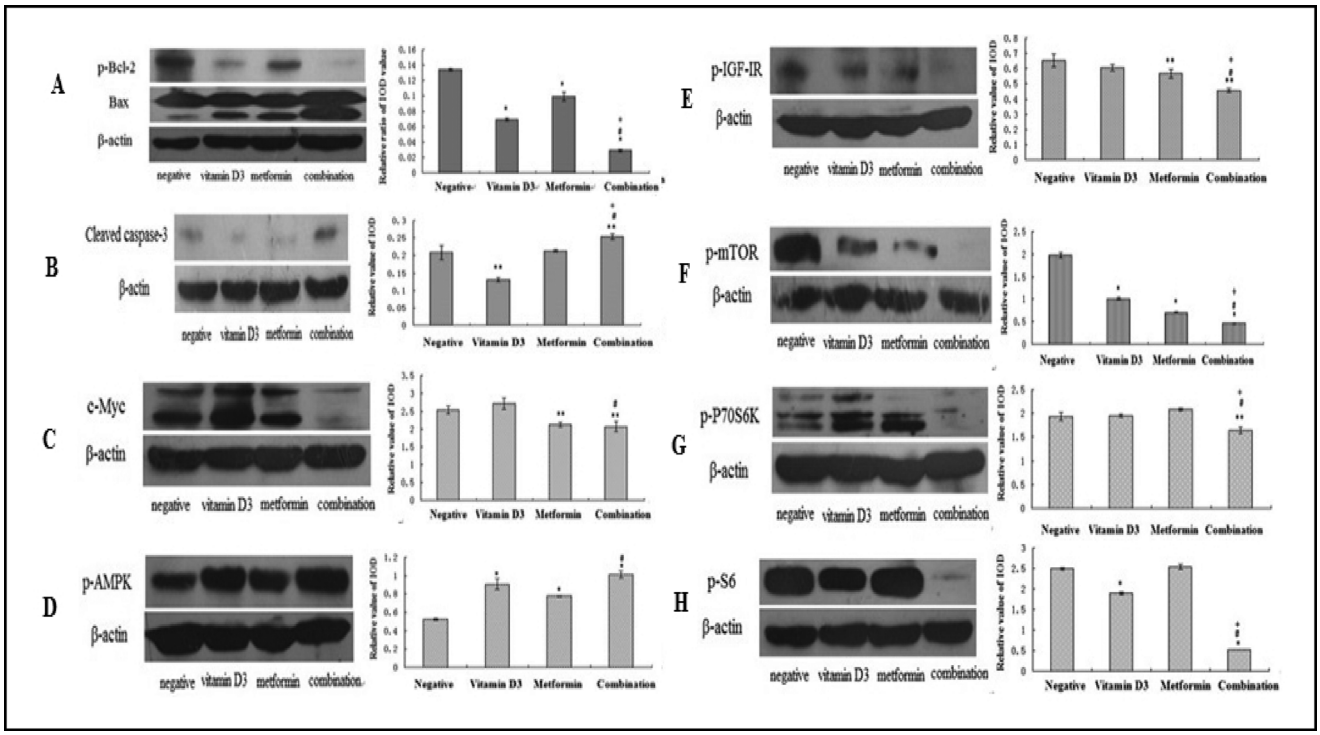


Fig. 2: Vitamin D3 enhances antitumor activity of metformin in MDA-MB-231 cells by inhibiting p-IGF-IR, activating p-AMPK and mTOR/S6P pathway. Western Bloting analysis for p-Bcl-2, Bax, Cleaved caspase-3, c-Myc, p-IGF-IR, p-AMPK, mTOR, p-70S6K, p-S6 and β-actin in each group. Data were means ± SD, n = 3 pre group. $^{*}P<0.01$, $^{**}P<0.05$*vs* control group; $^{\#}P<0.01$, $^{+}P<0.01$*vs* monotherapy treatment groups.

**Table 3: Relative value of IOD of cleaved caspase-3 in different treatment groups (24 h, $\bar{x} \pm S$, n = 3)**

| groups | IOD(Cleaved caspase-3/β-actin) |
|---|---|
| Negative control group | 0.209 ± 0.02 |
| Vitamin D3 group | 0.131 ± 0.0056** |
| Metformin group | 0.213 ± 0.0035 |
| Combination group | 0.254 ± 0.009**#+ |

$^{**}P<0.01$*vs* control group; $^{\#}P<0.01$*vs* vitamin D3 group; $^{+}P<0.05$*vs* metformin group.

**Table 4: Relative value of IOD of c-Myc in different treatment groups (24 h, $\bar{x} \pm S$, n = 3)**

| groups | IOD(c-Myc/β-actin) |
|---|---|
| Negative control group | 2.551 ± 0.11 |
| Vitamin D3 group | 2.713 ± 0.155 |
| Metformin group | 2.123 ± 0.065** |
| Combination group | 2.074 ± 0.137**# |

**$P<0.05$*vs* control group; $^{\#}P<0.05$*vs* vitamin D3 group.

treatment groups, there existed significant differences (Fig. 2B, Table 3).

### *2.5. Expression levels of c-Myc in the different treatment groups*

Compared with the negative control group, metformin treatment groups' protein expression levels of c-Myc was decreased, while the combination treatment group's decreased obviously; Compared with the negative group and monotherapy treatment groups, (280 μg/ml, 300 μg/ml, 320 μg/ml) significant differences existed (Fig. 2C, Table 4).

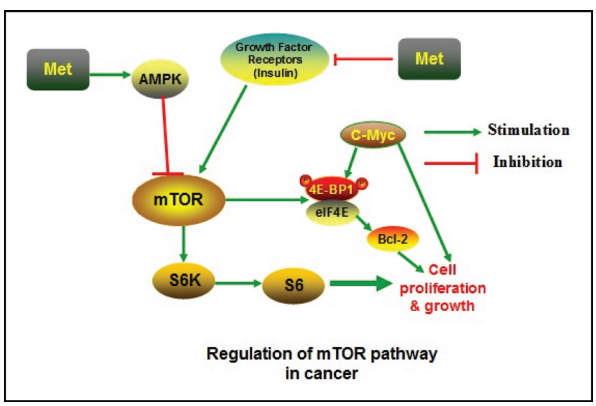


Fig. 3: Regulation of mTOR in cancer

### *2.6. Expression levels of p-IGF-IR in the different treatment groups*

Compared with the negative control group, metformin treatment groups' protein expression levels of p-IGF-IR were decreased, while the combination treatment group's decreased more obviously; Compared with the negative group and monotherapy treatment groups, significant differences existed (Fig. 2E, Table 5).

### *2.7. Expression levels of p-AMPK in the different treatment groups*

Compared with the negative control group, monotherapy treatment groups' protein expression levels of p-AMPK were increased, while the combination treatment group's increased more obviously; Compared with the negative group and monotherapy treatment groups, significant differences existed (Fig. 2D, Table 6).

**Table 5: Relative value of IOD of p-IGF-IR in different treatment groups (24 h, $\bar{x} \pm S$, n = 3)**

| groups | IOD(p-IGF-IR/β-actin) |
|---|---|
| Negative control group | 0.655 ± 0.042 |
| Vitamin D3 group | 0.608 ± 0.022 |
| Metformin group | 0.568 ± 0.027** |
| Combination group | 0.457 ± 0.015**#+ |

** $P < 0.05$ *vs* control group; # $P < 0.01$ *vs* vitamin D3 group; + $P < 0.01$ *vs* metformin group.

### *2.8. Expression levels of p-mTOR in the different treatment groups*

Compared with the negative control group, monotherapy treatment groups' protein expression levels of p-mTOR decreased, while the combination treatment group's decreased more obviously; Compared with the negative group and monotherapy treatment groups, significant differences existed (Fig. 2F, Table 7).

### *2.9. Expression levels of p-P70S6K in the different treatment groups*

Compared with the negative control group, monotherapy treatment groups' protein expression levels of p-P70S6K were not reduced but improved, while the combination treatment group's decreased obviously; Compared with the negative group and monotherapy treatment groups, significant differences existed (Fig. 2G, Table 8).

**Table 6: Relative value of IOD of p-AMPK in different treatment groups (24 h, $\bar{x} \pm S$, n = 3)**

| groups | IOD(p-AMPK /β-actin) |
|---|---|
| Negative control group | 0.522 ± 0.01 |
| Vitamin D3 group | 0.908 ± 0.06* |
| Metformin group | 0.779 ± 0.004* |
| Combination group | 1.015 ± 0.04*# |

* $P < 0.01$ *vs* control group; # $P < 0.01$ *vs* metformin group.

**Table 7: Relative value of IOD of p-mTOR in different treatment groups (24 h, $\bar{x} \pm S$, n = 3)**

| groups | IOD(p-mTOR /β-actin) |
|---|---|
| Negative control group | 1.977 ± 0.051 |
| Vitamin D3 group | 1.013 ± 0.023* |
| Metformin group | 0.709 ± 0.0046* |
| Combination group | 0.462 ± 0.0053*#+ |

** $P < 0.01$ *vs* control group; # $P < 0.01$ *vs* vitamin D3 group; + $P < 0.01$ *vs* metformin group.

**Table 8: Relative value of IOD of p-P70S6K in different treatment groups (24 h, $\bar{x} \pm S$, n = 3)**

| Groups | IOD(p-P70S6K /β-actin) |
|---|---|
| Negative control group | 1.016 ± 0.007 |
| Vitamin D3 group | 0.521 ± 0.028* |
| Metformin group | 0.453 ± 0.01* |
| Combination group | 0.23 ± 0.015*#+ |

** $P < 0.05$ *vs* control group; # $P < 0.01$ *vs* vitamin D3 group; + $P < 0.01$ *vs* metformin group.

**Table 9: Relative value of IOD of p-S6 in different treatment groups (24 h, $\bar{x} \pm S$, n = 3)**

| Groups | IOD(p-S6/β-actin) |
|---|---|
| Negative control group | 2.494 ± 0.032 |
| Vitamin D3 group | 1.894 ± 0.03* |
| Metformin group | 2.542 ± 0.054 |
| Combination group | 0.516 ± 0.0055*#+ |

* $P < 0.01$ *vs* control group; # $P < 0.01$ *vs* vitamin D3 group; + $P < 0.01$ *vs* metformin group.

### *2.10. Expression levels of p-S6 in the different treatment groups*

Compared with the negative control group, monotherapy treatment groups' protein expression levels of p-S6 had no significant change, while the combination treatment group's decreased obviously; Compared with the negative group and monotherapy treatment groups, significant differences existed (Fig. 2H, Table 9).

## 3. Discussion

Human breast cancer cell line MDA-MB-231 belongs to the triple negative breast cancer (TNBC) with characteristics of strong aggressive, highly malignant, easy metastasis and recurrence, low survival rate, poor prognosis. 15%-20% of breast cancer patients are TNBC, and in China nearly 1/4 of breast cancer patients are triple-negative. There is still no standard treatment for triple negative breast cancer (Perez et al. 2010).

The diagnosis of breast cancer falls into two broad categories, either estrogen receptor (ER)-positive or ER-negative, based on the expression level of ER in the cancer cells. ER is expressed in about 60% of all breast cancers. ER-positive breast cancer generally has a better prognosis, and is often responsive to antiestrogen therapy. In contrast, ER-independent breast cancers are more aggressive and unresponsive to antiestrogens (Nizamutdinova et al. 2008). Studies showed that 10 nM and 100 nM 1,25-dihydroxyvitamin D3 (calcitriol) inhibited MCF-7 cell growth by $10.8 \pm 2.4\%$ and $34.9 \pm 0.5\%$ after acting on cells for 72 h (Cho et al. 1991). In a preliminary experiment, calcitriol and metformin were used in monotherapy and combination, but MTT data showed that 0.01 ng/ml – 100 ng/ml (> 200 nM) calcitriol had no inhibitory effect on MDA-MB-231 cells after acting on cells for 48 h or 72 h. Besides, low, middle, and high concentrations of calcitriol combined with metformin also did not show synergistic effects. Its mechanisms need further study, and it is speculated that this might be related to estrogen receptor.

Many studies have shown that metformin has good anti-tumor effect *in vitro* and *in vivo*, and its mechanism may be related to signal pathways inhibiting the proliferation of tumor cells, such as the AMPK signaling pathway, mTOR signaling pathway, insulin-like growth factor signaling pathways and so on (Shackelford and Shaw 2009; Harris and Lawrence 2003). Metformin's inhibition on IGF-IR can indirectly inhibit the activity of m-TOR, and inhibit the activity of downstream S6K1 (p70S6 kinase) and 40S ribosomal protein S6 (p70S6, S6). Activated m-TOR can phosphorylate 4E-BP1 and then inactivate 4E-BP1, which leads to its dissociation from the eukaryotic translation initiation factor 4E (eIF-4E). Moreover, eIF-4E reduction in human tumor cells can robustly induce apoptosis and suppress the expressions of c-myc, Bcl-2 an so on (Sarfstein et al. 2013; Liu et al. 2011; Vignot et al. 2005; Graff et al. 2007).

The most active form of vitamin D3 (calcitriol) can enhance the activity of a variety of chemotherapeutic agents. Studies have

shown that metformin has a certain sensitivity to chemotherapy, and associating with chemotherapy drugs can inhibit the proliferation of a variety of breast cancer cell phenotypes, also metformin shows synergistic action when combined with chemotherapeutic agents (Shackelford and Shaw 2009; Harris and Lawrence 2003).

This experiment studied the effects of vitamin D3 combined with metformin on the apoptosis induction in breast cancer cell line MDA-MB-231. The results showed that 280 μg/ml – 320 μg/ml vitamin D3 combined with 15000 μg/ml metformin acting on the cell for 24 h had an obvious synergistic effect; inhibition and apoptosis rates increased significantly. The synergistic antitumor effect of a vitamin D3/metformin combination is not only achieved through inhibiting p-Bcl-2/Bax protein expression levels, promoting cleaved caspase-3 protein expression, inhibiting c-Myc protein expression levels, but also through the activation of p-AMPK and p-IGF-IR protein expression reduction and then inhibiting p-mTOR and its downstream proteins p-S6, p-P70S6K.

The development of tumors is a multifactorial, multi-step, multigene action complex process, therefore, a single molecular targeted therapy gradually shows its drawbacks, and seeking multi-targeted drugs is a hotspot of anti-cancer research. This experiment confirmed that vitamin D3 combined with metformin could inhibit the proliferation of human breast cancer MDA-MB-231 cells, and promote apoptosis. Its action may be related to the expression of apoptosis-related proteins. However, whether the combination therapy will be able to achieve the desired results *in vivo*, has to be explored in further studies, expecting that the experiments with combination chemotherapy drugs will provide an experimental basis for clinical treatment of triple negative breast cancer.

## 4. Experimental

### *4.1. Materials*

#### *4.1.1. Cell line*

Human breast cancer cell line MDA-MB-231 was purchased from Chinese Academy of Sciences, Shanghai Institute of cell biology library.

#### *4.1.2. Main reagents*

Vitamin D3 crystallization is provided by Zhejiang Garden Biochemical High-Tech Co., Ltd. Metformin hydrochloride was provided by Qufu maidesen Fine Chemical Co., Ltd. RPMI 1640 medium was purchased from Gibco, USA; Leibovitz's 15 medium was purchased from Invitrogen corporation; Trypsin was purchased from Gibco BRL Company; Newborn calf serum was purchased from Lanzhou Rong Ye Biological Technology Co., Ltd.; Thiazolyl blue (MTT), Hoechst 33342 dye were purchased from Sigma; p-Bcl-2, c-Myc, Cleaved caspase-3, Bax, p-IGF-IR, p-AMPK, p-mTOR, p-P70S6K, p-S6 antibody were purchased from Cell Signaling Technology (CST).

#### *4.1.3. Instruments*

$CO_2$ incubator (Shellab 2306 and Shellab 2323, U.S.A.); Inverted microscope (OLYMPUS); The type ELX800 enzyme-linked immunoassay instrument (U.S.A.); Fluorescence microscope (Olympus Japan); Electrophoresis apparatus (Junyi Dongfang JY-SCZ2 + type vertical electrophoresis).

### *4.2. Methods*

#### *4.2.1. Cell culture*

MDA-MB-231 cells were maintained in Leibovitz's 15 medium supplemented with 10% newborn calf serum, 100IU/ml penicillin, 100IU/ml streptomycin. All of the cultures were maintained at 37 °C in a humidified atmosphere. Logarithmic growth phase cells were used for experiments.

#### *4.2.2. Preparation of drugs*

The amount of metformin, was weighed and diluted with saline to the desired concentration, then filtered through a 0.22 μm microporous filter, for current use. Vitamin D3 was dissolved in ethanol for the preparation of 0.2 g/ml stock solution; A proper amount of stock solution was taken and diluted to the required concentration with RPMI 1640 complete medium.

#### *4.2.3. MTT assay*

The experiment included negative control group, blank control group, metformin monotherapy group, vitamin D3 monotherapy group, combination group, positive control group; each group had 9 holes; Logarithmic growth phase cells was adjusted to $8 \times 10^4$/ml cell concentration with RPMI 1640 complete medium then seeded in 96-well plates; Negative control group and drug-treated groups were added to each well with 90 μl cell suspension, and blank control group were added with 90 μl RPMI 1640 complete medium, then placed in 37 °C, 5% $CO_2$ incubator; after 24 h, adding drugs 10 μl/well, cultured for 24 h, adding MTT 10 μl/well, after 4 h, adding 10 %SDS 100 μl/well, shocking for 10 min, OD value of each well was measured at a wavelength of 570 nm, then calculating inhibition rate (IR). IR = (negative OD mean value - test OD mean value) / negative OD mean value × 100%, made an evaluation of drug effects on cell proliferation.

#### *4.2.4. Hoechst 33342 staining was used to observe the morphological changes of apoptosis*

Logarithmic growth phase cells was adjusted to $8 \times 10^4$/ml cell concentration with RPMI 1640 complete medium, then seeded in a 24-well plate, which were divided into the negative control group, vitamin D3 monotherapy group, metformin monotherapy group, combination therapy group, positive control group and blank control group, Each group had 3 holes; 360 μl single cell suspension was added into each hole; cultured for 24 h, 40 μl dilution solvent were added to blank control group and negative control group, 40 μl final concentration of 0.5 μg/ml mitoxantrone solution were added to the positive control group, 40 μl final concentration of 320 μg/ml vitamin D3 solution were added to vitamin D3 monotherapy group, 40 μl final concentration of 15000 μg/ml metformin solution were added to metformin monotherapy group, 40 μl mixed solution with final concentration of 320 μg/ml vitamin D3 solution and 15000 μg/ml metformin solution were added to the combination treatment group; Drugs acted on the cells for 24 h, the medium was aspirated, PBS rinsed three times, fixed with 4% paraformaldehyde for 30 min, PBS rinsed three times, added to final concentration of 5 μg/ml of Hoechst 33342 fluorescent dye, stained for 30 min, PBS rinsed three times, then observed the apoptotic cells under fluorescence microscope and made photographic records, calculated the apoptosis rates (%).

#### *4.2.5. Western Blot detection of the expression of apoptosis-related proteins*

Logarithmic growth phase cells was adjusted to $8 \times 10^4$/ml cell concentration with RPMI 1640 complete medium, 27 ml of cell suspension were added to 500 ml flasks; Cultured for 24 h, negative control group was added 3 ml dilution solvent, vitamin D3 monotherapy group was added 3 ml final concentration of 300 μg/ml vitamin D3 solution, metformin monotherapy group was added 3 ml final concentration of 15000 μg/ml metformin solution, the combination treatment group was added 3 ml mixed solution with final concentration of 300 μg/ml vitamin D3 solution and 15000 μg/ml metformin solution; After 24 h, the cells were collected, cells were washed with cold PBS three times, added 1 ml RIPA lysis buffer, cracked for 30 min on ice. After collecting the supernatant after centrifugation, protein concentration was measured by BCA method. Loaded about 30 μg protein samples, proteins were isolated with 10% SDS-PAGE electrophoresis, 220 mA wet transferred to PVDF membrane for 70 min, blocked for 1 h by 5% blocking solution, after washing the membrane, cultured the corresponding antibodies in 4 °C overnight, cultured with horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h, added ECL luminescent liquid 1 ml, exposure, developed, fixed, took pictures, used ImageJ software to analyze the optical density values of target bands.

#### *4.2.6. Statistics*

Experimental data are expressed in Mean ± SD, statistical analysis used two samples t test.

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