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Vitamin D3 enhances antitumor activity of metformin in human bladder carcinoma SW-780 cells

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Objective: To study the effects of vitamin D3 combined with metformin on the proliferation and apoptosis in human bladder cancer cell line SW-780 and its possible mechanism. **Methods:** MTT assay and fluorescence microscope observations were used to study the effects of vitamin D3 combined with metformin on the proliferation and apoptosis of SW-780 cells *in vitro*. Western blot was used to detect the expression of apoptosis-related proteins p-Bcl-2, Bax, Cyclin D1, c-Myc and related signaling pathways activated proteins p-IGF-IR, p-mTOR, p-P70S6K, p-S6. **Results:** MTT results showed that 320 µg/ml vitamin D3 combined with 620 µg/ml metformin acting on cells for 48 h had a significant synergistic effect on proliferation. Fluorescence microscope observations showed that compared with negative control group and monotherapy treatment group, the apoptosis features of combination treatment group were obvious and the apoptosis rate increased greatly. Western blot showed that compared with the negative control group and monotherapy treatment group, the expression levels of p-Bcl-2, Cyclin D1 and c-Myc in combination treatment group significantly decreased, whereas the expression level of Bax significantly increased, and the expression levels of p-IGF-IR, p-mTOR, p-P70S6K and p-S6 in combination treatment group significantly decreased. **Conclusion:** Vitamin D3 combined with metformin exhibited obvious inhibitory effects on the cell proliferation and apoptosis induction in SW-780 cells. The underlying anti-tumor mechanism might be related to inhibiting the expressions of p-Bcl-2, Cyclin D1, c-Myc, p-IGF-IR, p-mTOR, p-P70S6K, p-S6 and activating the expression of Bax.

1. Introduction

Urinary bladder cancer (UBC) is a common malignant tumor in urinary system tumors and bladder transitional cell carcinoma (BTCC) is the most common pathological type of bladder cancer, accounting for about 90%, and shows a rising trend. Therefore, the effective control and treatment of TCCB which will directly affect the related morbidity, mortality and the global medical cost, is urgently needed (Ploeg et al. 2009; Rezaianzadeh et al. 2012; Owusu et al. 2013; Martyn-Hemphill et al. 2013). However, TCCB continues to be a great challenge for treatment, although transurethral resection of bladder tumor (TURBT) can cure some patients, more than 50% of the patients develop metastases within two years, and eventually die of cancer. Because transitional cell carcinoma is a chemosensitive disease (Hussain and James 2003), systemic chemotherapy is the only method to cure patients with advanced metastatic tumors. However, conventional chemotherapy regimens cannot be tolerated for many patients especially the elderly, therefore, many researchers are trying to find new drugs and treatment programs in order to improve the efficacy and tolerability.

Metformin is a first-line drug for the treatment of type 2 diabetes. In recent years, many studies suggest that metformin also

has good anti-tumor effects *in vitro* and *in vivo*. Calcitriol is the active metabolite of the secosteroid hormone vitamin D and is well-known for its important role in bone and mineral metabolism. Calcitriol causes anti-proliferative effects through multiple mechanisms including the induction of cell cycle arrest, apoptosis and differentiation *in vitro* and *in vivo* in a variety of cancer cell types, besides, calcitriol can enhance the activity of a variety of chemotherapeutic agents (Yu et al. 2010). Combination therapy can not only lead to synergistic effects in terms of efficacy, but are mostly better tolerated by patients. We devoted to study the drug combination of metformin and vitamin D3 on the effects of proliferation and apoptosis in the bladder transitional cell line SW-780 *in vitro* and its molecular mechanism.

2. Investigations and results

2.1. Effect of vitamin D3 combined with metformin on the proliferation of SW-780 cells

Vitamin D3 320 µg/ml combined with 620 µg/ml metformin acted on cells for 24 h. Inhibitionrate of cell

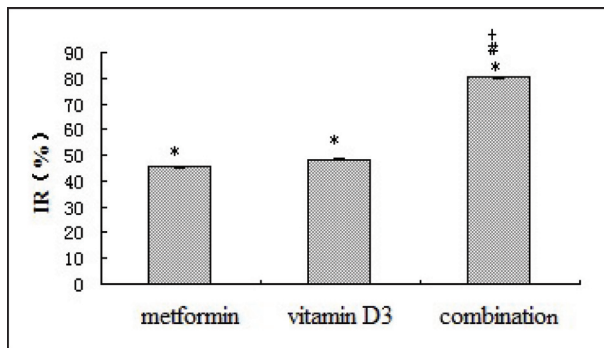


Fig. 1: Inhibitory effect of vitamin D3 and metformin alone and in combination on the proliferation of human bladder cancer cell line SW-780 (48 h, $\bar{x} \pm s$, $n = 9$), * $P < 0.05$ vs control group; # $P < 0.05$ vs vitamin D3 group; + $P < 0.05$ vs metformin group.

proliferation showed synergistic enhancement, the inhibition ratio of vitamin D3 monotherapy group, metformin monotherapy group, the combination treatment groups were $45.39\% \pm 0.4\%$, $48.34\% \pm 0.4\%$, $80.19\% \pm 0.13\%$, respectively (Fig. 1).

2.2. The effect of vitamin D3 combined with metformin on the apoptosis of SW-780 cells

After stained with Hoechst 33342, cells were observed under a fluorescence microscope. The negative control group appeared with normal morphology, light blue fluorescence, uniform staining, rare strong fluorescence. Compared with the negative control group, morphological changes of some cells appeared in the vitamin D3 and metformin monotherapy treatment groups, such as nuclear shrinkage, nuclei turned into a horseshoe, vacuolar cytoplasm, highly aggregated, marginalized, and even cracked into pieces, the formation of apoptotic bodies. Besides, the number of apoptotic cells in the combination group increased significantly, and the apoptotic characteristics were more obvious, the number of cells was significantly reduced (Fig. 2). Statistics showed that the negative control group apoptosis rate was $11.17 \pm 2.34\%$; the vitamin D3 treatment group apoptosis rate was $26.56 \pm 5.87\%$; the metformin treatment group apoptosis rate was $24.47 \pm 3.91\%$; the combined treatment group apoptosis rate was $65.93 \pm 4.15\%$; the cisplatin treatment group apoptosis rate was $29.05\% \pm 2.06\%$ (Fig. 3).

2.3. Expressions of p-Bcl-2 and Bax in SW-780 cells

Compared with the negative control group, the protein expression ratio of p-Bcl-2/Bax in the monotherapy treatment groups did not change significantly, while that of the combination treatment group obviously decreased. Compared with the negative control group and monotherapy treatment groups, significant differences existed (Fig. 4).

2.4. Expression of CyclinD1 in SW-780 cells

Compared with the negative control group, Cyclin D1 expression levels in the monotherapy treatment groups and the combination treatment groups all decreased, while they decreased in the combination treatment group. Compared with the vitamin D3 monotherapy treatment group, significant differences existed (Fig. 5).

2.5. Expression of c-Myc in SW-780 cells

Compared with the negative control group, c-Myc expression levels in the monotherapy treatment groups and in the combination treatment group all decreased, while they decreased in the combination treatment group. Compared with the negative control group and the monotherapy treatment groups, significant differences existed (Fig. 6).

2.6. Expression of p-IGF-IR in SW-780 cells

Compared with the negative control group, p-IGF-IR expression levels in the metformin treatment group and the combination treatment group both decreased, but increased in the vitamin D3 treatment group. When combination treatment group, negative control group and monotherapy treatment groups were compared, significant differences were seen (Fig. 7).

2.7. Expression of p-mTOR in SW-780 cells

Compared with the negative control group, p-mTOR expression levels of monotherapy treatment groups and the combination treatment group decreased. Compared with the negative control group and monotherapy treatment groups, combination treatment group's decreased more obviously, significant differences existed (Fig. 8).

2.8. Expression of p-P70S6K in SW-780 cells

Compared with the negative control group, p-P70S6K expression levels of monotherapy treatment groups and combination treatment group all decreased, while the combination treatment group's decreased obviously, and compared with the negative control group and monotherapy treatment groups, there existed significant differences (Fig. 9).

2.9. Expression of p-S6 in SW-780 cells

Compared with the negative control group, p-S6 expression levels of monotherapy treatment groups and combination treatment group all decreased, while the combination treatment group levels also decreased. Compared with the negative control group and monotherapy treatment groups, significant differences existed (Fig. 10).

3. Discussion

The human bladder carcinoma cell line SW-780 belongs to the bladder transitional cell carcinoma (BTCC). BTCC is the most common genitourinary malignant tumor after prostate cancer, and is one of the malignancies with the highest mortality and treatment costs (Martyn-Hemphill et al. 2013; Sadeghi and Garcia 2012). Patients with localised but muscle-invasive bladder transitional cell carcinoma are at high risk of relapse and death from metastatic disease after local treatment by cystectomy, radiation, or both. Because BTCC is quite sensitive to chemotherapy (Juffs et al. 2002), chemotherapy usually plays a vital role in adjuvant regimes. The traditional standard chemotherapy-MVAC scheme (methotrexate, vinblastine, doxorubicin, cisplatin) shows low cure rate, high relapse rate, significant toxicity, and many patients especially the elderly cannot tolerate the treatment (Hussain and James 2003; Bellmunt et al. 2003). Since drug therapy has poor curative effects and easily leads to tolerance, to find new anticancer drugs and their combination therapy is an important strategy for cancer therapy.

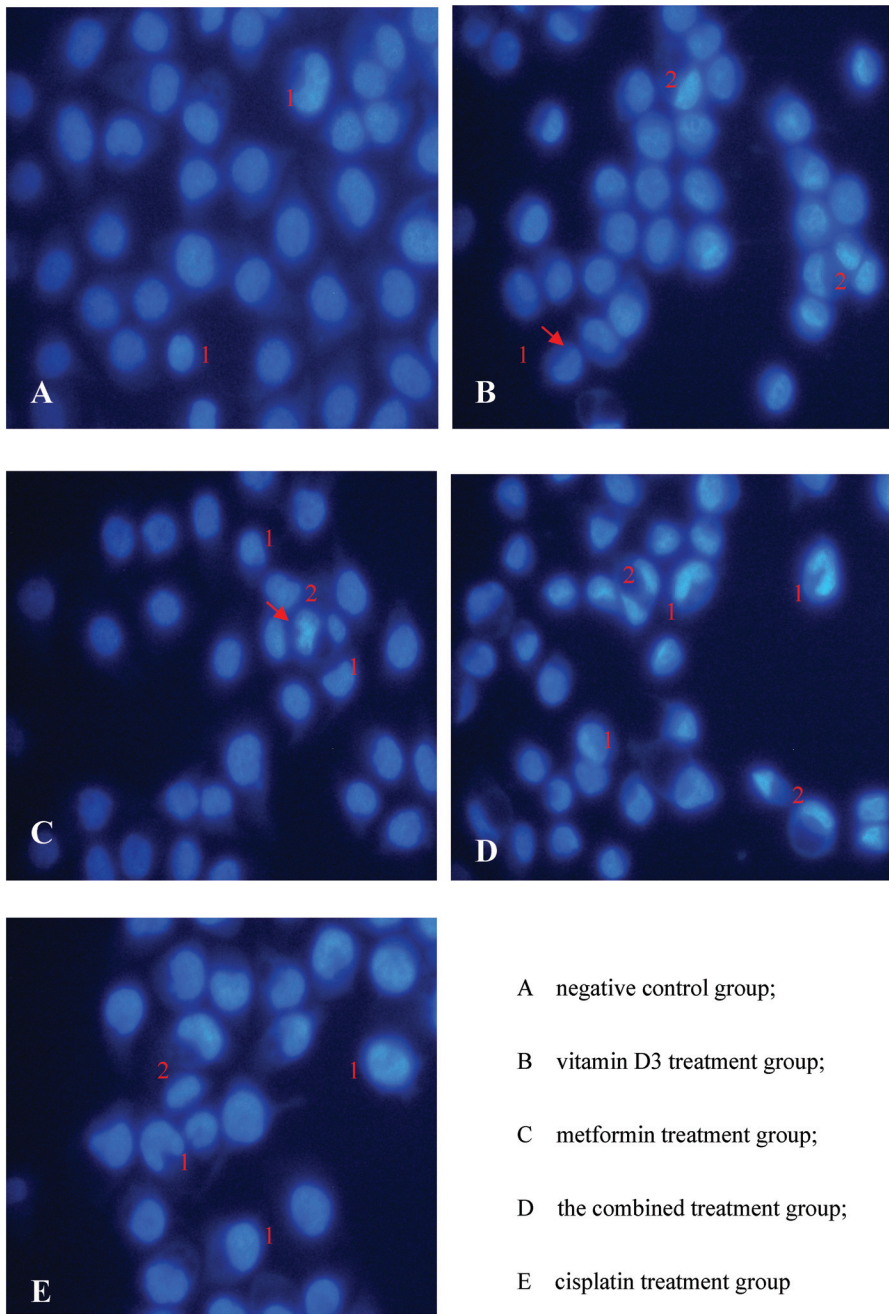


Fig. 2: The apoptotic morphology of SW-780 of different drug treatment groups observed under fluorescence microscope. Marks represent: 1 nuclear shrinkage, the nucleus turned into a horseshoe, vacuolar cytoplasm, highly aggregated, marginalized, which are the primary stage of apoptosis; 2 With the cell membrane invagination and chromatin broken into fragments, chromatin fragments and organelles are surrounded by reflexed cell membrane and gradually separate, finally the apoptotic bodies are formed.

As a traditional first-line oral hypoglycemic drug, metformin reduces patients' blood sugar level mainly through enhancing insulin sensitivity. Bowker and other authors have shown that type 2 diabetes patients who took metformin had significantly lower probability of suffering from malignant tumors than those who took sulfonylureas and insulin (Bowker et al. 2006). Insulin like growth factors (IGFs) play a crucial role in the insulin signaling pathway and are closely linked to cancer biology. The relationship between IGF-I signaling pathway and tumor is a hot research topic in recent years. IGF-I exerts its biological activity through its receptor (IGF-IR), and an increase in IGF-IR serum levels can promote tumorigenesis. Meanwhile, studies have shown that metformin can inhibit the activity of IGF-IR, reduce the expression of IGF-IR and inhibit IGF-IR-induced pathway's activation of PI-3K/AKT. mTOR is a downstream

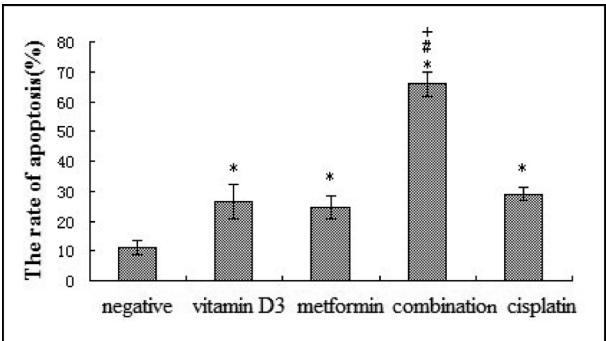


Fig. 3: The apoptosis rate of SW-780 of different treatment groups (48 h, $\bar{x} \pm s$, n=3) * $P < 0.01$ vs control group; # $P < 0.01$ vs vitamin D3 group; * $P < 0.01$ vs metformin group.

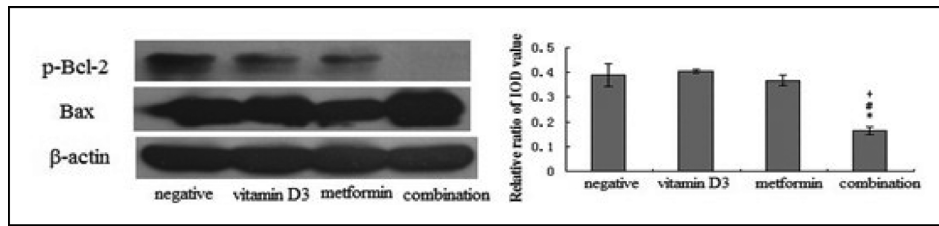


Fig. 4: Expressions of p-Bcl-2, Bax in the different treatment groups (48 h, $\bar{x} \pm s$, $n = 3$). * $P < 0.01$ vs control group; # $P < 0.01$ vs vitamin D3 group; + $P < 0.01$ vs metformin group

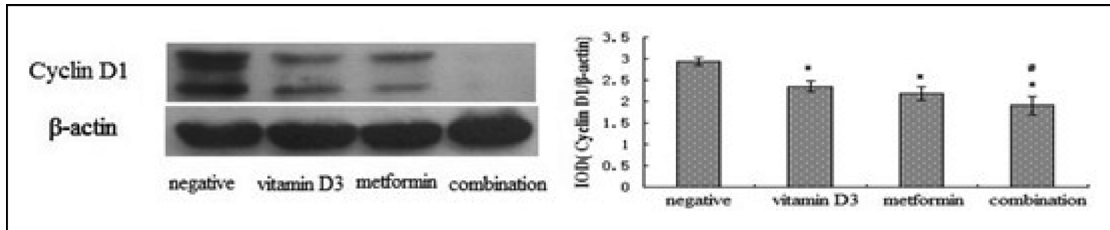


Fig. 5: Expression of Cyclin D1 in the different treatment groups (48 h, $\bar{x} \pm s$, $n = 3$). * $P < 0.01$ vs control group; # $P < 0.05$ vs vitamin D3 group.

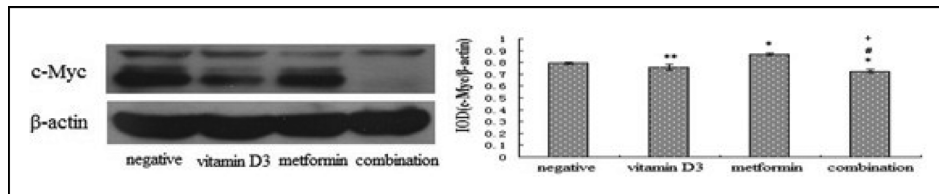


Fig. 6: The Expression of c-Myc in the different treatment groups (48 h, $\bar{x} \pm s$, $n = 3$). * $P < 0.01$, ** $P < 0.05$ vs control group; # $P < 0.05$ vs vitamin D3 group; + $P < 0.01$ vs metformin group.

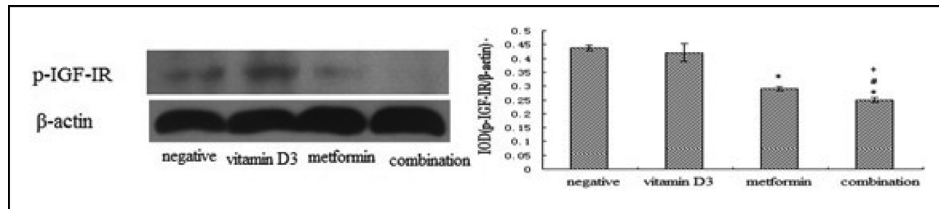


Fig. 7: The Expression of p-IGF-IR in the different treatment groups (48 h, $\bar{x} \pm s$, $n = 3$). * $P < 0.01$ vs control group; # $P < 0.01$ vs vitamin D3 group; + $P < 0.01$ vs metformin group

molecule of PI-3K/AKT pathway, and AKT can activate mTOR. Therefore, inhibition effects of metformin on IGF-IR activity can indirectly inhibit the activity of m-TOR, and inhibit the activity of S6K1 (p70S6 kinase) and 40S ribosomal protein S6 (p70S6, S6) (Sarfstein et al. 2013; Liu et al. 2011; Vignot et al. 2005).

mTOR regulates the translation of downstream proteins through phosphorylation of its downstream target proteins, mainly 40S ribosomal S6 kinase (p70S6K1) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1). 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 cyclin D1, c-myc, Bcl-2 (Graff et al. 2007).

In this study, cell proliferation was evaluated by MTT assay, changes of apoptotic morphology and the rate of apoptosis was determined by fluorescence microscopy observation, then

the molecular biological mechanisms of vitamin D3 enhancing anti-tumor activity of metformin were detected by western blot. The experimental results show that the mechanism of vitamin D3 enhancement anti-tumor effects of metformin on SW-780 cells might be mediated through the insulin signaling pathway that could indirectly regulate mTOR and its related protein expression. Compared with the negative control group and the monotherapy treatment group, the expression levels of p-IGF-IR, m-TOR, p-P70S6K, p-S6, p-Bcl-2, CyclinD1, c-Myc in combination treatment group were significantly reduced.

Bladder cancer is a disease with the highest costs from diagnosis to treatment among all diseases, but the relapse rate is high. Metformin is a clinically safe and effective antidiabetic drug that is widely used. Vitamin D3 is the active form of the highest biological metabolic rate in vitamin D. Both of them are readily available and inexpensive. Their synergistic roles in the treatment of bladder cancer still need further research *in vivo* or even clinical trials.

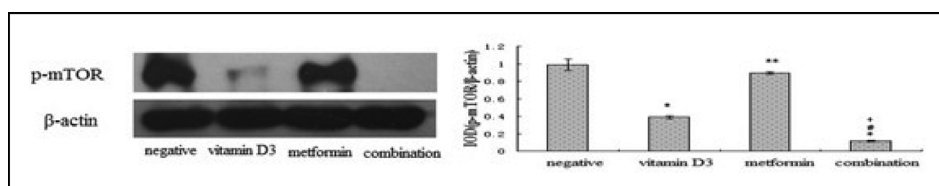


Fig. 8: Expression of p-mTOR in the different treatment groups (48 h, $\bar{x} \pm s$, $n = 3$). * $P < 0.01$, ** $P < 0.05$ vs control group; # $P < 0.01$ vs vitamin D3 group; + $P < 0.01$ vs metformin group.

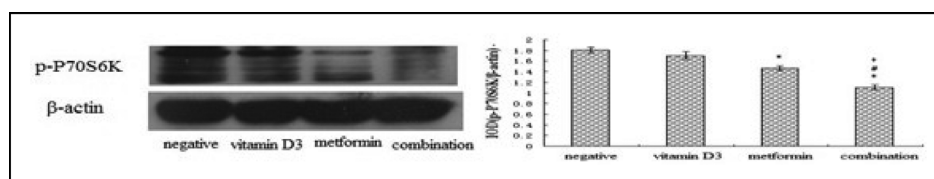


Fig. 9: Expression of p-P70S6K in the different treatment groups (48 h, $\bar{x} \pm s$, $n = 3$). * $P < 0.01$ vs control group; # $P < 0.01$ vs vitamin D3 group; + $P < 0.01$ vs metformin group

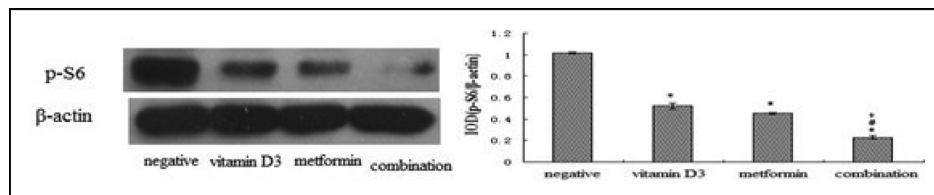


Fig. 10: Expression of p-S6 in the different treatment groups (48 h, $\bar{x} \pm s$, $n = 3$). * $P < 0.01$ vs control group; # $P < 0.01$ vs vitamin D3 group; + $P < 0.01$ vs metformin group

4. Experimental

4.1. Materials

4.1.1. Cell line

Human bladder cancer cell line SW-780 was purchased from the Chinese Academy of Sciences, Shanghai Institute of cell biology library.

4.1.2. Main reagents

Vitamin D3 crystallization was provided by Zhejiang Garden Biochemical High-Tech Co., Ltd. Metformin hydrochloride was provided by Qufu maidesen Fine Chemical Co., Ltd. RPMI 1640 medium and trypsin were purchased from Gibco, USA; Leibovitz's 15 medium was purchased from Invitrogen corporation; 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, Bax, c-Myc, Cyclin D1, p-IGF-IR, p-AMPK, p-mTOR, p-P70S6K, p-S6 antibody were purchased from Cell Signaling Technology (CST).

4.1.3. Instruments

CO2 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

SW-780 cells were cultured with Leibovitz's 15 medium supplemented with 10% newborn calf serum, 100 IU/ml penicillin, 100 IU/ml streptomycin in 37 °C, 5% CO2 incubator. The cells were passaged 1 time every 4 days. Logarithmic growth phase cells were used for experiments.

4.2.2. Preparation of drugs

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

4.2.3. MTT assay

Logarithmic growth phase cells were adjusted to 8×10^4 /ml cell concentration with RPMI 1640 complete medium and then seeded into 96-well plates, 90 μl cell suspension for each well, and placed in 37 °C, 5% CO2 incubator. After 24 h, drug solution (10 μl/well) was added, and cultured for 48 h. MTT solution (5 μg/ml, 10 μl/well) was added, after 4 h, 10 % SDS solution 100 μl/well was added and shocked for 10 min. Then, the OD value of each well was measured at a wavelength of 570 nm, inhibition rate (IR) was calculated.

4.2.4. Morphological changes

Logarithmic growth phase cells was adjusted to 8×10^4 /ml cell concentration with RPMI 1640 complete medium, then seeded into a 24-well plate, 360 μl cell suspension for each well, cultured in 37 °C, 5% CO2 incubator for

24 h; 40 μl dilution solvent were added to blank control group and negative control group, 40 μl final concentration of 1 μg/ml cisplatin solution were added to the positive control group, 40 μl final concentration of 300 μg/ml vitamin D3 solution were added to vitamin D3 monotherapy group, 40 μl final concentration of 630 μg/ml metformin solution were added to the metformin monotherapy group, 40 μl mixed solution with a final concentration of 300 μg/ml vitamin D3 solution and 630 μg/ml metformin solution were added to the combination treatment group. Cells were exposed to drugs for 48 h, rinsed with PBS three times, fixed with 4% paraformaldehyde for 30 min, rinsed with PBS three times, stained by the final concentration of 5 μg/ml of Hoechst 33342 fluorescent dye for 30 min, rinsed with PBS three times. Then, morphological changes of the cells were observed under a fluorescence microscope, photographic records were made and apoptosis rates were calculated (%).

4.2.5. Western blot analysis

The experiment included a negative control group, a vitamin D3 monotherapy group (320 μg/ml), a metformin monotherapy group (630 μg/ml), and a combination group. After 48 h, the cells were lysed by RIPA lysis buffer for 30 min in a low-temperature environment. Supernatants were collected after centrifugation, and protein concentration was measured by the BCA method. Loading about 50 μg protein samples, proteins were isolated with 10% SDS-PAGE electrophoresis. After electrophoresis, proteins were transferred to PVDF membranes, blocked for 1 h by 5% blocking solution at room temperature, and then cultured with the corresponding antibodies at 4 °C overnight. Membranes were washed by TBST three times, incubated with secondary antibody dilution (1:2000) for 1 h at room temperature, and washed by TBST. ECL luminescent liquid was added. After exposure, developing, and fixing, pictures were taken, and analyzed with ImageJ software to determine the optical density values of target bands.

4.2.6. Statistics methods

Experimental data are expressed in mean $\pm$ SD, statistical analysis used two samples t test.

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