ORIGINAL ARTICLE _____

Metformin inhibits proliferation and promotes apoptosis of HER2 positive breast cancer cells by downregulating HSP90

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Summary

Purpose: To investigate the effects and the possible molecular mechanisms of metformin on HER2 positive breast cancer cells.

Methods: SK-BR-3 HER2 positive breast cancer cells were treated with different concentrations of metformin. The growth inhibitory rate of the cells was calculated by MTT assay, apoptosis was detected by flow cytometry, and the expression level of heat shock protein 90 (HSP90) was performed by Western blot analysis. A control group consisted of cells treated with PBS.

Results: With increased concentrations of metformin, cell growth inhibitory rates increased. The growth inhibitory rates with 0.5 mM, 2mM or 8mM metformin were significantly higher compared with the control group (p<0.05). Apoptosis in the metformin treated cells was also significantly higher compared with the control group (p=0.003). The expression level of HSP90 in the metformin group was significantly lower than that in the control group.

Conclusion: Metformin can inhibit the proliferation and promote apoptosis of HER2 positive breast cancer cells,which is maybe related to inhibition of HSP90.

Key words: breast cancer, heat-shock protein90, HER2/neu, metformin

Introduction

Almost 25% of breast cancer cases bear amplification of HER2/neu gene or overexpress HER2 receptor protein. Owing to its poor differentiation and highly aggressive nature, HER2 positive breast cancer patients have usually poorer prognosis. Trastuzumab can obviously improve the prognosis of such patients [1] and objective response rates have been reported to range from 12 to 34% [2]. Nearly 15% of early breast cancer patients still suffer relapse or metastasis after treatment with trastuzumab [3]. Retrospective studies indicated that metformin could improve the prognosis of patients with HER 2 positive breast cancer who received the drug for type 2 diabetes mellitus [4,5]. Experimental studies *in vitro* confirmed that metformin has a role in the inhibition of proliferation in various solid tumors [6-9]. Yet, the molecular mechanisms of metformin on HER2 positive breast cancer remain largely unclear.

The aim of the present study was to investigate *in vitro* the role of metformin on HER2 positive breast cancer cells in relation to the proliferation rate, apoptosis and HSP90 expression.

Methods

Cell preparation and reagents

The breast cancer cell line SK-BR-3(ER-/PR-/HER2+++) was provided by the Breast Cancer Research Institution of Fudan University, China. Cells were cultured in DMEM with 10% FBS (Gibco, Oklahoma, USA), 1% glutamine, 100 IU/ml penicillin and 100 mg/l streptomycin. MTT kit and metformin were purchased from Sigma Co (Colorado, USA). Annexin V-FITC & PI Annexin V-EGFP Apoptosis Detection Kit were purchased from KGI chemical corporations in Nanjing (Nanjing, China), mouse anti-HSP90 antibody from Abcam (Cambridge,UK) and mouse anti-GAPDH antibody from SANTA (Santa Cruz, USA).

Cell treatment

Cells were divided into 4 groups. Group A: cells were treated with PBS; group B: cells were treated with 0.5mM/L metformin; group C: cells were treated with 2.0mM/L metformin; group D: cells were treated with 8.0mM/L metformin.

MTT assay

Cell viability was determined using the 3- (4,5-dimethyl-thiazolyl)-2, 5-diphenyltetrazoliumbromide (MTT) assay (Sigma-Aldrich; Carlsbad, CA). A, B, C and D group cells were plated in 96-well plates (1,500 cells per well) and incubated under normal culture conditions. After culture for 12, 36 and 72 h, the cells were treated with 0.5 mg/ml MTT for 4 h and lysed with dimethyl sulfoxide (DMSO). Absorbance rates were measured at 550-560 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

Annexin V-FITC/PI analysis

Detection of apoptosis was conducted using the Annexin V-FITC/PI apoptosis detection kit, according to the manufacturer's protocol. Briefly, all 4 groups of cells were harvested by trypsinization, washed in PBS and stained with annexin V-FITC conjugate and propidium iodide. Cells were then analyzed by flow cytometry (BD FACSCalibur™, USA) using BD CellQuest acquisition and analysis software.

Western blot analysis

The total volume of all of the formulations was 10 μl. The cells were diverted into a 1.5 ml Ep tube which contained RIPA schizolysis liquid with protease inhibitor. After being put in ice for 3-5 min, the mixture was swirled to make it fully dissolved, and put in ice for 30 min. Then the mixture was centrifuged (100 rpm) at 4°C for 20 min, the top clear liquid was collected and electrophoresed.

Statistics

Values were shown as mean ± SEM. The significance of differences between all groups was evaluated using one-way ANOVA with a post-hoc Student-Newman-Keuls multiple comparisons test. Statistical analyses were performed using SPSS Software (V.17.0, SPSS, USA), and a p-value < 0.05 was considered to be statistically significant.

Results

### Metformin inhibits the SK-BR-3 cells proliferation

As shown in Table 1, Figure 1 and 2, metformin could significantly inhibit the cancer cells' proliferation compared to the control PBS group (p<0.05). Moreover, with increasing metformin concentrations, the prolif-

**Table 1.** Inhibition of SK-BR-3 cells' proliferation after treatment with metformin

Group	Metformin (mM/L)	Inhibition rate of SK-BR-3 breast cancer cells (mean±SD)		
		12h	36h	72h
A	Control	0	0	0
B	0.5	5.70±0.23	6.52±0.14	7.74±0.09
C	2	7.44±0.33	16.04±0.27	22.50±0.14
D	8	8.37±0.24	22.64±0.31	30.86±0.06

SD: standard deviation
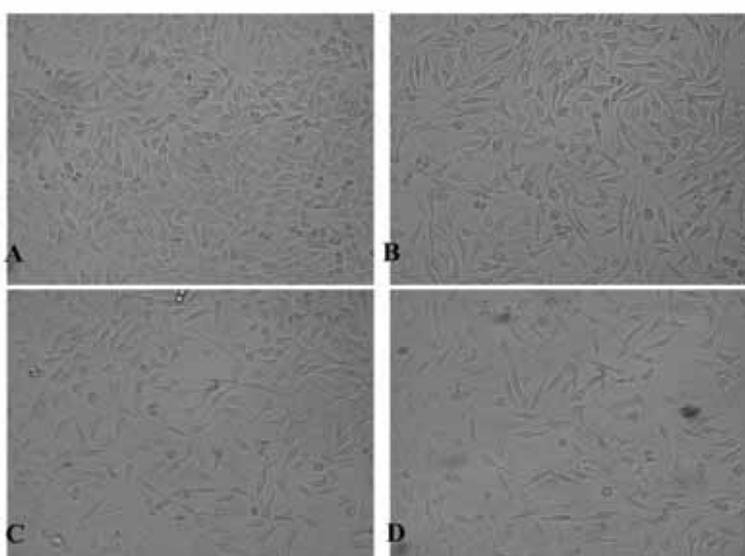
Figure 1. Inhibition of cells' proliferation after treatment with different concentrations of metformin at 72 h. **A:** PBS; **B:** 0.5mM metformin; **C:** 2mM metformin; **D:** 8mM metformin. Cells were visualized by phase contrast and fluorescence microscopy at x100.
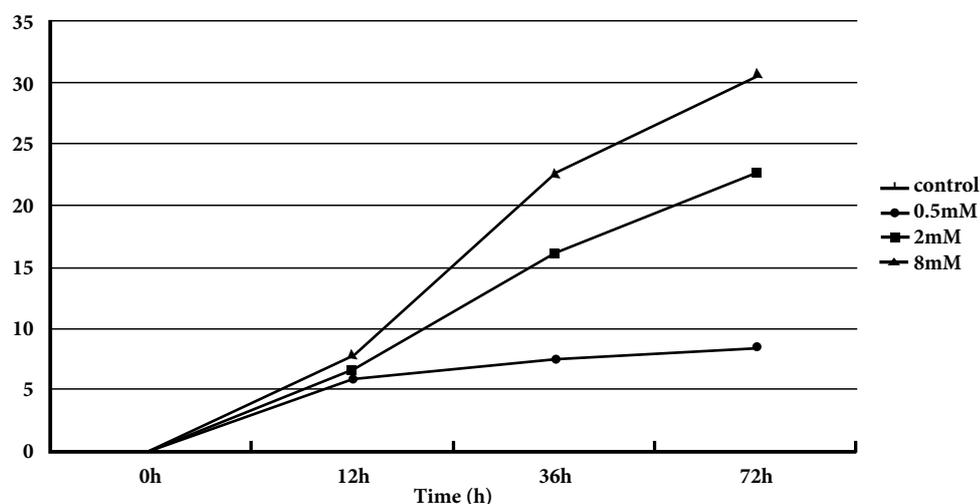
Figure 2. Inhibition of cell proliferation by metformin *in vitro*. There was statistically significant difference in the inhibition rate with 0.5 , 2 and 8 mM metformin vs the control group ($p < 0.05$). Moreover, with increasing metformin concentrations the proliferation rates gradually decreased ($p < 0.05$).
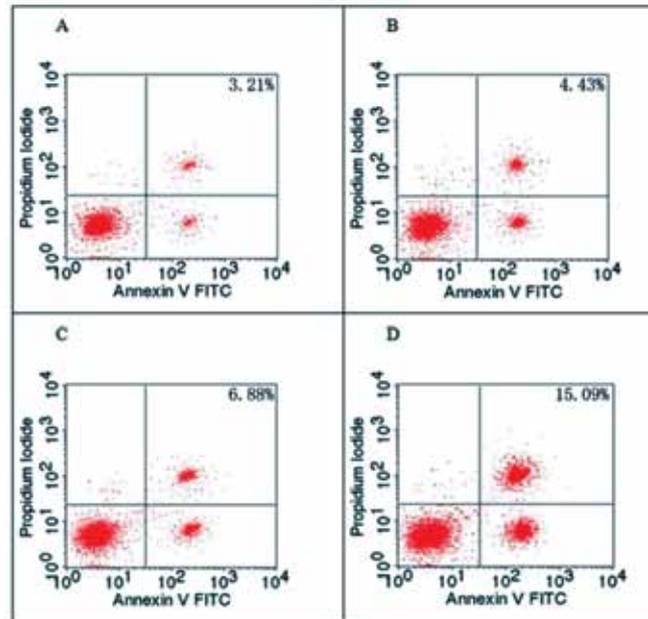
Figure 3. Metformin-induced breast cancer cell apoptosis. The apoptoptic rate in A, B, C and D groups was 3.21, 4.43, 6.88 and 15.09 %, respectively. The apoptotic rate in group D differed significantly compared to that in group A (p=0.003). The apoptotic rate in group D also showed significant difference compared to that in group B (p=0.008). **A**:control group; **B**:0.5 mM group; **C**:2 mM group; **D**:8 mM group.
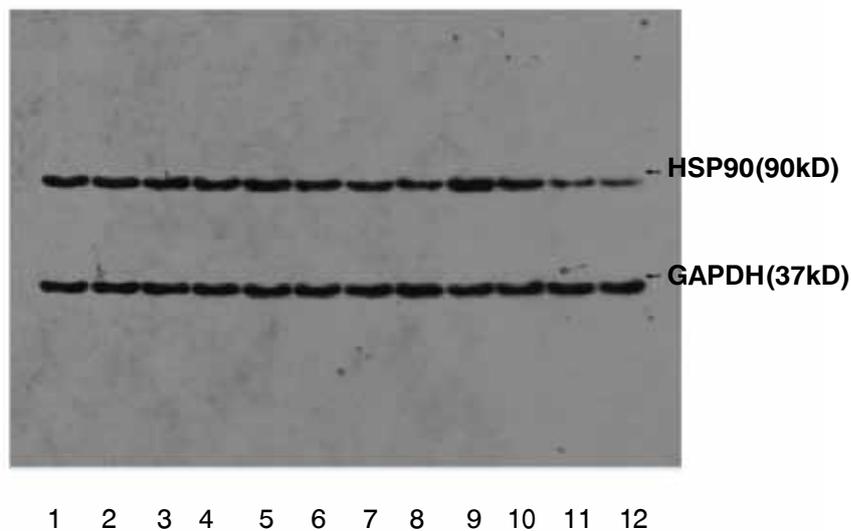
Figure 4. HSP90 protein expression was detected by Western blot at 0, 36, and 72h. 1, 5 and 9 channels are group A; 2, 6 and 10 channels are group B; 3,7 and 11 channels are group C; and 4, 8 and 12 channels are group D. HSP90 expression was significantly lower in B,C and D groups compared to that in group A. In addition, HSP90 expression at 72h was significantly lower (p<0.05) compared to that at 36h.

eration rates were gradually decreased.

Metformin improves the SK-BR-3 cells apoptosis
The apoptoptic rate in A, B, C and D groups was 3.21, 4.43, 6.88 and 15.09%, respectively. However, only the apoptotic rate in group D differed significantly compared to that in group A (p=0.003). Meanwhile, the apoptotic rate in group D also showed significant difference compared to that in group B (p=0.008) (Figure 3).

Metformin attenuates the HSP90 protein expression
HSP90 protein expression of SK-BR-3 cells after treatment with metformin is shown in Figure 4. HSP90 expression was significantly lower (p<0.05) in B, C, and D groups compared to that in A group. Moreover, the HSP90 expression at 72h was significantly lower compared to that at 36h (p<0.05).

Discussion

Metformin is widely used in the treatment of type 2 diabetes mellitus. A recent study [10] showed that metformin exerts anti-tumor properties in a wide spectrum of malignancies, including prostate, pancreatic, colorectal and breast cancers. The mechanisms of such activities may be through cell cycle arrest and initiation of cancer cell apoptosis by activating the apoptotic signal transduction pathway. A retrospective study by Jiralerspong et al. [4] revealed that patients with diabetes mellitus and breast cancer had a higher pathologic complete response rate with neo-adjuvant chemotherapy taking also metformin compared with patients not taking metformin (24 vs 8%, p=0.007). However, this study did not reveal any relation between patients suffering from different molecular types of breast cancer taking metformin and the curative effect of neo-adjuvant chemotherapy, so the effects of metformin on different molecular types of breast cancer still remain unclear.

Oliveira-Ferraros et al. [11] revealed that metformin can restrain related gene expression in M phase of breast cancer cell cycle by activating the AMPK pathway, thus breast cancer cell cycle was arrested in G2 phase. Dowling et al. [12] reported that metformin inhibits the growth of human breast cancer cells by activating the AMPK pathway, restraining the expression of mTOR and meddling the initial process of translation. Another study [13] used different concentrations of metformin in conjunction with estrogen receptors (ER) in breast cancer cell lines to observe metformin's role on cell proliferation and apoptosis. The results showed that metformin inhibition of ER positive breast cancer cells was stronger compared to ER negative breast cancer cells; this could be related to the low expression of hypoxia inducible factor 1α (HIF-1α). A previous study [9] confirmed that metformin inhibited the proliferation of triple-negative breast cancer cells by inhibiting the activation of EGFR pathway and promoting cell apoptosis. Vazquez and colleagues [14] found that metformin could prevent the proliferation of HER2 positive breast cancer cells by inhibiting the activation of mTOR effector p70S6K1 at low concentration, while it could significantly reduce the expression of HER2 at high concentration. However, this study did not reveal the relations between downstream key molecules expression and inhibition of the proliferation of HER2 positive breast cancer cells after treatment with metformin, leaving the mechanism of metformin activity on HER2 positive breast cancer cells unexplained.

Our results showed that with increasing concentrations of metformin the inhibition of proliferation of SK-BR cells gradually increased. The apoptotic rate in the metformin-treated groups was higher compared with the control group (p<0.05). It is rather clear that metformin can induce SK-BR-3 cell apoptosis *in vitro* and restrain cell proliferation. The activation of AKT and MAPK pathways and the high expression of HSP90 protein are common in HER2 positive breast cancer [15] and there is a positive correlation between HSP90 and breast cancer pathological stage, local recurrence and distant metastasis, all of which result in poor patient prognosis [16]. Our study reveals that metformin can restrain the expression of HSP90 protein on SK-BR-3 cells, and that the inhibitory effect is enhanced with increasing drug concentrations. Metformin can restrain the proliferation and promote apoptosis of cancer cells, and it is possible that metformin restrains the expression of HSP90 protein, which down-regulates the key downstream molecule activation of AKT and MAPK pathway [17].

In conclusion, metformin can inhibit the proliferation and promote apoptosis of HER2 positive breast cancer cells, which may be related to the inhibition of HSP90 protein expression of cancer cells.

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