

## RESEARCH ARTICLE

# Curcumin Inhibits TGF- $\beta$ 1-Induced MMP-9 and Invasion through ERK and Smad Signaling in Breast Cancer MDA-MB-231 Cells

Na Mo<sup>1</sup>, Zheng-Qian Li<sup>2</sup>, Jing Li<sup>1</sup>, You-De Cao<sup>1\*</sup>

### Abstract

**Objective:** To evaluate the effects of curcumin on matrix metalloproteinase-9 (MMP-9) and invasion ability induced by transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) in MDA-MB-231 cells and potential mechanisms. **Methods:** Human breast cancer MDA-MB-231 cells were used with the CCK-8 assay to measure the cytotoxicity of curcumin. After treatment with 10 ng/ml TGF- $\beta$ 1, with or without curcumin ( $\leq 10 \mu\text{M}$ ), cell invasion was checked by transwell chamber. The effects of curcumin on TGF- $\beta$ 1-stimulated MMP-9 and phosphorylation of Smad2, extracellular-regulated kinase (ERK), and p38 mitogen activated protein kinases (p38MAPK) were examined by Western blotting. Supernatant liquid were collected to analyze the activity of MMP-9 via zymography. Following treatment with PD98059, a specific inhibitor of ERK, and SB203580, a specific inhibitor of p38MAPK, Western blotting and zymography were employed to examine MMP-9 expression and activity, respectively. **Results:** Low dose curcumin ( $\leq 10 \mu\text{M}$ ) did not show any obvious toxicity to the cells, while 0-10  $\mu\text{mol/L}$  caused a concentration-dependent reduction in cell invasion provoked by TGF- $\beta$ 1. Curcumin also markedly inhibited TGF- $\beta$ 1-regulated MMP-9 and activation of Smad2, ERK1/2 and p38 in a dose- and time-dependent manner. Additionally, PD98059, but not SB203580, showed a similar pattern of inhibition of MMP-9 expression. **Conclusion:** Curcumin inhibited TGF- $\beta$ 1-stimulated MMP-9 and the invasive phenotype in MDA-MB-231 cells, possibly associated with TGF- $\beta$ /Smad and TGF- $\beta$ /ERK signaling.

**Keywords:** Curcumin - breast cancer - TGF- $\beta$ 1 - MMP-9 - MAPKs - invasion

*Asian Pacific J Cancer Prev*, 13 (11), 5709-5714

### Introduction

Breast cancer is one of most common malignant tumor in global female. And the leading cause of morbidity and mortality in breast cancer patients is cancer metastases, but not the primary tumor (Porter, 2009; Sanchez-Zamorano et al., 2011). It has been demonstrated that tumor cell invasion is the key event in the metastatic steps (Geiger et al., 2009). However, recent studies show that cancer progression toward metastasis is not entirely dependent on tumor cells themselves, but the result of the interactions of tumor cells and the primary tumor microenvironment (Allen et al., 2011). Cancer cells, specially those at the edges of the tumor, can attract and stimulate host cells to secrete many kinds of factors (TGF- $\beta$ 1, IL-1, TNF- $\alpha$ , etc.) in the tumor microenvironment. Those factors can generate a series of invasion-related biological effects through the tumor cell signal transduction pathways, and then cause the cells to separate from the primary lesion focus and result in local invasion and distant metastasis finally (Allen et al., 2011). So far, novel findings indicates that TGF- $\beta$ 1 is one of the factors which could be highly

relevant to breast cancer invasion and metastasis (Perera et al., 2010).

TGF- $\beta$ 1 is known to be one of the TGF- $\beta$  superfamily members that plays a dual role in breast cancer generation and progression. In the early stages, TGF- $\beta$ 1 acts as a tumor suppressor, nevertheless, it promotes cancer invasion and metastasis in later stages (Biswas et al., 2007). After binding to TGF- $\beta$ 1, the type II TGF- $\beta$ 1 receptor recruits and phosphorylates the type I receptor. The activated type I receptor subsequently initiates the activation of Receptor-associated Smads (R-Smads), then the latter forms heteromeric complexes with common mediator Smad4 (co-Smad4) and translocate into the nucleus. The Smad complexes regulate specific target genes by directly or indirectly combining and interacting with transcription factors, co-repressors, and co-activators (Smith et al., 2012). In addition to the classical TGF- $\beta$ /Smad signaling pathway, TGF- $\beta$ 1 can also directly activate non-Smad signaling pathways (Zhang, 2009). Previous reports have shown the direct function of MAPK pathways, including c-Jun N-terminal kinase (JNK), extracellular-regulated kinase ERK, and p38 MAPK, in

<sup>1</sup>Department of Pathology, Chongqing Medical University, Chongqing <sup>2</sup>Department of Anesthesiology, Peking University Third Hospital (PUTH), Beijing, China \*For correspondence: cydcyj@163.com

signal transduction of TGF- $\beta$ 1-regulated cell migration and invasion (Safina et al., 2007).

Researchers have demonstrated that the serum TGF- $\beta$ 1 level in early-stage breast cancer patients is uplifted and the high level of TGF- $\beta$ 1 has positive correlation with the effects of anti-tumor. However, it accelerates cancer invasion and formation of metastases in late-stage breast cancer (Cheung, 2007). Serra R et al. have found that radiation and chemotherapy can quicken tumor cells diffusion in mouse models of breast cancer. The experimental datas indicate that the levels of TGF- $\beta$ 1 in those mouse models have been rised remarkably. In contrast, there is no far metastasis in the mouse models with low level of TGF- $\beta$ 1 (Serra et al., 2005). Therefore, antagonism of TGF- $\beta$ 1 signaling may provide a therapeutic target for late-stage breast cancer, blocking metastasis without detrimental side effects.

Curcumin is a natural phenolic pigment extracted from the roots of turmeric. Numerous studies have reported that it has positive pharmacological effects, such as anti-tumor, anti-oxidation, anti-inflammation, anti-rheumatism and so on (Yan et al., 2012). The U.S. National Cancer Institute has listed it as the third-generation anti-cancer drug to study, so that curcumin will likely to be a promising clinical anti-cancer drug (Park et al., 2008). Nevertheless, the precise molecular mechanisms underlying its anti-tumor invasion and metastasis are not entirely clear (Park et al., 2008). Most of the reports pay more attention to the anti-fibrosis activity of curcumin on TGF- $\beta$ 1-stimulated organs (Tubulointerstitial, corneal, liver, etc.) fibrosis via blocking TGF- $\beta$  signing pathway (Smith et al., 2010; Yao et al., 2012; Zhang et al., 2012). Kim et al. have confirmed that curcumin inhibites TGF- $\beta$ 1-induced MMPs in mouse keratinocytes (Santibáñez et al., 2000; Santibáñez et al., 2002), but the effects on breast cancer MDA-MB -231 have not been reported.

Therefore, the present study investigated the effect of curcumin on the exogenous TGF- $\beta$ 1-stimulated expression and activity of MMP-9 in human breast cancer MDA-MB-231 cells. Furthermore, the underlying mechanisms were also probed.

## Materials and Methods

### Cell culture

MDA-MB-231 cells were incubated with RPMI-1640 medium (Gibco, California, USA) containing 10% fetal calf serum at 37°C in 5% CO<sub>2</sub> incubator. Cells were cultured in serum-free media for 24h to synchronize cell growth before the experiments, then the media were exchanged for fresh serum-free medium, treated with various agents at the concentrations specified, and cells were harvested at different time points for various analyses.

### Cytotoxicity assay

MDA-MB-231 cells were trypsinized and seeded in 96-well plates at  $5 \times 10^3$  cells/well. After 24 h, escalating doses of curcumin (Sigma, USA) were added, and incubated for another 24 h, 48h, and 72h respectively. Cells without any treatment were used as control. Then,

10  $\mu$ L CCK-8 (Dojindo, Tokyo, Japan) solution in culture medium was added to each well. Plates were incubated for an additional 2 h. The optical density of each well was measured using microplate absorbance reader at a 450 nm wavelength. Cell viability was calculated as follows. The cell survival rate (%) = [(A Treatment group- A blank wells) / (A negative control group-A blank wells)]  $\times$  100%.

### Invasion assay

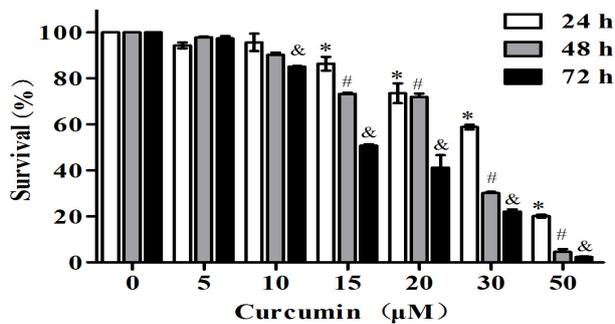
The MDA-MB-231 cells invasion behavior with or without indicated treatment was tested by Matrigel transwell system as described previously (Ye et al., 2012). After cultured in 6 well plates for 48h, the cells in different experimental groups were trypsinized, centrifuged, and resuspended at  $1 \times 10^6$  cells/mL in serum-free medium respectively. 100 $\mu$ l cell suspension per well were seeded onto the upper wells of transwells (8- $\mu$ m-diameter pores; Millipore), which precoated with Matrigel (0.5 mg/ML, BD Biosciences Discovery Labware). Lower chamber of the transwells contained the medium containing 10% FBS as chemoattractant. After 8 h of incubation, the cells on the upper chamber were carefully wiped with the cotton swab. The wells were washed 3 times with PBS, then fixed with 4% paraformaldehyde, and stained with crystal violet solution (Sigma Chemical, USA). The cells on the lower surface of the membrane were counted under a light microscope (magnification,  $\times$ 100). The experiments were performed three times, each time in triplicate.

### Gelatin zymography

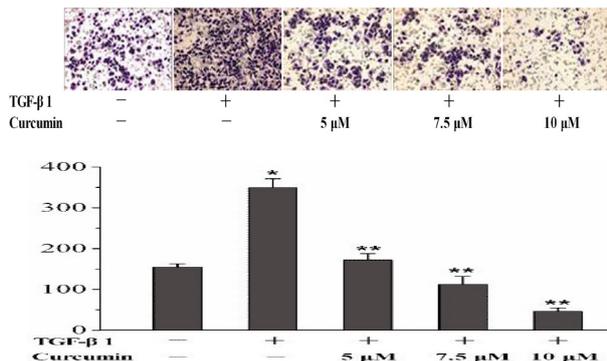
Cells in the logarithmic phase were seeded in 6-well plate at the desity of  $3 \times 10^5$  cells per well. After incubated in serum-free medium with or without curcumin (2.5, 7.5 and 10  $\mu$ M) and 10  $\mu$ M TGF- $\beta$ 1 treatment for 48h. The supernatants were collected, and gelatin zymography assay was performed as described formerly (Zayani et al. 2012). After electrophoresis, the gels were washed three times with renaturing buffer containing 50 mM Tris-HCl, 5 mM CaCl<sub>2</sub> and 2.5% Triton X-100 (v/v), pH 7.5 for 30 min, followed by a brief rinsing with washing buffer (50 mM Tris-HCl, 5 mM CaCl<sub>2</sub>), pH 7.5. Then the gels were incubated at 37°C for 42 h in developing buffer containing 50 mM Tris-HCl, 5 mM CaCl<sub>2</sub>, 0.2 M NaCl, and 0.02% Brij 35, pH 7.5. The gels were subsequently stained with 0.25% Coomassie Brilliant Blue (G250) followed by destaining with a solution containing 10% acetic acid and 20% methanol. Enzyme-digested regions were visualized as light bands against a dark background. Zones of enzymatic activity were regarded as negatively stained bands.

### Western blotting

Cells in experimental groups were collected and lysed in RIPA buffer respectively. Supernatants of the cell lysates were used in the western blot analysis for MMP-9 (Bioss, Beijing, China),  $\beta$ -actin (Beyotime, Beijing, China), and phosphorylation levels of ERK1/2, Smad2, p38MAPK (Cell signing technology, USA). After electrophoresis and transmembrane, the PVDF membranes (Millipore, USA) containing the proteins were blocked with 5% bovine serum albumin in TBST buffer (0.01%



**Figure 1. The Cell Toxicity of MDA-MB-231 Cells Treated by Curcumin.** \*P<0.05 vs. 0  $\mu$ M Cur (24h), #P<0.05 vs. 0  $\mu$ M Cur (48h), &P<0.05 vs. 0  $\mu$ M Cur (72h)



**Figure 2. Effect of Curcumin on TGF- $\beta$ 1-induced Invasion Ability of MDA-MB-231 Cells in vitro (crystal violet  $\times$ 100)** (a) MDA-MB-231 cells were incubated with different doses of Cur, with or without TGF- $\beta$  (10 ng/ml) for 48h, then checked invasion ability by Transwell assay. (b) Quantitation of the cells which invasive matrigel to lower surface of the membrane by cell counting under microscope ( $\times$ 100) (mean $\pm$ SEM from 3 independent tests). \*P<0.05 vs. control, \*\*P<0.05 vs. TGF- $\beta$ 1 alone

Tween 20 in TBS) at room temperature (RT) for about 2h, following by incubated with the primary antibody (i.e., specific antibodies against the target proteins described above) in TBST at 4°C overnight. The membranes were washed three times in TBST and subsequently covered with anti-rabbit HRP-conjugated goat anti-rabbit IgG (Bioss, Beijing, China) or goat anti-mouse IgG (Beyotime, Beijing, China) at RT for 2h in TBST buffer. After a 2h incubation, all blots were washed three times and ECL reagents (Beyotime, Beijing, China) were used for development.

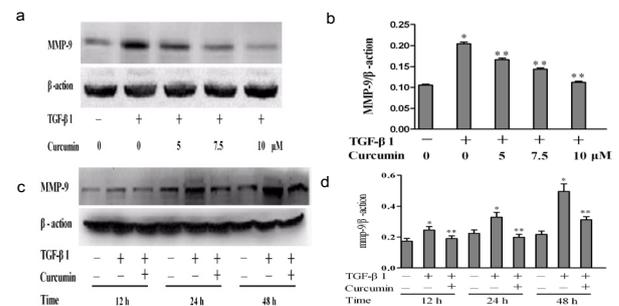
#### Statistical analysis

Data are presented as means  $\pm$  SEM and analyzed for statistical significance using independent samples T-test. All Statistical analysis was performed with SPSS for Windows (version 14.0; SPSS, Chicago, IL). Statistical significance was considered as P<0.05.

## Results

### The cytotoxicity effect of curcumin on the growth of MDA-MB-231 cells

To evaluate cytotoxicity of curcumin on the growth of breast cancer cells, MDA-MB-231 cells were treated with 5, 10, 15, 20, 30 and 50  $\mu$ M curcumin for 24h, 48h, 72h respectively and then cell viability was detected by



**Figure 3. Effect of Curcumin on MMP-9 Protein Expression Activated by TGF- $\beta$ 1** (a, b) Cell were incubated with different doses of curcumin and with or without TGF- $\beta$ 1 for 48h, and the MMP-9 Protein expression was checked by Western blotting. (mean  $\pm$  SEM from 3 separate tests) \*P<0.05 vs. control, \*\*P<0.05 vs. TGF- $\beta$ 1 alone. (c, d) MDA-MB-231 cells were treated with 10 $\mu$ M Cur, followed by TGF- $\beta$ 1 for different times, and the MMP-9 Protein expression was analyzed by Western blotting (mean  $\pm$  SEM from 3 separate tests) \*P<0.05 vs. control, \*\*P<0.05 vs. TGF- $\beta$ 1 alone

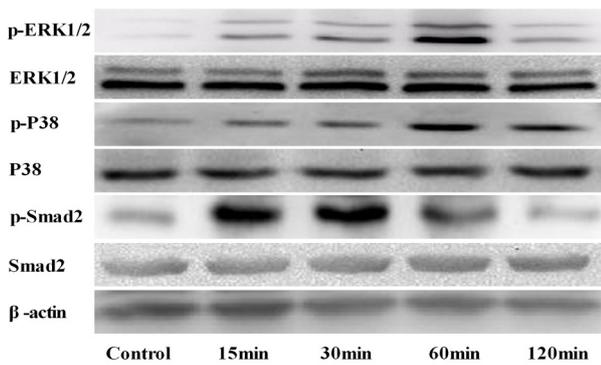
CCK-8 assay. As shown in Figure 1, low-dose curcumin ( $\leq$ 10  $\mu$ M) did not affect the viability of MDA-MB-231 cells, and survival rates of all the low-dose groups had exceeded 90% (except 10  $\mu$ M curcumin for 72 h). But when the concentrations was above 10 $\mu$ M, curcumin time- and dose- dependently inhibited the growth of MDA-MB-231 cells. Therefore, the cells were treated with selected doses ( $\leq$  10 $\mu$ M) for no more than 48 hours in subsequent experiments.

### Effects of curcumin on TGF- $\beta$ 1-induced invasiveness of MDA-MB-231 cells

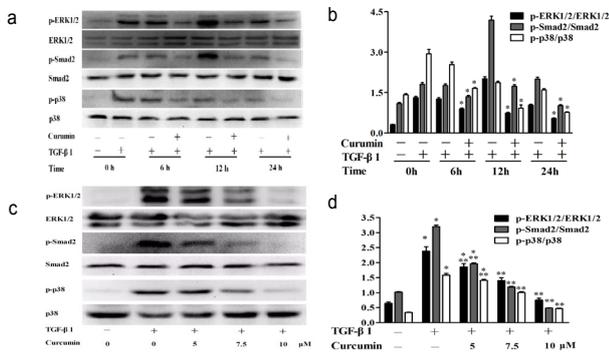
We next examined the effect of curcumin on TGF- $\beta$ 1-induced cell invasion in MDA-MB-231 breast cancer cells using the transwell chamber assay. Our results showed that the invasiveness of MDA-MB-231 cells was increased by TGF- $\beta$ 1 treatment (Figure 2). On the other hand, TGF- $\beta$ 1-stimulated invasiveness of cells was decreased by curcumin in a dose-dependent manner (Figure 2). These results were inconsistent with the results of the wound healing assay (data did not shown). This suggests that curcumin can prevent the TGF- $\beta$ 1-induced invasion in MDA-MB-231 cells.

### Effects of curcumin on TGF- $\beta$ 1-Mediated MMP-9 protein expression and activity in MDA-MB-231 cells

We examined whether curcumin involved with the TGF- $\beta$ 1-induced MMP-9 protein expression. After pretreatment with different concentrations of curcumin for 30 min, the cells were cultured with TGF- $\beta$ 1 and various doses of curcumin for 48h. Western blot analyses revealed that TGF- $\beta$ 1-induced MMP-9 protein expression was significantly decreased by curcumin in a dose-dependent manner. The level of MMP-9 protein expressions was increased to 1.93-fold of the control level by 10 nM TGF- $\beta$ 1 treatment, while the TGF- $\beta$ 1-induced MMP-9 protein expressions was decreased to 81.5%, 70.4%, 55.0% of the 10 nM TGF- $\beta$ 1 group level by 5, 7.5 and 10 $\mu$ M curcumin treatment, respectively (Figure 3A, 3B). So, 10  $\mu$ M curcumin has the maximal inhibitory effect. After pretreatment with 10 $\mu$ M curcumin



**Figure 4. Effects of TGF-β1 on p-smad2, p-ERK1/2, p-p38 Expression in Different Time Periods.** MDA-MB-231 cells were treated with TGF-β1 (10 ng/ml) for the indicated times, and the phosphorylation of Smad2<ERK1/2 and p38 was analyzed by Western blotting. The same membrane was retested with anti-Smad2<ERK1/2 and p38 antibody

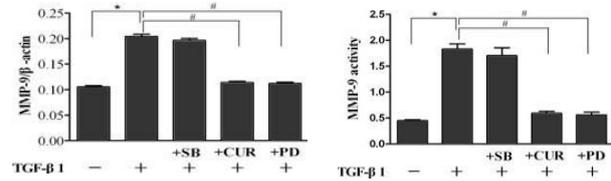
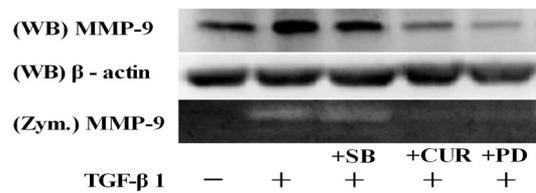


**Figure 5. Effects of Curcumin on p-smad2, p-ERK1/2, p-p38 Expression Activated by TGF-β1.** (a) (b) MDA-MB-231 cells were treated with 10μM Cur for different times, with or without TGF-β1 for an additional 15min, and the phosphorylation of Smad2, ERK1/2, p-p38 was analyzed by Western blotting (mean ± SEM from 3 separate tests) \*P<0.05 vs. TGF-β1 alone. (c) (d) Cells were incubated with different doses of Cur for 12h, and with or without TGF-β1 for an additional 15min, and the phosphorylation of Smad2, ERK1/2, p-p38 was checked by Western blotting. (mean ± SEM from 3 independent tests) \*P<0.05 vs. control, \*\*P<0.05 vs. TGF-β1 alone

for 30 min, the cells were treated with 10 nM TGF-β1 and 10μM curcumin for 12h, 24h, 48h, respectively. Our results showed that the TGF-β1-induced MMP-9 protein expressions were evidently suppressed by curcumin in a time-dependent way. The TGF-β1-induced MMP-9 protein expressions was decreased by 77.5%, 60.4%, 58.4% of the 10 nM TGF-β1 group level in 12h, 24h, 48h respectively. Therefore, curcumin had the best inhibitory effect at 48h. As exhibited in Figure 3 C and D, treatment with 10 nM TGF-β1 for 24h led to increase enzymatic activity of MMP-9. In contrast, curcumin dose-dependently inhibited this effect, with 10 μM curcumin showing optimum inhibitory effect.

*Effect of TGF-β1 on phosphorylation of Smad2, ERK and p38MAPK in breast cancer MDA-MB-231 cells*

As shown in Figure 4, 10μM TGF-β1 stimulated phosphorylation of Smad2, ERK and p38MAPK as early as 15 minutes while p-Smad2 peaked at 30 minutes. And the p-ERK and p-p38MAPK reached maximum at 60 min. The levels of total Smad2, ERK and p38 did not altered.



**Figure 6. Effects of PD98059, SB202580, and Curcumin on TGF-β1-induced MMP-9 in MDA-MB-231 Cells.** The MMP-9 expression and enzymatic activity were analyzed by Western blot and zymography, respectively (\*P<0.05 vs. control #P<0.05 vs. TGF-β1 alone)

*Effect of curcumin on activation of Smad and MAPK Pathway by TGF-β1*

Cells were treated with 10μM curcumin for 0, 6, 12, and 24 h, with or without inducing of TGF-β1 for an additional 15 min. Western blot analyses indicated that TGF-β1 markedly increased activation of Smad2, ERK1/2, and p38MAPK at 12h and 24h, but did not increase levels of total Smad2, ERK1/2, p38MAPK. Curcumin at 10μM exhibited significantly inhibitive effect on TGF-β1-induced phosphorylation of Smad2, ERK1/2, p38MAPK at different intervals, and the 12 h treated group was more effective. Compared with the same time point of TGF-β1 alone treatment group, the difference was significant (P <0.001) as shown in Figure 5 A and B.

Next, cells were incubated with different concentrations (0, 5, 7.5, 10 μM) of curcumin for 12 h, followed by treatment of TGF-β1 for an additional 15 min. The results revealed that TGF-β1 at 10μM dependently induced p-Smad2, p-ERK1/2 and p-p38MAPK when compared with constant levels of total Smad2, ERK1/2, p38 MAPK. Curcumin significantly inhibited TGF-β1-induced phosphorylation of Smad2, ERK1/2 and P38MAPK in MDA-MB-231 cells at protein levels, and the inhibition effect was characterized by a concentration-dependent way, and the dose of 10 μM was regarded as the best inhibition effect (Figure 5 C, D)

*Effect of PD98059, SB202580, curcumin on MMP-9 enzymatic activity and expression by TGF-β1*

Cells were treated with curcumin (10μM), PD98059 (30μM), SB202580 (20μM) for 48 h, combination with or without treatment of TGF-β1 (10 ng/ml). The inhibitory effect of PD98059 on MMP-9 was similar to curcumin, whereas SB202580 group shown no significant change when compared with the TGF-β1 alone group (Figure 6).

**Discussion**

About one-third of women with breast cancer develop distant metastasis and ultimately die worldwide each year (Allen et al., 2011; Nasser et al., 2012). Thus, metastatic breast cancer has been thought to be the principal challenge for the effective treatment and prevention

(Hortobagyi et al., 2002). Extensive evidence now indicates that degradation of extracellular matrix assisting cancer cell to invade neighbouring tissue, blood vessels and spread to other sites is the essential process of distant metastases in invasive breast cancer (Hassan et al., 2012).

Reportedly, MMP-9 plays a significant role in breast cancer invasion and metastasis via degrading type IV collagen-rich extracellular matrix (Chen et al., 2011), and also be regulated by TGF- $\beta$ 1, epidermal growth factor (EGF), fibroblast growth factor (FGF), Nerve growth factor (NGF), vascular endothelial growth factor (VEGF), etc., which secreted by cancer cells and/or host cells in the tumor microenvironment (Chou et al., 2006; Belotti et al., 2008; Blair et al., 2011). Among those factors, TGF- $\beta$ 1 generated by autocrine and paracrine is high related to malignant tumor (Na et al., 2010). And TGF- $\beta$  signaling pathway is of great importance of breast cancer invasiveness and metastasis (Imamura et al., 2012). Based on these reports, compounds that can suppress TGF- $\beta$ 1-induced MMP-9 are applied for the treatment of metastatic breast cancer. Many researchers focus mostly on the anti-fibrotic effects of curcumin via inhibiting TGF- $\beta$  signaling pathway (Smith et al., 2010; Yao et al., 2012; Zhang et al., 2012). However, whether curcumin can suppress TGF- $\beta$ 1-stimulated MMP-9 expression was not clear.

In accordance with previous reports (Yodkeeree et al., 2010), invasion assay in our study shown that curcumin at a nontoxic concentration markedly decreased invasiveness in response to TGF- $\beta$ 1 in a dose-dependent manner. Our results demonstrated that the levels of MMP-9 protein expression and enzymatic activity were significantly increased stimulated by TGF- $\beta$ 1 in the MDA-MB-231 breast cancer cells, and this increase can be inhibited obviously by curcumin. So, we came to the conclusion that TGF- $\beta$ 1-stimulated MMP-9 expression and activity might mediate cell invasion during wound healing and curcumin inhibited this process in MDA-MB-231 cells.

In classical TGF- $\beta$ /Smad pathway, TGF- $\beta$ 1 signal through TGF- $\beta$ /receptor serine- threonine kinases. The activated receptor complex phosphorylated receptor-regulated Smads (R-Smads), the latter forms heteromeric complexes with Smad4 that translocate into the nucleus and regulate transcription of specific target genes (Zhang, 2009). Besides TGF- $\beta$ /Smad pathway, TGF- $\beta$ 1 can also directly activate non-Smad signaling pathways, including the MAPKs (JNK, ERK, p38) (Smith et al., 2012). In other words, different transcriptional responses to TGF- $\beta$ 1 depend on activation of either or both Smad and Smad-independent pathways. So far, several study revealed that TGF- $\beta$ 1 might regulate MMP-9 expression through rapidly activating intracellular Smads and MAPKs signaling pathway (Chou et al., 2006). However, the previous study have shown that TGF- $\beta$ /MAPKs pathway which participate in regulating expression and activity of MMP-9 has specificity in different types of cells (Kim et al., 2005; Dziembowska et al., 2007; Szuster-Ciesielska et al., 2011). For example, Hsieh et al. have found that TGF- $\beta$ 1 induced MMP-9 expression is associated with the ERK signaling pathway in gastric cancer cells (Hsieh et al., 2010). Nevertheless, this process is inconclusive in breast cancer MDA-MB-231 cells. Some researchers

have also used kinase inhibitors to implicate both p38 and ERK signaling pathways in TGF- $\beta$ 1-mediated regulation of MMP-9 (Iiunga et al., 2004). However, other results have revealed that p38 inhibitor in minimal effective dose has no effects on TGF- $\beta$ 1-induced MMP-9, while excess p38 inhibitors do. The subsequent research have found that excess p38 inhibitors not only decreased phosphorylation of p38, but also can effectively block the activation of TGF- $\beta$  receptors kinases (Safina et al., 2007). Our results indicated that non-toxic dose of curcumin ( $\leq 10\mu\text{M}$ ) markedly inhibited phosphorylation of Smad2, ERK1/2, p38 mediated by TGF- $\beta$ 1 in a dose- and time-dependent way. Furthermore, PD98059 and curcumin had the similar inhibitory effects on TGF- $\beta$ 1-induced MMP-9. Nevertheless, the lowest effect concentration of SB203580 did not affect regulation of MMP-9. These results were consistent with the results have reported by Safina A et al.. Accordingly, our findings argued that curcumin perhaps down-regulated TGF- $\beta$ -induced MMP-9 via a mechanism involving ERK, Smad2 but not p38MAPK in MDA-MB-231 cells.

Moreover, previous studies have revealed that the p38 pathway might enhance cell metastasis ability by regulating actin remodeling factor HSP27 (Hedges et al., 1999) and affecting actin polymerization and cell contractility (Srinivasan et al. 2008). ERK may regulate cell motility by preventing formation of extensive actin stress fibers via suppression of tropomyosin induction by TGF- $\beta$ 1 or through inhibition of RhoA-Rho kinase pathway (Bakin et al., 2004; Helfman et al., 2005). So, perhaps activation of ERK, Smads and p38 MAPK signaling pathways is all required for the suppression of curcumin on TGF- $\beta$ 1-mediated cell migration. This speculative conclusion will also be our future research directions.

Our data revealed that the change of ERK1/2 and Smad2 phosphorylation was the key point of the anti-invasion effect of curcumin. Although our results also found that curcumin was able to reduce TGF- $\beta$  RII expression in MDA-MB-231 cells (data does not show). However, as the lack of specific protein phosphatase inhibitors, we cannot exclude the possibility that curcumin inhibits TGF- $\beta$ 1-stimulated MMP-9 via directly suppressing activation of the TGF- $\beta$  receptors which acts as an upstream regulator of ERK and Smad pathway.

In conclusion: In the present study, we explored the inhibition action of curcumin on TGF- $\beta$ 1-induced invasion in human breast cancer MDA-MB-231 cells and disclosed the potential mechanisms of the anti-invasion and metastasis effect. We demonstrated that (1) Curcumin dose-dependently inhibited the invasion ability induced by TGF- $\beta$ 1 in MDA-MB-231 cells. (2) Curcumin inhibited TGF- $\beta$ 1-induced MMP-9 protein expression and activity in MDA-MB-231 cells in a time- and concentration- way. (3) Curcumin time- and dose-dependently inhibited Smad2, ERK1/2, p38MAPK phosphorylation induced by TGF- $\beta$ 1. (4) PD98059 and curcumin had the similar suppression effect on TGF- $\beta$ 1-induced MMP-9 protein expression and activity. All together, these findings highlight the profitable effect of curcumin, and it serves as an anti-MMP-9 factor through inhibition of the TGF- $\beta$ /Smad and TGF- $\beta$ /Erk signaling pathway.

## References

- Allen M, Louise Jones J (2011). Jekyll and Hyde: the role of the microenvironment on the progression of cancer. *J Pathol*, **223**, 162-76.
- Bakin AV, Safina A, Rinehart C, et al (2004). A critical role of tropomyosins in TGF-beta regulation of the actin cytoskeleton and cell motility in epithelial cells. Daroqui C, Darbary H, Helfman DM. *Mol Biol Cell*, **15**, 4682-94.
- Belotti D, Calcagno C, Garofalo A (2008). Vascular endothelial growth factor stimulates organ-specific host matrix metalloproteinase-9 expression and ovarian cancer invasion. *Mol Cancer Res*, **6**, 525-34.
- Biswas S, Guix M, Rinehart C, et al (2007). Inhibition of TGF-beta with neutralizing antibodies prevents radiation-induced acceleration of metastatic cancer progression. *J Clin Invest*, **117**, 1305-13.
- Blair KJ, Kiang A, Wang-Rodriguez J, et al (2011). EGF and bFGF promote invasion that is modulated by PI3/Akt kinase and Erk in vestibular schwannoma. *Otol Neurotol*, **32**, 308-14.
- Chen P, Lu N, Ling Y, Chen Y, et al (2011). Inhibitory effects of wogonin on the invasion of human breast carcinoma cells by downregulating the expression and activity of matrix metalloproteinase-9. *Toxicology*, **282**, 122-8.
- Cheung KL (2007). Endocrine therapy for breast cancer: an overview. *Breast*, **16**, 327-43.
- Chou YT, Wang H, Chen Y (2006). Cited2 modulates TGF-beta-mediated upregulation of MMP9. *Oncogene*, **25**, 5547-60.
- Dziembowska M, Danilkiewicz M, Wesolowska A (2007). Cross-talk between Smad and p38 MAPK signalling in transforming growth factor beta signal transduction in human glioblastoma cells. *Biochem Biophys Res Commun*, **354**, 1101-6.
- Hassan ZK, Daghestani MH (2012). Curcumin effect on MMPs and TIMPs genes in a breast cancer cell line. *Asian Pac J Cancer Prev*, **13**, 3259-64.
- Hortobagyi GN (2002). The status of breast cancer management: challenges and opportunities. *Breast Cancer Res Treat*, **75**, S61-5, discussion S57-9.
- Hsieh HL, Wang HH, Wu WB, et al (2010). Transforming growth factor- $\beta$ 1 induces matrix metalloproteinase-9 and cell migration in astrocytes: roles of ROS-dependent ERK and JNK-NF- $\kappa$ B pathways. *J Neuroinflammation*, **8**, 88.
- Hedges JC, Dechert MA, Yamboliev IA, et al (1999). A role for p38(MAPK)/HSP27 pathway in smooth muscle cell migration. *J Biol Chem*, **274**, 24211-9.
- Helfman DM, Pawlak G (2004). Myosin light chain kinase and acto-myosin contractility modulate activation of the ERK cascade downstream of oncogenic Ras. *J Cell Biochem*, **95**, 1069-80.
- Iiunga K, Nishiura R, Inada H (2004). Co-stimulation of human breast cancer cells with transforming growth factor-beta and tenascin-C enhances matrix metalloproteinase-9 expression and cancer cell invasion. *Int J Exp Pathol*, **85**, 373-9.
- Imamura T, Hikita A, Inoue Y (2012). The roles of TGF- $\beta$  signaling in carcinogenesis and breast cancer metastasis. *Breast Cancer*, **19**, 118-24.
- Geiger TR, Peeper DS (2009). Metastasis mechanisms. *Biochim Biophys Acta*, **1796**, 293-308.
- Kim HS, Luo L, Pflugfelder SC, Li DQ (2005). Doxycycline inhibits TGF-beta1-induced MMP-9 via Smad and MAPK pathways in human corneal epithelial cells. *Invest Ophthalmol Vis Sci*, **46**, 840-8.
- Na D, Lv ZD, Liu FN, et al (2010). Transforming growth factor beta1 produced in autocrine/paracrine manner affects the morphology and function of mesothelial cells and promotes peritoneal carcinomatosis. *Int J Mol Med*, **26**, 325-32.
- Harhra NA, Basaleem HO (2012). Trends of breast cancer and its management in the last twenty years in aden and adjacent governorates, yemen. *Asian Pac J Cancer Prev*, **13**, 4347-51.
- Park J, Ayyappan V, Bae EK, et al (2008). Curcumin in combination with bortezomib synergistically induced apoptosis in human multiple myeloma U266 cells. *Mol Oncol*, **2**, 317-26.
- Perera M, Tsang CS, Distel RJ, et al (2010). TGF-beta1 interactome: metastasis and beyond. *Cancer Genomics Proteomics*, **7**, 217-29.
- Porter PL (2009). Global trends in breast cancer incidence and mortality. *Salud Publica Mex*, **51**, S141-6.
- Safina A, Vandette E, Bakin AV (2007). ALK5 promotes tumor angiogenesis by upregulating matrix metalloproteinase-9 in tumor cells. *Oncogene*, **26**, 2407-22.
- Sanchez-Zamorano LM, Flores-Luna L, Angeles-Llerenas A et al (2011). Healthy lifestyle on the risk of breast cancer. *Cancer Epidemiol Biomarkers Prev*, **20**, 912-22.
- Santibáñez JF, Guerrero J, Quintanilla M, et al (2002). Transforming growth factor-beta1 modulates matrix metalloproteinase-9 production through the Ras/MAPK signaling pathway in transformed keratinocytes. *Biochem Biophys Res Commun*, **16**, 267-73.
- Santibáñez JF, Quintanilla M, Martínez J (2000). Genistein and curcumin block TGF-beta 1-induced u-PA expression and migratory and invasive phenotype in mouse epidermal keratinocytes. *Nutr Cancer*, **37**, 49-54.
- Serra R, Crowley MR (2005). Mouse models of transforming growth factor beta impact in breast development and cancer. *Endocr Relat Cancer*, **12**, 749-60.
- Smith AL, Robin TP, Ford HL (2012). Molecular Pathways: Targeting the TGF-beta Pathway for Cancer Therapy. *Clin Cancer Res*, **18**, 4514-21.
- Smith MR, Gangireddy SR, Narala VR, et al (2010). Curcumin inhibits fibrosis-related effects in IPF fibroblasts and in mice following bleomycin-induced lung injury. *Am J Physiol Lung Cell Mol Physiol*, **298**, L616-25.
- Srinivasan R, Forman S, Quinlan RA, et al (2008). Regulation of contractility by Hsp27 and Hic-5 in rat mesenteric small arteries. *Am J Physiol Heart Circ Physiol*, **294**, H961-9.
- Szuster-Ciesielska A, Plewka K, Daniluk J (2011). Betulin and betulinic acid attenuate ethanol-induced liver stellate cell activation by inhibiting reactive oxygen species (ROS), cytokine (TNF-alpha, TGF-beta) production and by influencing intracellular signaling. *Toxicology*, **280**, 152-63.
- Yan G, Graham K, Lanza-Jacoby S (2012). Curcumin enhances the anticancer effects of trichostatin A in breast cancer cells. *Mol Carcinog*.
- Yao QY, Xu BL, Wang JY, et al (2012). Inhibition by curcumin of multiple sites of the transforming growth factor-beta1 signalling pathway ameliorates the progression of liver fibrosis induced by carbon tetrachloride in rats. *BMC Complement Altern Med*, **12**, 156.
- Ye B, Jiang LL, Xu HT, et al (2012). Expression of PI3K/AKT pathway in gastric cancer and its blockade suppresses tumor growth and metastasis. *Int J Immunopathol Pharmacol*, **25**, 627-36.
- Yodkeeree S, Ampasavate C, Sung B, et al (2010). Curcumin suppresses migration and invasion of MDA-MB-231 human breast cancer cell line. *Eur J Pharmacol*, **627**, 8-15.
- Zayani Y, Allal-Elasmi M, Jacob MP, et al (2012). Abnormal circulating levels of matrix metalloproteinases and their inhibitors in diabetes mellitus. *Clin La*, **58**, 779-85.
- Zhang SS, Gong ZJ, Li WH, et al (2012). Antifibrotic effect of curcumin in TGF-beta 1-induced myofibroblasts from human oral mucosa. *Asian Pac J Cancer Pre*, **13**, 289-94.
- Zhang YE (2009). Non-Smad pathways in TGF-beta signaling. *Cell Res*, **19**, 128-39.