

# Role of TG2 and TGF- $\beta_1$ in the pathogenesis of human breast cancer

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**Abstract.** The present study analyzed the role of transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ) and tissue transglutaminase (TG2) in breast cancer, as well as their protein levels in MCF-7 cells treated with cisplatin. In addition, the present study investigated the effects of TG2 and TGF- $\beta_1$  in MCF-7 cells following TGF- $\beta_1$  and TG2 inhibition or TGF- $\beta_1$  induction. The protein levels of TG2 and TGF- $\beta_1$  in breast cancer tissues and in MCF-7 cells treated with cisplatin, TG2 and TGF- $\beta_1$  inhibitors or 10 ng/ml TGF- $\beta_1$  were analyzed by immunohistochemical staining, immunofluorescence and western blotting. The results revealed that the expression levels of TG2 and TGF- $\beta_1$  in breast cancer tissues were significantly higher compared with those in paracancerous tissues. The fluorescence intensity of TG2 and TGF- $\beta_1$  in MCF-7 cells treated with cisplatin was lower compared with that in untreated MCF-7 cells. Using bioinformatics analysis, the present study predicted that TGF- $\beta_1$  may be associated with TG2. In addition, the expression levels of TGF- $\beta_1$  and TG2 in MCF-7 cells treated with inhibitors of TGF- $\beta_1$  and TG2 were lower compared with those in untreated MCF-7 cells. By contrast, the expression levels of TGF- $\beta_1$  and TG2 in MCF-7 cells treated with TGF- $\beta_1$  were higher compared with those in untreated MCF-7 cells. Therefore, the present study demonstrated that TGF- $\beta_1$  and TG2 may serve an important role in breast cancer tissues and in MCF-7 cells. In addition, it was revealed that TG2 and TGF- $\beta_1$  may have a synergistic role in MCF-7 cells.

## Introduction

Breast cancer is the most commonly occurring type of cancer among women (1). The Global Health Organization reported that 508,000 women succumbed to breast cancer in 2011 (2). There are a number of factors involved in the occurrence and development of breast cancer, such as tissue transglutaminase (TG2) and transforming growth factor- $\beta$  (TGF- $\beta$ ) family members (3,4).

TGF- $\beta_1$  is a member of the transforming growth factor superfamily that is widely involved in various pathophysiological processes, such as inflammation, trauma and organ fibrosis (5). TG2 is an enzyme that is upregulated in epithelial malignancies and participates in  $\text{Ca}^{2+}$ -dependent protein post-translational modifications and cross-linking via the acyl-transfer reaction between glutamine and lysine residues (6). It has been reported that TG2 serves an important role in the epithelial-to-mesenchymal transition (EMT) (6). In addition, upregulation of TGF- $\beta$  is associated with metastasis, cell invasiveness and EMT in ovarian cancer (7,8). However, to the best of our knowledge, the synergistic role of TG2 and TGF- $\beta_1$  in regulating the occurrence and development of breast cancer has been reported.

Cisplatin is a broad-spectrum anticancer drug that is commonly used in ovarian, prostate, testicular and lung cancer, nasopharyngeal carcinoma, esophageal cancer, malignant lymphoma, head and neck squamous cell carcinoma and thyroid cancer (9). However, whether the expression of TG2 and TGF- $\beta_1$  is regulated by cisplatin remains to be elucidated.

The present study aimed to analyze the role of TG2 and TGF- $\beta_1$  in breast cancer. In addition, the present study aimed to investigate the protein levels of TGF- $\beta_1$  and TG2 in MCF-7 cells treated with cisplatin and the effect of TG2 and TGF- $\beta_1$  in MCF-7 cells treated with TGF- $\beta_1$  and TG2 inhibitors or TGF- $\beta_1$ .

## Materials and methods

**Tissue samples.** A total of 30 pairs of breast cancer and paracancerous tissue samples were obtained from the China-Japan Union Hospital (Changchun, China) between March 2018 and March 2019. The median age is 38 years (range, 28-45 years).

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**Key words:** breast cancer, tissue transaminase, transforming growth factor- $\beta_1$ , cisplatin, cell death

The study was approved by the Ethics Committee of the China-Japan Union Hospital. The samples were obtained with signed informed consent from the patients or their family.

**Cell culture.** MCF-7 cells were gifted from Jilin University School of Pharmacy. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 U/ml penicillin G and 100  $\mu$ g/ml streptomycin in an incubator at 37°C and 5% CO<sub>2</sub>. (all from Invitrogen; Thermo Fisher Scientific, Inc.).

**Reagents.** All materials for the SDS-PAGE were purchased from Bio-Rad Laboratories, Inc. The monoclonal antibody against  $\beta$ -actin (1,200; cat. no. AAPR201-100) was purchased from Sigma-Aldrich; Merck KGaA. Rabbit polyclonal antibodies against TGF- $\beta_1$  (1:2,000; cat. no. RAB-0238) and TG2 (1:2,000; cat. no. CTA-DE056) were obtained from Cell Signaling Technology, Inc. The TG2 inhibitor (MDC) and the TGF- $\beta_1$  inhibitor (ITD) were purchased from Sigma-Aldrich; Merck KGaA.

**Hematoxylin and eosin (HE) staining.** The sections (3  $\mu$ m) of breast cancer tissue were fixed in 4% paraformaldehyde followed by dehydration using a gradient ethanol series (80 and 95%). The section was subsequently stained with HE at 25°C (10 min). Images were captured on a fully automatic photomicrography device (magnification x200; five field of views; Olympus PM-10AO; Olympus Corporation).

**Immunohistochemistry** Immunohistochemical staining was performed using a SABC kit (Biyuntian Biotechnology Co., Ltd.). 0.3% hydrogen peroxide formaldehyde solution was added to the paraffin sections and incubated at 37°C. The sections of breast cancer tissues and paracancerous tissues were washed with PBS and incubated with 10% bovine serum albumin (Thermo Fisher Scientific, Inc.) for 15 min. Rabbit anti-human TGF- $\beta_1$  and TG2 polyclonal antibodies (1:300) were added and incubated at 4°C for 12 h. The next day, color rendering was performed using 3,3'-diaminobenzidine and images were captured on a fully automatic photomicrography device (magnification x200; five field of views; Olympus PM-10AO; Olympus Corporation).

**MTT assay.** MCF-7 cells were seeded in 96-well plates at  $1 \times 10^3$  per well, treated with 1 or 2 mg/l cisplatin (German Pharmaceutical Co., Ltd.) and incubated in an incubator at 37°C at 5% CO<sub>2</sub> for 24 h. Next, 10  $\mu$ l MTT was added to each well and incubated for 30 min at 37°C. The absorbance was measured at 450 nm using a microplate reader.

**Western blotting.** The protein expression levels of TG2 and TGF- $\beta_1$  in MCF-7 cells were assessed by western blotting. Tissue samples were homogenized in a PIPA buffer (Qiagen, Inc.). Protein concentrations were determined using a bicinchoninic acid kit (Pierce; Thermo Fisher Scientific, Inc.). Protein (20  $\mu$ g/lane) was separated using 12% SDS-PAGE and transferred to a PVDF membrane. The membrane was incubated with primary antibodies against TG2 (1:2,000), TGF- $\beta_1$  (1:2,000) and  $\beta$ -actin (1:2,000) at 4°C overnight. The membranes were incubated with horseradish peroxidase-conjugated anti-rabbit

IgG antibody (cat. no. ZB-2301; 1:2,000; Beijing Noble Technology Co., Ltd). TG2 and TGF- $\beta_1$  were detected using ECL development solution (Pierce; Thermo Fisher Scientific, Inc.). TG2 and TGF- $\beta_1$  expression levels were determined using Quantity One v4.6.2 software (Bio-Rad Laboratories, Inc.).

**Immunofluorescence.** The fluorescence intensity of TG2 and TGF- $\beta_1$  in MCF-7 cells was assessed via immunofluorescence. MCF-7 cells ( $5 \times 10^4$ ) were treated with cisplatin (1 mg/l), TGF- $\beta_1$  (150  $\mu$ mol/l) and TG2 inhibitors (8.31  $\mu$ mol/l), and incubated at 37°C and 5% CO<sub>2</sub> for 24 h. Rabbit monoclonal primary antibodies against TG2 (1:300) and TGF- $\beta_1$  (1:300) were added to the cells and incubated overnight at 4°C. Following overnight incubation with fluorescein-conjugated IgG (cat. no. ZB-2301; Beijing Noble Technology Co., Ltd.) antibody at 4°C. TG2 and TGF- $\beta_1$  expression levels were determined using Quantity One v4.6.2 software (Bio-Rad Laboratories, Inc.).

**Molecular docking.** The crystal structure of TG2 [in complex with GTP; Protein Data Bank (PDB) ID, 4PYG] and TGF- $\beta_1$  (in complex with scFv GC1009; PDB ID, 4KV5) were obtained from the PDB (<http://www.rcsb.org/pdb>). The protein files of TG2 and TGF- $\beta_1$  were prepared by removing water molecules and other ligands. Molecular docking studies and docking analysis were performed using the PatchDock server (<http://bioinfo3d.cs.tau.ac.il/PatchDock/>). Analysis and visualization of interactions in the docked complexes obtained by PatchDock server were analyzed by PyMOL (<https://pymol.en.softonic.com/>).

**Statistical analysis.** Quantitative data are presented as the mean  $\pm$  standard deviation and were analyzed using SPSS 19.0 software (IBM Corp.). Student's t-test was used to compare two groups; one-way ANOVA followed by Dunnett's test was used to compare all treatment groups against an untreated control group. The  $\chi^2$  test was used to analyze the associations between protein expression and patient characteristics.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**HE staining.** In the present study, tissues from 30 patients with breast cancer were evaluated using HE staining (Table I). These patients were recruited at the China-Japan Union Hospital of Jilin University. Clear structures and contours of the tissues were detected in paracancerous tissues (Fig. 1A). However, in the breast cancer samples, moderately heteromorphic and moderately differentiated cells were observed in the epithelial tissue (Fig. 1B). In addition, moderate atypia and interstitial fibrosis were observed in the breast cancer tissues (Fig. 1B).

**Immunohistochemical staining.** In the present study, the expression levels of TG2 and TGF- $\beta_1$  were assessed via immunohistochemical staining. The results revealed that the expression levels of TG2 and TGF- $\beta_1$  in the cell membrane and cytoplasm in the paracancerous tissues were low. However, the expression levels of TG2 and TGF- $\beta_1$  were significantly higher in the breast cancer tissues compared

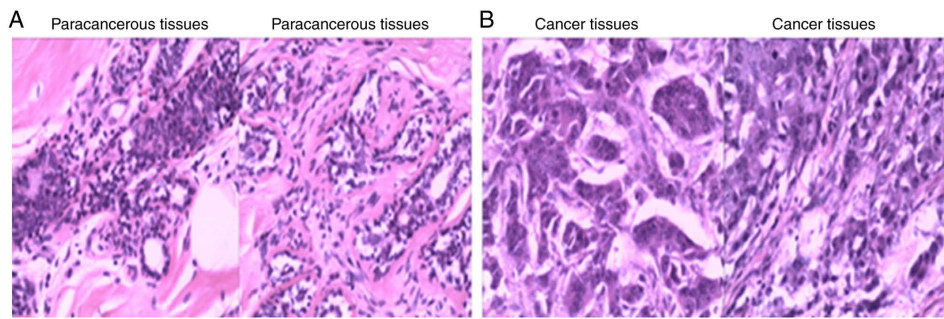


Figure 1. Hematoxylin and eosin staining of breast cancer and paracancerous tissues. (A) Paracancerous tissues. (B) Cancer tissues. Magnification x200.

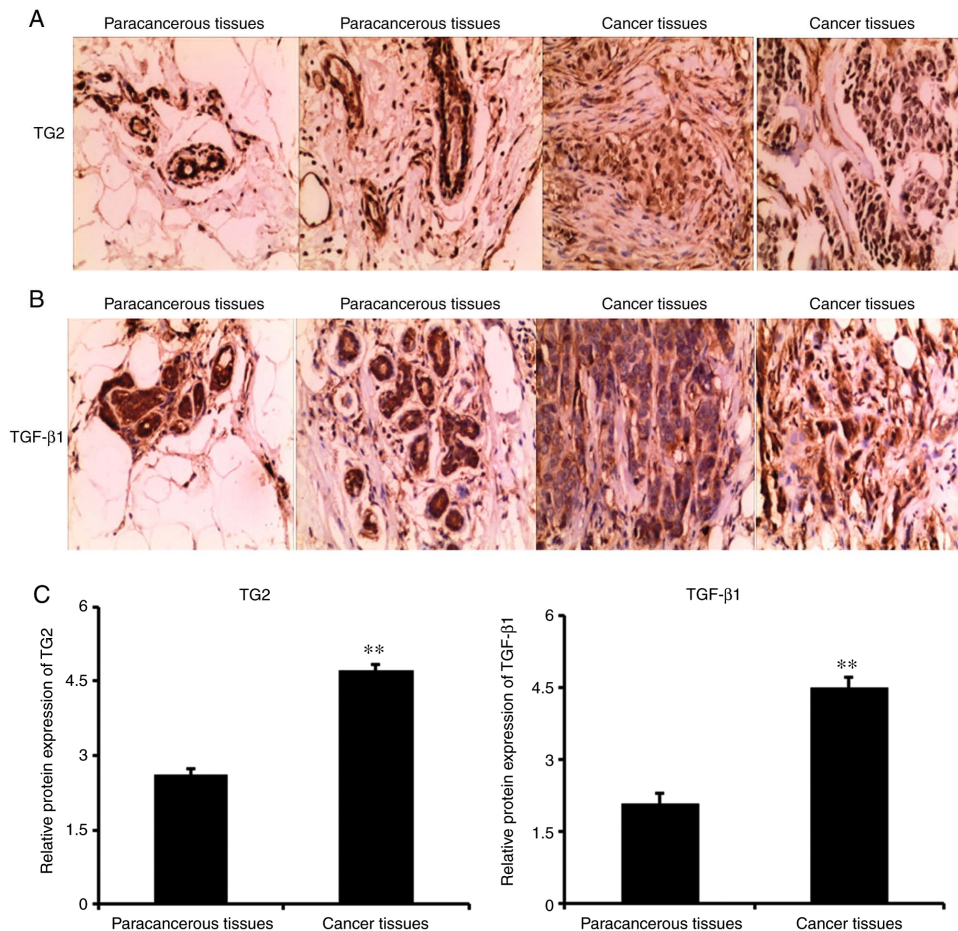


Figure 2. Immunohistochemical staining of TG2 and TGF-β<sub>1</sub>. (A and B) Expression of TG2 and TGF-β<sub>1</sub> in paracancerous and cancer tissues. (C) Expression levels of TG2 and TGF-β<sub>1</sub> in paracancerous and breast cancer tissues. Magnification, x200. \*\*P<0.01 vs. paracancerous tissues. TGF-β<sub>1</sub>, transforming growth factor β<sub>1</sub>; TG2, tissue transglutaminase.

with those in the paracancerous tissues (P<0.05; Fig. 2A-C). TG2 and TGF-β<sub>1</sub> were primarily expressed in the tumor and interstitial regions.

**MTT.** The viability of MCF-7 cells treated with cisplatin was analyzed by MTT assay. The results revealed that cell survival in untreated MCF-7 cells was lower compared with that in cells treated with 1 and 2 mg/l cisplatin (P<0.05; Fig. 3).

**Immunofluorescence.** In the present study, immunofluorescence was used to determine the fluorescence intensity of TG2 and TGF-β<sub>1</sub> in MCF-7 cells. The results demonstrated that

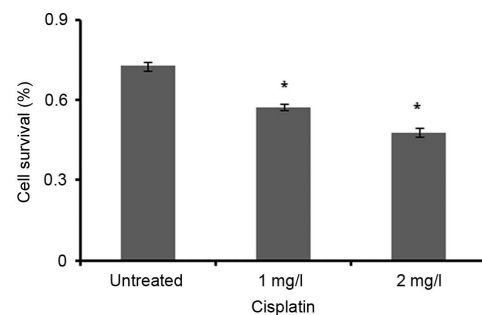


Figure 3. Survival of cisplatin-treated MCF-7 cells. \*P<0.05, 1 and 2 mg/l cisplatin-treated MCF-7 cells vs. untreated MCF-7 cells.

Table I. Associations between patient clinicopathological characteristics and the expression of TG2 and TGF- $\beta_1$ .

Characteristic	TG2-positive	TG2-negative	P-value	TGF- $\beta_1$ -positive	TGF- $\beta_1$ -negative	P-value
Age, years, n						
35-45	8	6		10	5	
45-55	9	7		10	5	
Stage, n						
II	30			30		
Infiltration depth (%)	17 (56.7%)	13 (43.3%)	0.025	20 (66.7%)	10 (33.3%)	<0.001
Nucleus and nuclear membrane, n	15	11		2	1	
Serosa, n	2	2		18	9	

TGF- $\beta_1$ , transforming growth factor- $\beta_1$ ; TG2, tissue transglutaminase.

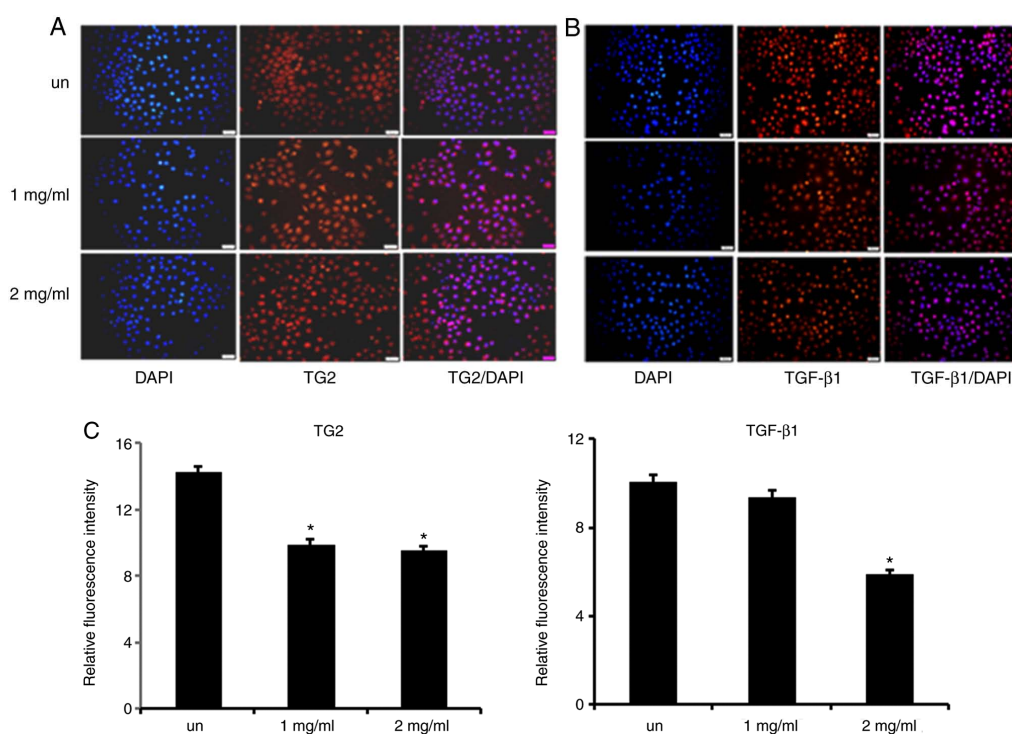


Figure 4. (A and B) Immunofluorescence of TG2 and TGF- $\beta_1$ . (C) Fluorescence intensity of TG2 and TGF- $\beta_1$  in untreated MCF-7 cells and MCF-7 cells treated with 1 and 2 mg/l cisplatin. Magnification x200. \*P<0.05 vs. untreated cells. TGF- $\beta_1$ , transforming growth factor- $\beta_1$ ; TG2, tissue transglutaminase; un, untreated cells.

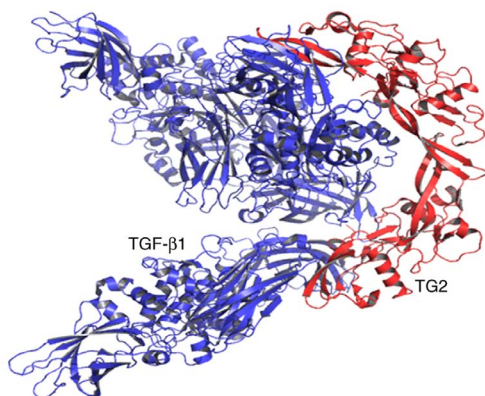


Figure 5. Molecular docking structure of TG2 and TGF- $\beta_1$ . Red, TG2; blue, TGF- $\beta_1$ . TGF- $\beta_1$ , transforming growth factor- $\beta_1$ ; TG2, tissue transglutaminase.

the fluorescence intensity of TG2 and TGF- $\beta_1$  decreased in cisplatin-treated MCF-7 cells compared with untreated MCF-7 cells (P<0.05; Fig. 4A-C).

*Molecular docking of TG2 with TGF- $\beta_1$ .* In order to further understand and characterize the interactions between TG2 and TGF- $\beta_1$ , the binding interaction of TG2 with TGF- $\beta_1$  was modeled by molecular docking. The present results found that there were ten sites that prove TGF- $\beta_1$  may be associated with TG2 (Table II; Fig. 5).

*TG2 and TGF- $\beta_1$  fluorescence intensity following TGF- $\beta_1$  induction and TG2 or TGF- $\beta_1$  inhibitors.* In the present study, the fluorescence intensity of TG2 and TGF- $\beta_1$  in MCF-7 cells treated with the inhibitors of TGF- $\beta_1$  and TG2 was assessed by



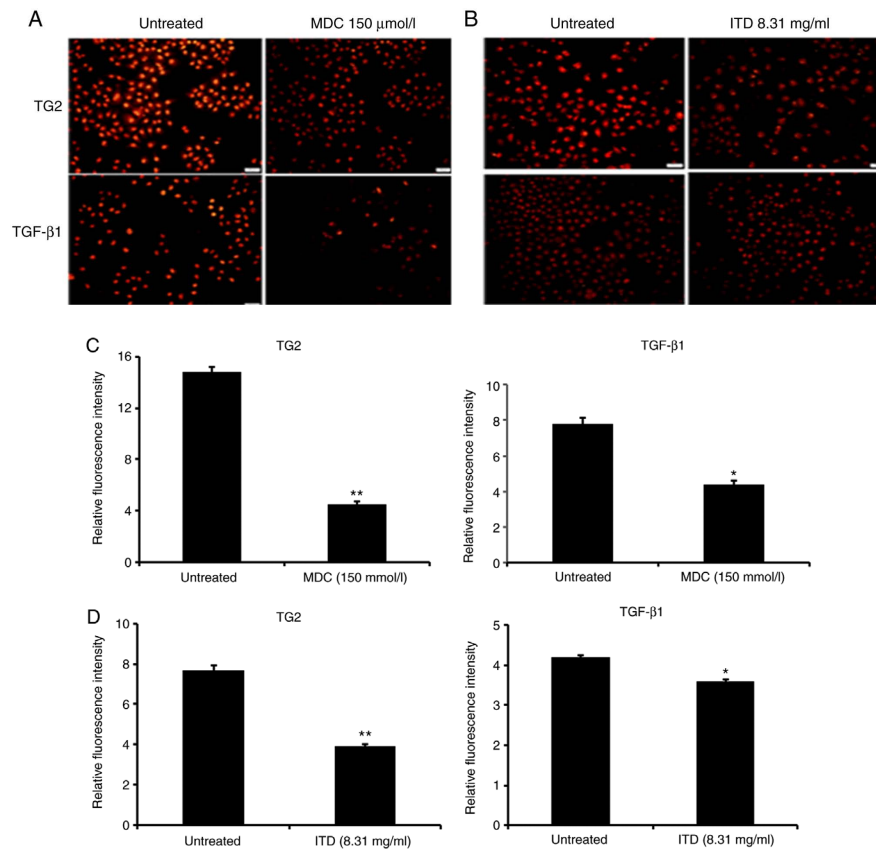


Figure 6. (A and B) Fluorescence of TG2 and TGF-β<sub>1</sub> in MCF-7 cells treated with TG2 and TGF-β<sub>1</sub> inhibitors. (C and D) Fluorescence intensity of TG2 and TGF-β<sub>1</sub> in MCF-7 cells treated with TG2 and TGF-β<sub>1</sub> inhibitors. Magnification x200. \*P<0.05, \*\*P<0.01 vs. untreated cells. TGF-β<sub>1</sub>, transforming growth factor β<sub>1</sub>; TG2, tissue transglutaminase; ITD, TGF-β<sub>1</sub> inhibitor; MDC, TG2 inhibitor.

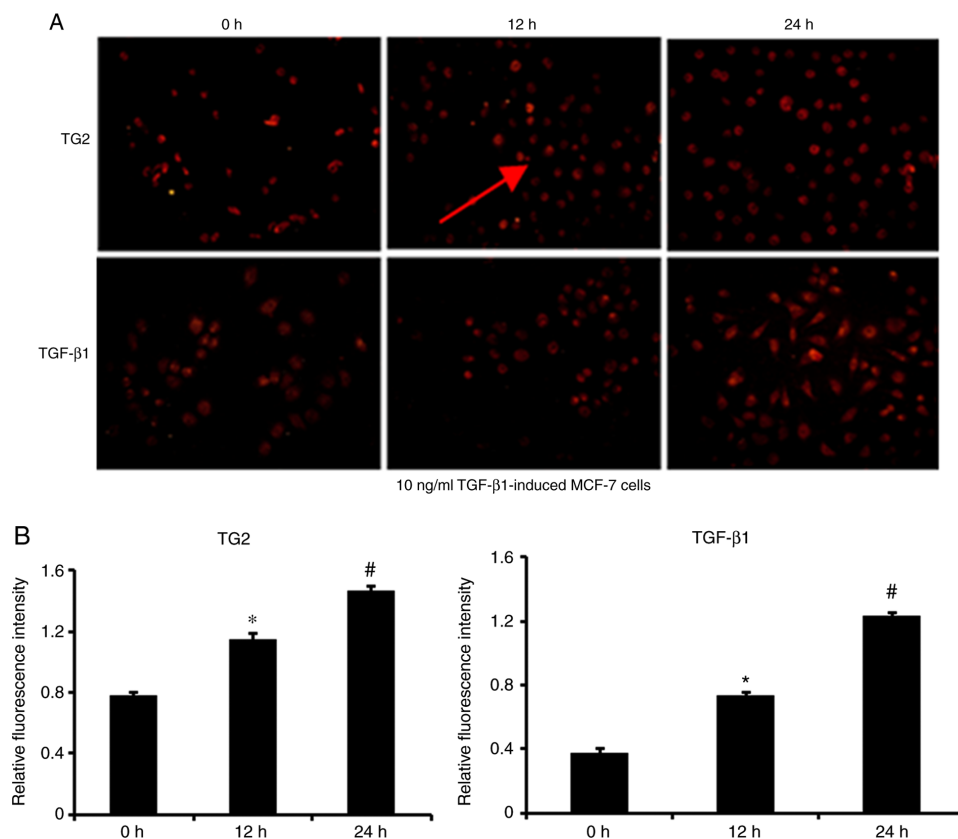


Figure 7. (A and B) Fluorescence of TG2 and TGF-β<sub>1</sub> in untreated MCF-7 cells and MCF-7 cells treated with 10 ng/ml TGF-β<sub>1</sub>. Magnification, x200. \*P<0.05 and #P<0.01 vs. 0 h. TGF-β<sub>1</sub>, transforming growth factor β<sub>1</sub>; TG2, tissue transglutaminase.

Table II. Binding scores of junction for TG2 with TGF- $\beta_1$ .

Solution no.	Score	Area ( $\text{\AA}$ )
1	20,950	3614.3
2	20,368	3214.5
3	20,160	3012.9
4	19,952	2918.5
5	19,554	3492.0
6	19,460	3311.2
7	19,428	2974.3
8	19,288	2760.4
9	19,254	2881.1
10	19,096	2987.4

TGF- $\beta_1$ , transforming growth factor- $\beta_1$ ; TG2, tissue transglutaminase.

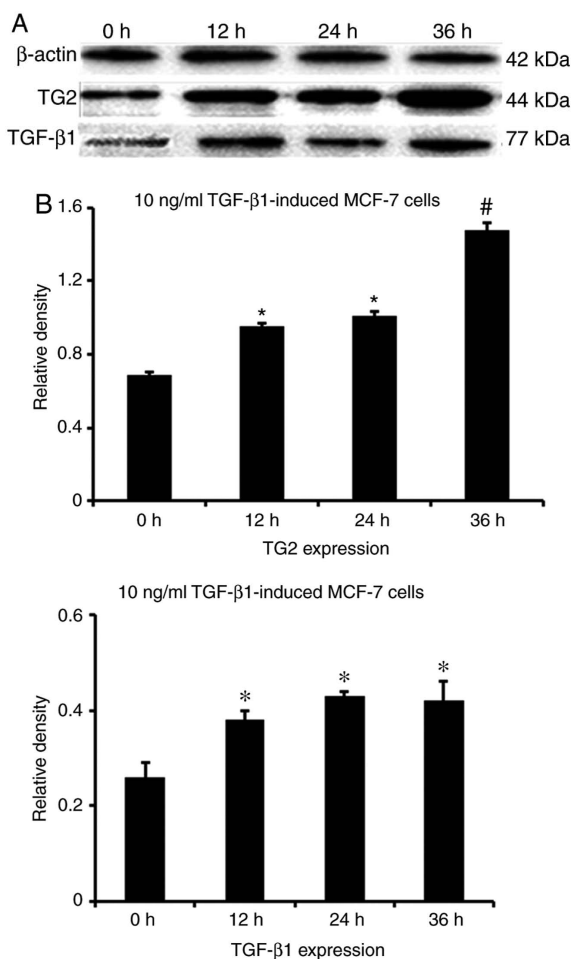


Figure 8. (A) Protein expression of TG2 and TGF- $\beta_1$ . (B) Quantification of the expression of TG2 and TGF- $\beta_1$  in untreated MCF-7 and MCF-7 cells treated with 10 ng/ml TGF- $\beta_1$ . Magnification x200. \* $P < 0.05$  and # $P < 0.01$  vs. 0 h. TGF- $\beta_1$ , transforming growth factor- $\beta_1$ ; TG2, tissue transglutaminase.

immunofluorescence. The results demonstrated that the fluorescence intensity of TG2 and TGF- $\beta_1$  in MCF-7 cells treated with the TGF- $\beta_1$  inhibitor (8.31  $\mu\text{mol/l}$ ) and the TG2 inhibitor (150  $\mu\text{mol/l}$ ) was lower compared with that in untreated MCF-7 cells ( $P < 0.05$ ; Fig. 6). In addition, the fluorescence and

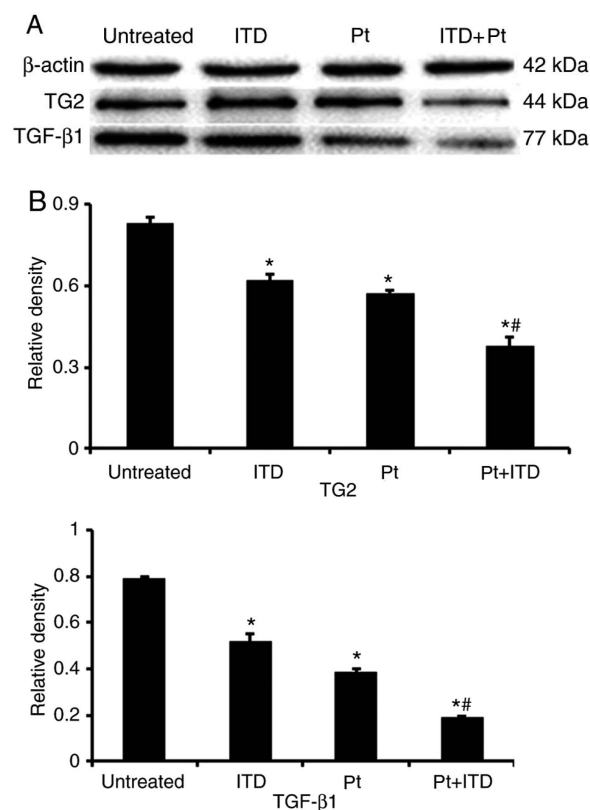


Figure 9. (A) Protein expression of TG2 and TGF- $\beta_1$  in MCF-7 cells treated with ITD, Pt and ITD+Pt. (B) Quantification of the expression levels of TG2 and TGF- $\beta_1$  in untreated MCF-7 and MCF-7 cells treated with ITD, Pt and ITD+Pt. Magnification x200. \* $P < 0.05$  vs. untreated; \*\* $P < 0.01$  vs. ITD (TGF- $\beta_1$ ) and Pt. TG2, tissue transglutaminase; ITD; TG2 inhibitor; Pt, cisplatin.

protein levels of TG2 and TGF- $\beta_1$  in MCF-7 cells induced with TGF- $\beta_1$  were higher compared with that in untreated MCF-7 cells ( $P < 0.001$ ; Figs. 7 and 8). In Fig. 9, the expression levels of TG2 and TGF- $\beta_1$  in MCF-7 cells treated with ITD (TGF- $\beta_1$ ), Pt (cisplatin), and ITD plus Pt were lower compared with those in untreated MCF-7 cells ( $P < 0.05$  and  $P < 0.001$ ).

## Discussion

The results of the present study demonstrated that TG2 and TGF- $\beta_1$  served an important role in the development and progression of breast cancer. The protein levels of TG2 and TGF- $\beta_1$  were evaluated in MCF-7 cells treated with cisplatin. In addition, the present study investigated the role of TG2 and TGF- $\beta_1$  in MCF-7 cells treated with inhibitors of TGF- $\beta_1$  and TG2.

TG2 is involved in the occurrence and development of Parkinson's disease (10,11). A previous study have demonstrated the association of TG2 with tumor growth, EMT and metastasis (12). TG2 also exhibits  $\text{Ca}^{2+}$ -independent guanosine triphosphate hydrolase, protein kinase and disulfide isomerase activity (13). TG2 knockdown decreases the proliferative, migratory and colony forming abilities of breast cancer cells (14). With the occurrence and development of cancer cachexia, the role of the TGF- $\beta$  signaling pathway changes, inducing the EMT process and promoting tumor invasion and metastasis (15). It has been demonstrated that the level of the TGF- $\beta$  signaling pathway activity is associated with lung

fibrosis and patient mortality (16). The results of the present study revealed that the expression levels of TG2 and TGF- $\beta_1$  in human breast cancer were higher compared with those in paracancerous tissues. These results suggested that TGF- $\beta_1$  and TG2 may serve an important role in the occurrence and development of breast cancer.

To further analyze the role of TGF- $\beta_1$  and TG2 in breast cancer, the present study analyzed the expression levels of TGF- $\beta_1$  and TG2 in MCF-7 cells treated with cisplatin. Cisplatin is a selective drug used for the treatment of prostate cancer (17). A large proportion of patients develop resistance to cisplatin, inducing tumor relapse and limiting its clinical usefulness (17). In the present study, the results demonstrated that the fluorescence intensity of TG2 and TGF- $\beta_1$  in untreated MCF-7 cells was higher compared with that in MCF-7 cells treated with cisplatin. These results suggested that cisplatin may exert its chemotherapeutic effects in MCF-7 cells by regulating the levels of TG2 and TGF- $\beta_1$  protein.

TG2 was associated with TGF- $\beta_1$  in the occurrence and development of various diseases (8). To clarify the effect of TG2 and TGF- $\beta_1$  in MCF-7 cells, the present study investigated the effect of TG2 and TGF- $\beta_1$  in the MCF-7 cells treated with inhibitors of TGF- $\beta_1$  and TG2. The results demonstrated that the fluorescence intensity of TG2 and TGF- $\beta_1$  in MCF-7 cells treated with a TG2 or TGF- $\beta_1$  inhibitor was lower compared with that in untreated MCF-7 cells. By contrast, the fluorescence intensity of TG2 and TGF- $\beta_1$  in MCF-7 cells treated with TGF- $\beta_1$  was higher compared with that in untreated MCF-7 cells. Therefore, TG2 and TGF- $\beta_1$  may serve a role in the occurrence and development of breast cancer.

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#### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

#### Authors' contributions

HCG conceived the study and drafted the manuscript. YC and ZHW acquired the data. YZH, YQL and GHY analyzed the data and revised the manuscript. All authors read and approved the final manuscript.

#### Ethics approval and consent to participate

The study was approved by the Ethics Committee of China-Japan Union Hospital (Changchun, China; approval no. 2018120506). Signed informed consents were obtained from the patients and/or the guardians.

#### Patient consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

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