Original article

Genistein Down-Regulates the Expression of uPA and the Activity of PTK in MDA-MB-453 Breast Cancer Cells

Xiaoping Yu, MD, Min Wei, MD, Mantian Mi, MD, Jundong Zhu, MD

From Department of Public Health (Drs Yu and Wei), School of Preclinical Medicine, Chengdu Medical College, 601# Tianhui Road, Rongdu Avenue, Chengdu 610083, Sichuan province, China; Department of Nutrition and Food Hygiene (Drs Yu, Mi and Zhu), the Third Military Medical University, 30 Gaotanyan Street, Shapingba District, Chongqing 400038, China

ABSTRACT This study is to investigate the effects of genistein on the expression of urokinase-type plasminogen activator (uPA) and the activity of protein tyrosine kinase (PTK) in MDA-MB-453 cells, to further explore the molecular mechanism of genistein's anti-angiogenesis in HER-2/neu over-expressing breast cancer. The Western blot, immunoprecipitate, reverse transcription- polymerase chain reaction (RT-PCR) and kinase activity analysis techniques were used to measure the expression of uPA, the protein phosphorylation of HER-2/neu receptor, and the activity of protein tyrosine kinase (PTK) in MDA-MB-453 cells treated by 5× 10-5 mol/l genistein for 24, 48, 72 h, respectively. We found that the expression of uPA, the level of protein phosphorylation of HER-2/neu receptor, and the activity of PTK were significant decreased after tested cells treated with genistein, which had a time-dependence. Therefore, it can be concluded that genistein inhibits the activity of PTK, the protein phosphorylation of HER-2/neu receptor, and down-regulates the expression of uPA at transcription and translation levels in breast cancer cells, which might be a part of molecular mechanism of genistein's anti-angiogenesis in HER-2/neu over-expressing breast cancer.

KeyWords: Genistein; Urokinase-type plasminogen activator; Potein tyrosine kinase; MDA-MB-453 breast cancer cell

Soya bean has redundant nutritive value, which contains not only high-grade protein but also a kind of un-trophic component (e.g. genistein). Genistein, as an important composition of isoflavones, has general biological effects, such as anti-tumor, anti-virus, anti-eumycete, anti-oxidization, anti-mutation, anti-hypertension, and anti-hyperplasia, in which, it is one of the investigative hot spots that genistein inhibits the angiogenesis of tumor (1). The angiogenesis is the foundation for tumor further growth and metastasis, and is regulated by tumour cells and the angiogenesis related factors. The uPA, one of angiogenesis related factors, plays a key role in tumoral angiogenesis (2). Previous studies have demonstrated that breast cancers with HER-2/neu over-expressing possess the characteristics of rapid growth, powerful ability of angiogenesis, easy metastasis, and bad prognosis (3). And previous results from our laboratory implied that genistein effectively inhibits the angiogenesis of xenograft tumor with HER-2/neu over-expression breast cancer cells in BLAB/c nude mouse (4, 5). However, the molecular mechanism of genistein's anti-angiogenesis has not yet been fully substantiated by experimental evidence. Herein, we conducted this study to investigate whether genistein regulates the uPA expression and the activity of protein tyrosine kinase (PTK) in MDA-MB-453 cells with HER-2/neu over-expressing, to further explore its possible molecular mechanisms involving anti-angiogenesis.

This work is supported by the National Natural Science Foundation of China (No.30771794), the Key Project of Chinese Ministry of Education (No: 208123). Sichuan Provincial Youth Fund of Science and Technology Department (No.08ZQ026-051), Sichuan Provincial Emphasis Item of Education Department (No. 07ZA015), and Fund of Chengdu Medical College (No. CYZ07-001).

Corresponding author: Mamtian Mi, MD. Department of Nutrition and Food Hygiene, The Third Military Medical University, 30 Gaotanyan Street, Shapingba District, Chongqing 400038, China. Tel: +86-23-68752292, Fax: +86-23-68752292. Email: mimt2005@sina.com.

ISSN: 1538-5124/$ - see front matter © 2009 U.S. Chinese Journal of Lymphology and Oncology. All rights reserved.

Materials and methods

Cell culture and treatments

The human originated MDA-MB-453 breast cancer cells, with estrogen receptor negative-expression and HER-2/neu overexpression, were purchased from Shanghai Cell biology Institute of Chinese Academy of Sciences and cultured in Dulbecco's modified Eagle's medium (Invitrogen), supplemented with 10% fetal bovine serum (Hyclone), and 100 U/ml penicillin and l00 mg/ml streptomycin (Invitrogen) in a humidified atmosphere of 5% CO_2 at 37℃. MDA-MB-453 cells were grown in 25-cm^2 culture flask and incubated without (control) or with 50μ mol/l genistein (Sigma) pre-dissolved into dimethyl sulfoxide (DMSO) for 24, 48, and 72h, as our previous method (4, 5). And the equal volume solvent DMSO was added in control group.

The protein expression of uPA with Western blot

To analyze uPA protein expression, total protein extracts of MDA-MB-453 cells incubated with/out 50μ mol/l genistein for each experimental condition were made by homogenization in 5 volumes of ice-cold Tris-buffered saline (0.15mol/l of sodium chloride and 20mmol/l of Tris-HCl, pH7) containing 1% Triton X-100, 1mmol/l phenylmethylsulfonylfluoride and 1mg/l aprotinin, and were measured with Bradfod method (4). Extracts containing

40μ g of total protein were loaded onto 12% SDS-PAGE using a protein assay (Bio-Rad Laboratories, Hercules, CA), and the separated proteins were electrophoretically transferred to a nitrocellulose filter membrane. The membrane was blocked in Tris-buffered saline with 0.1% Tween-20/5% nonfat milk and probed with 1:400 diluted antibody (anti-uPA or GAPDH, DAKO Co.) overnight, followed by a horseradish peroxidase linked secondary antibody (1: 1,000 dilution). Specific protein bands were revealed by 3,3'-diaminobenzidine (DAB, Zhongshan Co., China) and further quantified by scanning the gray area with imaging analytical system (Bio-Rad Laboratories, Hercules, CA).

Analysis of HER-2/neu receptor phosphorylation with immuno-precipitation

20μ l extracts containing 80μ g of total protein was pretreated with 30μ l 0.1g/ml protein A-sepharose suspension (0.1g/ml Protein A-sepharose, 10mmol/l Tris·Cl (pH7.4), 0.15 mol/l NaCl, 10μ g/ml Aprotinin, 0.4% TritonX-100 (V/V)) for 1-2h, then was centrifuged and the supernatant was added with 5μ l p-Tyr antibody at 0℃ to stay overnight. Thereafter, the supernatant was added 30μ l 0.1g/ml protein A-sepharose suspension to precipitate antigen antibody complex for 1h, and the complex was rinsed with RIPA buffer (25mmol/l Tris·Cl (pH8.0), mol/l NaCl 0.15, 1mmol/l DTT, 0.5mmol/l EDTA, 1.0% NP-40 (V/V), 0.1% SDS, 1μ g/ml aprotinin, 100μ g/ml phenylmethyl sulfonylfluoride (PMSF), 2μ g/ml leupeptin, 0.1mmol/l Na3VO4, and 0.5% sodium deoxycholate) for 3 times, at last rinsed with 0.1% NP-40 in 10mmol/l Tris·HCl (pH7.4) for one time. Then, the immunoprecipitated p-Tyr protein sample product was loaded onto 12% SDS-PAGE (Bio-Rad Laboratories, Hercules, CA), and followed by above Western blot method analysis with 1:200 diluted antibody (anti-HER-2/neu, DAKO Co.).

The PTK activity of HER-2/neu receptor by kinase activity analysis technique

The PTK activity of HER-2/neu receptor was strictly performed as described by the operation of PTK activity analytical reagent provided by Promega Company. The results were shown by pmol/min/?g protein.

uPA mRNA expression analyzed with RT-PCR

uPA mRNA expression were analyzed by semi-quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR). Total RNA of MDA-MB-453 cells treated with/out 50μ mol/l genistein was extracted with Trizol reagent (Invitrogen, Carlsbad, CA). β -actin gene was used as an internal control. Specific primers for uPA and β -actin according to the literature (4), were as follows: for uPA, sense, 5'- TTC TCT GCG TCC TGG TCG TGA -3' and anti-sense, 5'- CTC CGG TTG TCT GGG TTC CTG -3', which afforded a 316bp fragment; for β -actin, sense, 5'- AGG GGC CGG ACT CGT CAT ACT -3' and anti-sense, 5'- GGC GGC ACC ACC ATG TAC CCT -3', which produce a 202bp fragment. All primers were gained from Shanghai bioengineering Corporation. The amplification system (total valume 50 μ l) was constituted of 3 μ l cDNA template, 3 μ l sense and anti-sense primer, 2 μ l MgCl2 buffer, 1 μ l dNTP, 5 μ l 10× PCR buffer, 1 μ l Taq DNA polymerase, and 32 μ l sterilizing H2O. The reaction parameter was set as 30 s at 94℃ , 50 s at 58℃ for VEGF or at 60℃ for β -actin, 1 min at 72℃ , total 40 cycles for VEGF or 35 cycles for β -actin, 10min at 72℃ after the last cycle. PCR products were visualized with electrophoresis in 2% agarose gels stained with ethidium bromide (EB), and the relative expression of VEGF to β -actin was further quantified with imaging analytical system (Bio-Rad Laboratories, Hercules, CA).

Data analysis

Results were expressed as means and standard deviation. The data of relative levels of uPA expression, the level of phosphoryla-

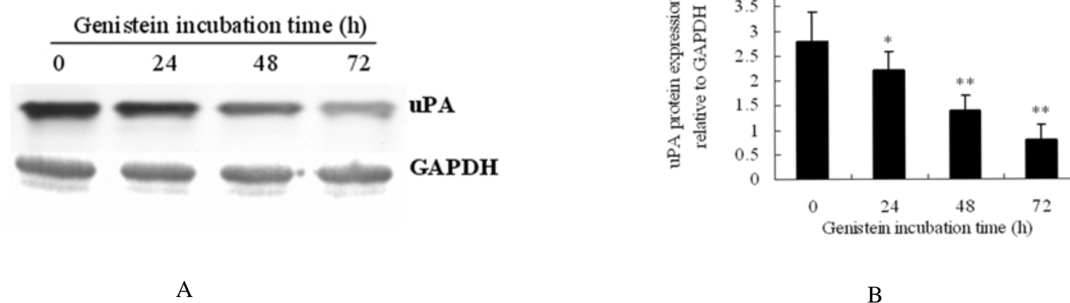

Fig.1 Genistein inhibited expression of uPA protein in MDA-MB-453 cells measured with Western blot. (A). the representative dying straps of anti- uPA antibody with Western blot; (B). the relative levels of uPA protein expression, normalized for corresponding glyceralde-3-phosphate dehydrogenase (GAPDH) levels, were obtained by gray scale. Values are means for three determinations for each time point, with standard deviations represented by vertical bars. *P<0.05, **P<0.01 Mean value was significantly differ from control represented by 50μmol/l genistein incubation 0h (Student´s t test).

tion and PTK activity of HER-2/neu receptor between control and 50μ mol/l genistein-loaded MDA-MB-453 cells was compared by single-factor analysis of variance (ANOVA) and post hoc Students's t tests. Difference was considered significant if $P < 0.05$. SPSS version 10.0 was used for statistical analysis.

Results

Genistein decreased expression of uPA protein in MDA-MB-453 cells measured with Western blot

The results from Western bolt were judged by the degree of brown strap on the nitrocellulose. That is, the deeper brown implied the corresponding protein with higher expression. As shown in Fig.1 (A), on the nitrocellulose, the brown strap of uPA in the control was deeper than that of cells incubated with 50μ mol/l genistein. Additionally, a gradual light strap was observed with the incubation time from 24 to 72h. The relative level of uPA protein expression to GAPDH was shown as Fig.1 (B). These results demonstrated that genistein inhibited uPA protein expression of MDA-MB-453 cells in a time-dependent pattern, which suggests that genistein down-regulates VEGF protein expression at translational level.

Genistein decreased the level of HER-2/neu receptor phosphorylation in MDA-MB-453 cells

As shown in Fig.2, on the nitrocellulose, the brown strap after immunoprecipitation in the control was deeper than that of 50μ mol/l genistein-loaded cells for 24, 48, and 72h, and the strap color got more light with the genistein-treated time, which indicated that the level of HER-2/neu receptor phosphorylation was gradually decreased by 50μ mol/l genistein incubation in a time-dependent pattern. Above results suggest that genistein down-regulates the level of HER-2/neu receptor phosphorylation in MDA-MB-453 cells.

Genistein decreased the PTK activity of HER-2/neu receptor in MDA-MB-453 cells

As seen in Fig.3 (n=3), the PTK activity of HER-2/neu receptor in MDA-MB-453 cells was (0.238± 0.032) pmol/min/?g. After the tested cells treated with 5× 10-5mol/l genistein for 24, 48, and 72h, the activity of HER-2/neu receptor were (0.155 ± 0.029), (0.117 ± 0.025), (0.048 ± 0.015) pmol/min/ μ g, respectively. It suggests that genistein down-regulates the PTK activity of HER-2/neu receptor in MDA-MB-453 cells ($p<0.01$), and the down-regulated effect is more powerful with genistein-loaded time.

Genistein down-regulated expression of uPA mRNA determined with RT-PCR technique

To analyze weather the alteration of uPA protein is relative to level of mRNA expression, the uPA mRNA of MDA-MB-453 cells incubated with/out genistein was determined with RT-PCR technique (Fig.4). The brightness of 2% agarose gels electrophoresis strip in 50μ mol/l genistein-loaded MDA-MB-453 cells was descendent than that of in control, and the down-regulation of uPA mRNA by genistein was gradual light with incubation time from 24 to 72h (Fig.3(A)). The relative level of uPA mRNA expression to β -actin was shown as Fig.4 (B). These results suggest that genistein also down-regulates uPA mRNA expression in MDA-MB-453 cells in time-dependent manner.

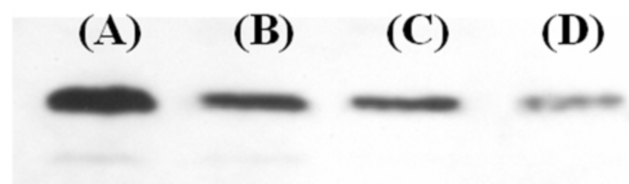

Fig.2 Genistein down regulated the level of HER-2/neu receptor phosphorylation in MDA-MB-453 cells. The levels of HER-2/neu receptor phosphorylation were detected with PTK activity analytical reagent provided by Promega Company in MDA-MB-453 cells treated with genistein for 0 (A), 24 (B), 48 (C) and 72h (D).Marker 1.

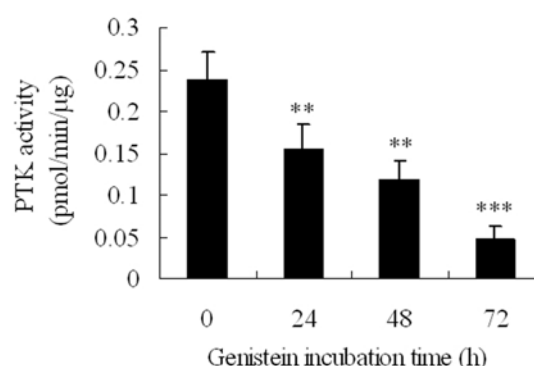

Fig.3 Genistein down-regulates the PTK activity of HER-2/neu receptor in MDA-MB-453 cells by kinase activity analysis technique. **P<0.01, **P<0.001 Mean value was significantly differ from control represented by 50μmol/l genistein incubation 0h (Student´s t test).

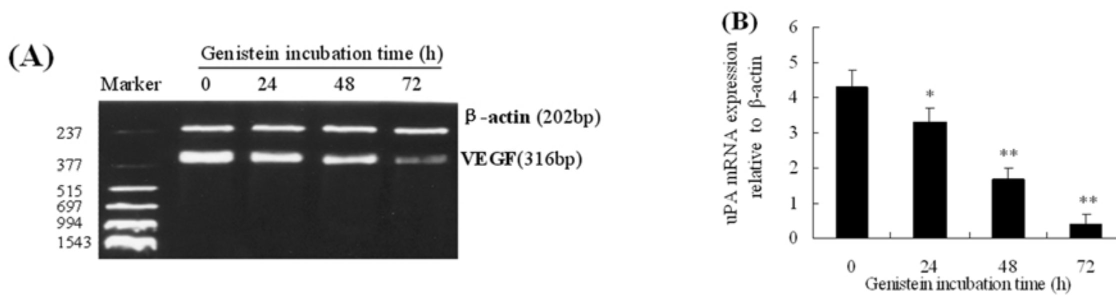

Fig.4 Genistein down-regulated expression of uPA mRNA in MDA-MB-453 cells with RT-PCR. (A). the representative amplification straps of special primer for uPA in 2% agarose gels stained with ethidium bromide (EB) with RT-PCR; (B). the relative levels of uPA mRNA expression, normalized for corresponding β-actin levels, were obtained by gray scale. Values are means for three determinations for each time point, with standard deviations represented by vertical bars. *P<0.01, **P<0.05, ***P<0.001 Mean value was significantly differ from control represented by 50μmol/l genistein incubation 0h (Student´s t test).

Discussion

The type of proteolytic enzyme affecting the ability of neovascularization is not the same manner in different tumors. Previous studies have demonstrated that the uPA expression is higher in the breast cancer with high microvessel density than that in low microvessel density, which suggests that the level of uPA expression plays a pivotal role in angiogenesis of breast cancer (2). As a serine protease secreted by the tumour cells or interstitial cells, uPA combines together with the uPA receptor (uPAR) in tumour cellular member and further be activated to become double-strands activated uPA. The activated uPA activates plasminogen to be fibrinolysin degrading extracellular matrix and basement membrane components, which is the premise condition of tumoral angiogenesis. Ryan BM et al have demonstrated that the disease free survival period of mammary cancer patient with higher uPA content in breast cancer homogenate is shorter than that with lower uPA content by ELISA method (6), afterward have displayed a similar result in total life span by multiple factor Cox model analysis, which suggests the uPA expression is negative to the disease free survival period and life span of mammary cancer patient, and is relative to the prognosis.

The results from many investigations have suggested that HER-2/neu expression is relative to the uPA expression. The protein coded by HER-2/neu gene is a membrane receptor containing transmembrane PTK, and belongs to the members of epidermal growth factor receptor (EGFR), therefore has the intrinsic PTK activity. HER-2/neu over-expression activates PTK activity and switches the signal transduction pathway mediated by PTK (7). It has been demonstrated that EGF can activate proto-oncogene ets-1 and ets-2, and up-regulates uPA expression in SK-BR-3 breast cancer cell with HER-2/neu over-expression (8). The higher HER-2/neu expression is concomitance with higher uPA expression in gastric carcinoma from clinical research (9). The positive correlation between HER-2/neu and uPA expression is independence with the therapeutic measure in nonsmall-cell lung cancer (NSCLC) (10). Heregulin, as an antagonist of HER-2/neu receptor, can down-regulates uPA expression via ras, PI3K, extracellular signal-regulated kinase 1/2 (ERK1/2) and signal transductor and transcription activator (STAT) pathway (11). Above researches have suggested that there is a close positive correlation between HER-2/neu and uPA expression.

Genistein, an un-trophic component mainly distributed in soy bean, has effective anti-tumoral role (1,4,5). It has been demonstrated that uPA expression in breast cancer depends on the PTK signal pathway, and the inhibition of PTK activity decreases the uPA expression and secretion (12). Genistein down-regulates the expression of uPAR mRNA and protein in ovarian cancer (13), which is further confirmed by cDNA micro-array, RT-PCR and Western blot techniques in prostatic carcinoma PC-3 cells (14). The erbB-2/neu gene regulates uPA expression via indirect style, which has been demonstrated by transfection technique and clinic mammary cancer samples (15). In the present experiment, we found that there is an obvious decrease of the tyrosine phosphorylation and the PTK activity of HER-2/neu receptor, and a significant down-regulation of uPA mRNA and protein expression in breast cancer cells with estrogen receptor negative (ER-) by 5× 10-5mol/l genistein, which suggests genistein decreases uPA expression of breast cancer cells with HER-2/neu over-expression through down-regulating the level of tyrosine phosphorylation and PTK activity of HER-2/neu receptor. Together with our previous results (4,5), it can be conducted that genistein can indirectly decreases uPA expression at transcriptional and translational level by blocking downstream signal pathway mediated by PTK activity of HER-2/neu receptor.

References

1. Banerjee S, Li Y, Wang Z, Sarkar FH. Multi-targeted therapy of cancer by genistein. Cancer Lett 2008; 269: 226-42.
2. Annecke K, Schmitt M, Euler U, Zerm M, Paepke D, Paepke S, et al. uPA and PAI-1 in breast cancer: review of their clinical utility and current validation in the prospective NNBC-3 trial. Adv Clin Chem 2008; 45:31-45.

3. Jerusalem G, Rorive A, Gennigens C, Sautois B, Mievis C, Fillet G. Targeted therapies in breast cancer: current status and perspectives. Rev Med Liege 2007; 62: 2-5.
4. Yu X, Mi M, Zhu J. Effect of genistein on angiogenesis in breast cancer xenograft tumor. China Public Health 2003; 19: 168-70.
5. Yu X, Mi M, Zhu J. Genistein Inhibits the Expression of Vascular Endothelial Growth Factor in MDA-MB-453 Breast Cancer Cells. U.S. Chinese Journal of Lymphology and Oncology 2008; 7: 8-13.
6. Ryan BM, Konecny GE, Kahlert S, Wang HJ, Untch M, Meng G, et al. Survivin expression in breast cancer predicts clinical outcome and is associated with HER2, VEGF, urokinase plasminogen activator and PAI-1. Ann Oncol 2006; 17: 597-604.
7. Urban P, Vuaroqueaux V, Labuhn M, Delorenzi M, Wirapati P, Wight E, et al. Increased expression of urokinase-type plasminogen activator mRNA determines adverse prognosis in ErbB2-positive primary breast cancer. J Clin Oncol 2006; 24: 4245-53.
8. Watabe T, Yoshida K, Shindoh M, Kaya M, Fujikawa K, Sato H, et al. The Ets-1 and Ets-2 transcription factors activate the promoters for invasion-associated urokinase and collagenase genes in response to epidermal growth factor. Int J Cancer 1998; 77: 128-37.
9. Lagarde SM, ten Kate FJ, Richel DJ, Offerhaus GJ, van Lanschot JJ. Lecular prognostic factors in adenocarcinoma of the esophagus and gastroesophageal junction. Ann Surg Oncol 2007; 14: 977-91.
10. Kasimir-Bauer S, Schleucher N, Weber R, Neumann R, Seeber S. Evaluation of different markers in non-small cell lung cancer: prognostic value of clinical staging, tumour cell detection and tumour marker analysis for tumour progression and overall survival. Oncol Rep 2003; 10: 475-82.
11. Natarajan J, Berrar D, Dubitzky W, Hack C, Zhang Y, DeSesa C, et al. Text mining of full-text journal articles combined with gene expression analysis reveals a relationship between sphingosine-1-phosphate and invasiveness of a glioblastoma cell line. BMC Bioinformatics 2006; 7:373.
12. Shida D, Fang X, Kordula T, Takabe K, Lé pine S, Alvarez SE, et al. Cross-talk between LPA1 and epidermal growth factor receptors mediates up-regulation of sphingosine kinase 1 to promote gastric cancer cell motility and invasion. Cancer Res 2008; 68: 6569-77.
13. Kobayashi H, Suzuki M, Kanayama N, Terao T. A soybean Kunitz trypsin inhibitor suppresses ovarian cancer cell invasion by blocking urokinase upregulation. Clin Exp Metastasis 2004; 21: 159-66.
14. Skogseth H, Follestad T, Larsson E, Halgunset J. Transcription levels of invasion-related genes in prostate cancer cells are modified by inhibitors of tyrosine kinase. APMIS 2006; 114: 364-71.
15. Liu B, Edgerton S, Yang X, Kim A, Ordonez-Ercan D, Mason T, et al. Low-dose dietary phytoestrogen abrogates tamoxifen-associated mammary tumor prevention. Cancer Res 2005; 65: 879-86.