A systematic study of Girdin on cell proliferation, migration and angiogenesis in different breast cancer subtypes

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Abstract. Breast cancer has one of the highest incidences in females worldwide. Girdin is a novel actin-binding protein, that induces cell migration and angiogenesis. However, a systematic study of Girdin function in distinct subtypes of breast cancer has not been reported to date. Therefore, the present study aimed to investigate the role of Girdin on cell proliferation, migration and angiogenesis in different subtypes of breast cancer. For this purpose, the breast epithelial MCF-7, breast ductal T47D and breast metastatic MDA-MB-231 cancer cell lines were selected. Girdin small interfering RNA (siRNA) was transfected into MCF-7, T47D and MDA-MB-231 cells. Girdin knockdown suppressed cell viability and migration in the different cancer cells tested. Girdin knockdown also suppressed mRNA expression of vascular endothelial growth factor (VEGF) and activation of phosphatidyl inositol 3-kinase (PI3K) and RAC-α serine/threonine-protein kinase (Akt) in the subtypes tested. In conclusion, these data indicate that Girdin knockdown suppressed cell viability and migration and may suppress angiogenesis via the PI3K/Akt signaling pathway, in various breast cancers subtypes. The present study therefore suggests a role for Girdin as a novel therapeutic target for breast cancer, independent of subtype.

Introduction

Breast cancer has one of the highest incidences in females worldwide, and it is the primary cause of mortality in female patients with cancer (1). At present, various breast cancer treatment methods have limitations, for example; surgery does not preclude hematopoietic or lymphatic dissemination leading to distant metastasis (2). A major advancement in the treatment of breast cancer has been targeted therapy (3). Therefore, research focusing on the molecular mechanisms of the development and metastasis of breast cancer is required.

Girdin is a novel actin-binding protein [a structural schematic which was first published by Jiang et al (4) in 2008, is depicted in Fig. 1], that induces cell migration and angiogenesis (5). Cell migration is a physiological activity of cells; however, is also involved in the pathological processes of cancer invasion and metastasis (6). A previous study has demonstrated that Girdin promotes DNA synthesis in tumor cells and inhibits their apoptosis (5). Girdin has also been demonstrated to induce migration and invasion of endothelial cells and thus promote angiogenesis (7). However, a systematic study of the role of Girdin in distinct subtypes of breast cancer has not been reported to date. Therefore, three cell lines, representing different subtypes of breast cancer, were selected for use in the present study: The epithelial MCF-7; the ductal T47D; and the metastatic MDA-MB-231 breast cancer cell lines. The present study aimed to investigate the role of Girdin on cell proliferation, migration and angiogenesis in the various subtypes of breast cancer cells, and potentially provide insights on novel therapeutic targets for breast cancer.

Materials and methods

Cell culture. MCF-7, T47D and MDA-MB-231 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). The present study was performed in accordance with the Experimental Guidelines of Harbin Medical University (Harbin, China) and ethical approval was obtained from Harbin Medical University. MCF-7 cells were cultured in RPMI 1640 medium (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich; Merck KGaA). T47D and MDA-MB-231 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Sigma-Aldrich; Merck KGaA) supplemented with 10% FBS. All cells were cultured with 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in an incubator with 5% CO₂ at 37°C.

Small interfering RNA (siRNA) transfection. Girdin siRNA (sc-94984) and non-targeting negative control siRNA (SIC002) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA) and GenePharma Co., Ltd. (Shanghai, China) respectively. Transfection was performed as described

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previously (8). Cells were seeded into 6-well plates at a density of 1x10^4 cells/well and grown to 60-80% confluency prior to transfection. siRNAs were transfected into cells using siRNA Transfection Reagent (Santa Cruz Biotechnology, Inc.), according to the manufacturer's protocol. Cells were incubated for a further 48 h following transfection and subsequently used for experiments.

Reverse transcription-semi-quantitative polymerase chain reaction (RT-sqPCR). Total RNA was extracted by TRIzol (Sigma-Aldrich; Merck KGaA) and relative mRNA was normalized to 18S ribosomal RNA. The following primers (Hokkaido System Science Co. Ltd, Sapporo, Japan) were used: Girdin, forward 5'-CCAGGCACTGAAGCGAACA-3' and reverse 5'-CGAGCATCCGGAAGCAAAT-3'; vascular endothelial growth factor (VEGF), forward 5'-TTGCTCTTGTGCTCTTACA-3' and reverse 5'-AAATGCTTCTCCGCTCTGTA-3'; and 4% paraformaldehyde in PBS for 10 min at room temperature and 18S, forward 5'-CCAACCCGTGAGACCCATT-3' and reverse 5'-CCATCCATAAGGTAAGGC-3'. Reverse transcription was performed using a Transcriptor First Strand cDNA Synthesis kit (Roche Applied Science, Madison, WI, USA). A total of 200 ng RNA was used as input for the RT reaction, and 2 µl input cDNA from the RT product was used for the sqPCR. PCR was performed using SYBR Premix Ex Taq II (Takara Bio, Inc., Otsu, Japan) and the ABI 7300 Fast real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were: Holding stage 95˚C for 30 sec (1 cycle) and cycling stage 95˚C for 3 sec and 60˚C for 31 sec (40 cycles). Ethidium bromide (Sigma-Aldrich; Merck KGaA) and relative mRNA was normalized to 18S ribosomal RNA. The following primers were used for experiments.

MTT Assay. Cell viability was determined by a colorimetric MTT assay according to the method described previously (9). Absorbance at 550 nm was measured using a MTP-800 microplate reader (Corona Electric, Ibaraki, Japan). Absorbance at 690 nm was also measured as a control, to compensate for interfering absorbance from potential cell debris or the microtiter plate. The % of viable cells was calculated as follows: Optical density (OD) of treated sample/OD of untreated control x100.

Migration assay. The migration assay was performed using 48-well migration transwell chambers with polycarbonate membranes (Sigma-Aldrich; Merck KGaA), according to the method described previously (10). To prepare the migration chambers, the upper wells were coated with 0.01% collagen and incubated for 30 min at 37˚C. Then, the cells (5x10^5 cells/well) were seeded on the upper chamber of the transwells in RPMI 1640 medium (for MCF-7 cells) or DMEM (for T47D and MDA-MB-231 cells). DMEM with 10% fetal calf serum was added to the lower wells of the chambers as a chemoattractant. Following incubation at 37˚C for 24 h, the cells that had migrated to the lower surface of the filters were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature and stained with crystal violet. Cell migration was defined as the number of cells that had migrated to the lower filter surface. Four non-overlapping fields per filter were selected and the migrated cells were counted under a microscope (Olympus Corporation, Tokyo, Japan) at a magnification of x100. The average number of the cells from four fields was presented as the results of the migration assay.

Western blot analysis. MCF-7, T47D and MDA-MB-231 cells were lysed by lysis buffer (1 M Tris-HCl, pH 7.4; 1 M NaCl; 20% Triton X-100; 10% SDS; and 0.5 M EDTA; Sigma-Aldrich Merck KGaA). The protein concentration was determined using the Bio-Rad Protein Assay. Electrophoresis was performed using a vertical slab 12% SDS-PAGE, as described previously (11). A total of 20 µg of protein was loaded per gel lane. Protein transfer to a membrane (Immobilon™-P, Merck KGaA) was performed electrophotorectically, as described previously (12) with certain modifications, using a Semi Dry Electroblotter (Sartorius AG, Goettingen, Germany) for 90 min, with an electric current of 15 V. The membrane was treated with Block Ace™ (4%; Bio-Rad, Hercules, CA, USA) for 30 min at 22˚C. Primary antibody incubations were performed overnight at 4˚C. Rabbit polyclonal antibody against phosphatidylinositol 3-kinase (PI3K; SAB5500162; 1:100; Sigma-Aldrich; Merck KGaA) and RAC-α serine/threonine-protein kinase (Akt; SAB4500797; 1:1,000; Sigma-Aldrich; Merck KGaA) in PBS containing 0.03% Tween-20 (PBST) for 1 h at 22˚C. Following washing in PBST, the secondary antibody incubation was performed using horseradish peroxidase-conjugated anti-rabbit goat IgG (A0545; 20 ng/ml; Sigma-Aldrich; Merck KGaA) for 30 min at 22˚C. Following washing in PBST, the enhanced chemiluminescence (ECL) reaction was performed on the membranes using the ECL Plus Western Blotting Detection System (GE Healthcare Life Sciences, Shanghai, China). ImageJ (version 1.38; National Institutes of Health) was used for the quantification of western blots.

Statistical analysis. The paired Student's t-test was used to analyze the data. Analyses were conducted using SPSS software (version 17.0; SPSS, Inc., Chicago, IL, USA) and data were expressed as the mean ± standard deviation. Each experiment was repeated at least three times. P<0.05 was considered to indicate a statistically significant difference.

Results

Girdin knockdown decreases viability in breast cancer cells. To investigate the effect of Girdin knockdown on the viability of breast cancer cells, MCF-7, T47D and MDA-MB-231 cells were seeded onto 6-well plates, grown to 60-80% confluency, and then transfected with either a Girdin-specific siRNA or a non-specific negative control siRNA. Following 48 h, the efficiency of Girdin siRNA knockdown was evaluated using RT-sqPCR (Fig. 2). The results demonstrated that Girdin was efficiently silenced in all three cell lines tested, compared with the control siRNA-transfected cells (Fig. 2). Girdin knockdown increases viability following Girdin knockdown compared with control siRNA-transfected cells (P<0.01; Fig. 3).

Girdin knockdown suppresses migration in breast cancer cells. The migration abilities of MCF-7, T47D and MDA-MB-231
were examined following Girdin knockdown using a transwell chamber migration assay. Girdin deficient MCF-7, T47D and MDA-MB-231 cells exhibited significantly suppressed migration compared with the control siRNA-transfected cells (P<0.01; Fig. 4).

Girdin knockdown suppresses VEGF expression in breast cancer cells. Breast cancer cells frequently express VEGF, an endothelial growth and chemotactic agent that promotes angiogenesis, and subsequently cancer metastasis. The effect of Girdin knockdown was therefore investigated on VEGF expression in breast cancer cells lines. Girdin deficient MCF-7, T47D and MDA-MB-231 cells exhibited significantly decreased VEGF mRNA expression levels, compared with control siRNA-transfected cells (P<0.01; Fig. 5).

Effect of Girdin knockdown on PI3K and Akt expression in breast cancer cells. The protein expression levels of PI3K and Akt in Girdin deficient MCF-7, T47D and MDA-MB-231 cells were measured by western blot analysis. Expression of PI3K and Akt proteins was significantly downregulated in MCF-7, T47D and MDA-MB-231 cells following Girdin knockdown, compared with the control siRNA-transfected cells. (P<0.01; Fig. 6).

Discussion

To the best of our knowledge, the present study demonstrated for the first time the effect of Girdin silencing on different subtypes of breast cancer. Breast cancer has one of the highest incidences in females worldwide, and it is the primary cause of mortality in female patients with cancer (1). Breast cancer has long been a leading cause of mortality in women of both developed and developing countries (13). Breast cancer is a heterogeneous disease (14). There are multiple methods of breast cancer classification. Based on tissue typing, breast cancers are
Girdin, first discovered by Japanese scholars in 2005 (5), is a novel actin binding protein that induces cell migration and angiogenesis (16). Girdin maintains the structure of actin (17), and it induces invasion and metastasis of tumor cells and angiogenesis (4,7). The present study is consistent with these previous reports, as it demonstrated that Girdin deficiency suppressed viability and migration of different subtypes of breast cancer cells.

Girdin may be important in angiogenesis of breast tumors as well.

PI3K phosphorylates phosphatidylinositol lipids in response to various growth factors (20). The PI3K/Akt signaling pathway is important in modulating cell growth, cell survival and cytoskeletal rearrangement (21). Since cancer cell proliferation and migration is regulated by the PI3K/Akt signaling pathway (22,23), the present study aimed to further investigate the association between Girdin and PI3K/Akt. PI3K and Akt protein expression levels were significantly decreased in Girdin deficient MCF-7, T47D and MDA-MB-231 cells compared with the control siRNA-transfected cells. The present data demonstrated that Girdin knockdown suppressed viability, migration and angiogenesis in distinct subtypes of breast cancer, potentially via the PI3K/Akt signaling pathway.

According to the model described in Fig. 7, Girdin knockdown suppressed cell viability, migration and angiogenesis in different subtypes of breast cancers, including breast epithelial, breast ductal and metastatic breast cancer, by downregulating expression of PI3K and Akt. A previous study has indicated...
that Girdin is important in combining with G protein (24). Although the present study provides evidence that Girdin may be important in regulating cancer cell viability, migration and angiogenesis, the underlying mechanism remains unclear and requires further investigation in the future. In conclusion, the present study suggested that Girdin may serve as a potential novel target for the development of novel clinical treatments for breast cancer.

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References


