



Biphasic expression of RhoGDI2 in the progression of breast cancer and its negative relation with lymph node metastasis

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Abstract. *RhoGDI2* has been shown to be a metastasis-related gene in several cancers. In human breast cancer, little clinical study of *RhoGDI2* has been reported. In this study, we investigated the expression level of *RhoGDI2* by immunohistochemistry, as well as the correlation of *RhoGDI2* with clinicopathological parameters in 71 breast cancer specimens. We also examined *RhoGDI2* expression at mRNA and protein levels of four human breast cancer cell lines differing in *in vivo* metastasis. Along with the extent of mammary epithelia proliferation and carcinogenesis, a biphasic pattern of *RhoGDI2* expression (increase and then decrease) was observed, which was also found in these examined cells. Furthermore, univariate and multivariate analysis revealed that reduced expression of *RhoGDI2* in the most malignant epithelia was significantly associated with lymph node metastasis ($P < 0.01$). Our results suggest that *RhoGDI2* may be implicated in the progress of malignancy and act as a metastasis-related marker in breast cancer.

Introduction

Breast cancer remains the most commonly diagnosed cancer for women in the world, and the second largest cause of their cancer deaths (1). The ability to detect and cure localized breast cancer has been improved appreciably in recent years. However, if metastases develop, the prognosis of survival is vague. While the primary tumor of breast cancer is still small and difficult to detect, cancer cells may have already spread to distant sites. Therefore, clinical markers are necessary to distinguish histological lesions and disseminated cells with a high probability to cause metastasis from those remaining indolent (2,3).

Cancer spreading mainly depends on cell migration ability (4). Rho GTPases are well-known regulators of cell migration by affecting cytoskeleton organization and signal transductions of different stimuli (5-7). Several members of Rho GTPases have been found to associate with malignant transformation and metastasis in recent years (8,9). Rho GTPases work as molecular switches by cycling between a GTP-bound 'on' state and GDP-bound 'off' state. Several regulators tightly regulate the cycling between these two states. One of these regulators is GDP dissociation inhibitors (RhoGDIs), which include RhoGDI, RhoGDI2 and RhoGDI3. RhoGDIs bind to Rho GTPases and keep the Rho proteins in their GDP-bound inactive state incapable of interacting with effector targets or other regulatory proteins (10,11). Since they function to inhibit the activity of Rho GTPases and subsequently cell motility, RhoGDIs may also regulate the malignancy of mammary cells. Overexpression of RhoGDI has been shown to increase the estrogen receptor transcriptional activation (12), which plays a critical role in the development and progression of breast cancer. Reduced expression of RhoGDI and RhoGDI3 in breast tumor tissues correlated with nodal metastasis (13). Recent study by Zhang and Zhang showed that knockdown of RhoGDI2 in breast cancer cells blocked cell motility and invasion (14). All these data suggest that RhoGDI2 is involved in breast cancer. However, there has been little clinical study of their relationship.

In the present study we analyzed the expression pattern of RhoGDI2 in 71 cases of breast cancer by immunohistochemistry and investigated the correlation of RhoGDI2 expression with clinicopathological parameters. In addition, we evaluated the *RhoGDI2* expression at mRNA and protein levels in four breast cancer cell lines that differ in *in vivo* metastatic potential. Our results indicated that the expression of RhoGDI2 might have an important effect on the progression and metastasis in human breast cancer.

Materials and methods

Patients and tumor specimens. Seventy-one patients diagnosed as having breast cancer from June 2005 to June 2006 at Shanghai Xinhua Hospital were enrolled in this study. Each patient was treated by lumpectomy, with or without axillary dissection. Those who had undergone radio- or chemotherapy prior to surgery were excluded. Cancer specimens for immunohistochemistry were taken from the representative cancerous

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Table I. Correlation of the expression of RhoGDI2 protein with patient demographics and molecular marker data in breast cancers.

Feature	Number of patients n=71 (%)	RhoGDI2 (n=71)		P value ^a
		Negative (n=39)	Positive (n=32)	
Age at surgery (years)				NS ^b
<50	24 (33.8)	14	10	
≥50	47 (66.2)	25	22	
Histological type				NS
Ductal	59 (83.1)	34	25	
Lobular/other	12 (16.9)	5	7	
Stage of disease				0.017 ^c
I	24 (33.8)	9	15	
II	34 (47.9)	20	14	
III and IV	13 (18.3)	10	3	
Tumor maximal diameter (cm)				NS
≤2	36 (50.7)	18	18	
>2 and ≤5	32 (45.1)	19	13	
>5	3 (4.2)	2	1	
Lymph node status				0.009
Negative	46 (64.8)	20	26	0.008 ^c
Positive	25 (35.2)	19	6	
ER status				NS
Negative	11 (15.5)	8	3	
Positive	60 (84.5)	31	29	
PR status				NS
Negative	18 (25.4)	13	5	
Positive	53 (74.6)	26	27	
c-erbB-2 status				NS
Negative	11 (15.5)	8	3	
Positive	60 (84.5)	31	29	

^aP was computed using the χ^2 test. ^bNS, not significant. ^cSignificant on general liner model analysis.

lesions including adjacent non-cancerous tissue that was used for intraspecimen comparison of RhoGDI2 expression. Routine fixation in formalin and paraffin was performed for histological assessment. Clinical data obtained by retrospective review of the medical records are shown in Table I. The median age of patients at time of surgery was 55.8 (range, 24 to 90) years. All tumors were staged according to the AJCC staging system (15). All studies conformed to the tenets of the Declaration of Helsinki.

Immunohistochemistry. The expression of RhoGDI2 was detected on paraffin sections using the ABC method. Briefly, deparaffinized sections were boiled in 1 mM TE buffer (pH 8.0) for 2 min in an autoclave for antigen demasking. After incubation with 3% hydrogen peroxide and 15% goat serum (Vector

Laboratories) for 60 min respectively, endogenous avidin and biotin were blocked using an Avidin/Biotin blocking kit (Vector Laboratories). Then slides were incubated with mouse monoclonal antibody against RhoGDI2 (2 ug/ml; clone 97A1015, Upstate) overnight. This was followed by incubation with a biotinylated goat anti-mouse secondary antibody (3 ug/ml; Vector Laboratories) for 30 min and the ABC Elite complex (Vectastain® Elite ABC Kit Standard, Vector Laboratories) for 30 min. Staining was visualized by using the DAB method (Vector Laboratories) for 5 min. Counterstaining was performed lightly with Harris hematoxylin. All incubations were performed at room temperature in a humidified chamber.

For each specimen, cells of connective tissue (except endothelial cells) were used as internal negative control and epithelia of duct and lobule were taken as positive control.

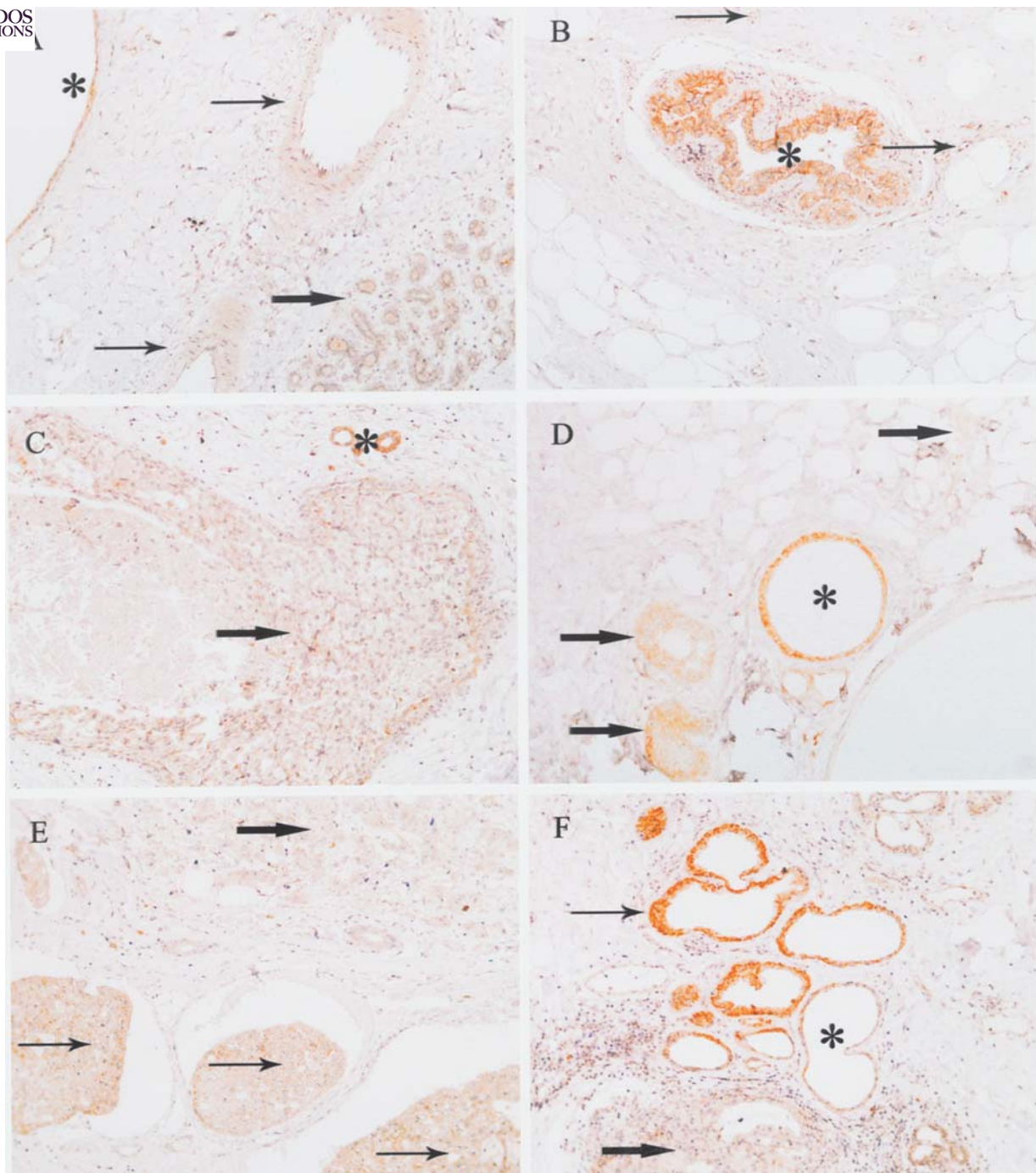


Figure 1. Immunohistochemical staining in representative breast tissues with RhoGDI2 monoclonal antibody (x200 magnification). (A) RhoGDI2 immunostaining in the ductal (star), lobular (dark arrow) epithelia and endothelial cells of artery (light arrow). (B) A hyperplastic duct showing strong staining (star). Note that moderate immunostaining was detected in the endothelial cells of capillary vessels (light arrow). (C) Section of ductal carcinoma *in situ* immunostained for RhoGDI2. The staining intensity of *in situ* cancer cells (dark arrow) was weaker than that of normal ductal epithelia (star). (D) The staining of invasive cancer cells (dark arrow) was weaker than that of the normal ductal epithelia (star). The epithelial cells of the duct in the lower part were shed. (E) Cells *in situ* (in the ducts, light arrow) were homogeneously immunoreactive and the invasive cancer cells (dark arrow) showed reduced expression. (F) A section containing multiple components was immunostained for RhoGDI2. The intraductal hyperplasia (light arrow) showed strongest staining, while the normal ductal epithelia (star) and the infiltrating cancer cells (dark arrow) presented weak and identical staining.

The qualitative analysis of RhoGDI2 status was performed by comparing the staining intensity between the most malignant and the normal epithelial cells in the same section. The case was estimated to be positive (defined as stronger than, or as strong as, normal adjacent epithelial cells of duct and lobule in the same section) (Fig. 1F), and negative (defined as weaker than normal adjacent epithelial cells of duct and lobule in the

same section) (Fig. 1C and D) (16). In detail, the RhoGDI2 status was assessed by comparing the staining intensity between the invasive and normal cells in invasive carcinoma (Fig. 1D and F). Concerning *in situ* carcinoma, the staining intensities of *in situ* and normal tissue were compared (Fig. 1C). The examination was carried out in a blinded fashion by two professional investigators, without knowledge of the patient

Table II. Correlation of RhoGDI2 expression with the number of involved axillary lymph nodes.

Number of involved lymph nodes	RhoGDI2 (n=71)		P value ^a
	Negative	Positive	
0	20	26	0.019
1-3	7	3	
4-9	4	3	
≥10	8	0	

^aP was computed using χ^2 test.

information. Occasional disagreements were discussed to reach a consensus. In the case of persistent differences, the sections were studied by a third independent observer and the majority decision was thus considered.

Cell lines and cell culture. The human breast cancer cell lines examined in this study were MCF7, MDA-MB-231, MDA-MB-468 and MDA-MB-435. They were kindly provided by Shanghai Institute of Materia Medica, Chinese Academy of Sciences. Cells were grown in DMEM medium (Gibco™) supplemented with 10% (v/v) fetal bovine serum (BioChrom AG) and antibiotics (100 units/ml penicillin and 100 ug/ml streptomycin; Cambrex BioScience, Walkersville). Cells were maintained in an atmosphere of 5% CO₂ at 37°C. For expression analysis, cells between 70 and 90% confluence were used, with viability >95% examined by Trypan blue staining. All cells tested negative for Mycoplasma contamination.

RNA extraction and semi-quantitative RT-PCR. Total RNA was extracted using Trizol® reagent (Invitrogen™ life technologies) according to the manufacturer's instructions. After complete treatment of RNA with RNase-Free DNase (Promega) for 45 min at 37°C, a cDNA library was generated using M-MLV reverse transcriptase (Promega) and oligo (dT) primers. PCR primers for *RhoGDI2* were designed as forward: 5' ACAGA AGTCCCTGAAAGAGC 3' and reverse: 5' AATGTGGCAG TGTGAAGAG 3'. In parallel, the β -actin gene was amplified to serve as an internal control using a set of primers 5' AGCG AGCATCCCCAAAGTT 3' and 5' GGGCACGAAGGCTC ATCATT 3'. RT-PCR products were analyzed in 1% agarose gel.

Western blotting. Protein extracts were prepared from cultured cells in 100-mm dishes. All extraction procedures were performed on ice with ice-cold reagents. After being washed thrice with PBS, cells were solubilized in lysis buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, and 1 mM EDTA) containing protease inhibitor cocktail (1:25; complete protease inhibitor cocktail tablets in glass vials, Roche). Protein concentrations were estimated by the Bradford method. Equal amounts of proteins were separated by 12% SDS-PAGE, transferred to PVDF membranes (Millipore) and probed with monoclonal antibody against RhoGDI2 (97A1015 at 1 ug/ml), followed by incubating with a peroxidase-conjugated goat

anti-mouse antibody (1 ug/ml in TBST; Rockland). Enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech Inc) was used for signal detection. Loading of protein samples was assessed by re-blotting with monoclonal anti- β -actin antibody (Santa Cruz).

Statistical analysis. Correlations of RhoGDI2 expression with categorical variables were examined by χ^2 analysis. To simultaneously examine the effect of more than one factor on RhoGDI2 protein expression, multivariate analysis (general liner model) was performed. Statistical analysis was performed with SPSS 12.0 software for Windows. P<0.05 was considered to be statistically significant. All tests were two-sided.

Results

Expression of RhoGDI2 in breast cancer tissues. As shown in Fig. 1A and B, RhoGDI2 immunoreactions were observed in benign and carcinomatous breast epithelial and myoepithelial cells. In addition, endothelial cells of vasculature also appeared immunopositive. RhoGDI2 expression was negative in all cases of fibroblasts, nerve fibers and adipocytes, which served as internal negative controls. Similar to the report by Theodorescu *et al* (16), RhoGDI2 was predominantly distributed in the cytoplasm, while it was also occasionally seen in the nucleus. According to the multi-step model of carcinogenesis in the breast, there is a transition from normal epithelium to invasive carcinoma step by step via non-atypical and atypical hyperplasia and *in situ* carcinoma (17,18). Sections of breast cancer often include multi-morphological areas: normal epithelia, hyperplasia, *in situ* and invasive lesions. In order to determine the RhoGDI2 expression level during breast cancer progression, we compared the immunostaining intensity among these areas. Our results indicated that the intensity of RhoGDI2 expression was almost homogeneous in each lesion. Furthermore, the RhoGDI2 expression pattern changed along with the extent of cellular proliferation and carcinogenesis (normal → hyperplastic → *in situ* → invasive) (Figs. 1C-F). In detail, RhoGDI2 expression levels in hyperplastic lesions mostly increased compared to normal epithelia (22 breast cancer specimens out of 23 totals with both components) (Fig. 1B and F). Only 50% of specimens showed an increased expression level in *in situ* lesions compared to hyperplastic lesions (5 breast cancer specimens out of 10 totals again with both components). As histological entity progressed from *in situ* to invasive (Fig. 1E), this ratio reduced to 18.2% (6/33). The increased frequency of each phase in 10 cases with co-existing normal, hyperplasia, *in situ*, and invasive components was 100%, 50% and 30%, successively. These data showed a biphasic (increase and then decrease) pattern of RhoGDI2 expression throughout the breast cancer progression.

Association of RhoGDI2 expression status with clinico-pathological parameters. RhoGDI2 expression was observed in 71 breast cancer samples by immunohistochemistry. The correlation of RhoGDI2 expression levels with the clinico-pathological features and other molecular marker data of the patients are shown in Table I. By comparing the staining intensity between the most malignant and the normal epithelia in the same section, RhoGDI2 expression was down-regulated

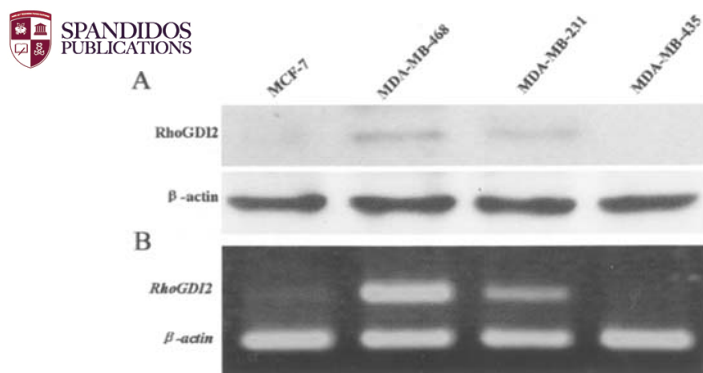


Figure 2. Expression levels of RhoGDI2 in four human breast cancer cell lines. (A) Western blot analysis of RhoGDI2 expression among four cultured cell lines using anti-RhoGDI2 specific antibody. Loading of protein samples was assessed by reprobing the blots with monoclonal anti- β -actin antibody. MCF7, MDA-MB-468, MDA-MB-231, and MDA-MB-435 reflect a stepwise increase in invasion and metastasis. (B) RT-PCR analysis of *RhoGDI2* expression in the cell lines as described in (A). β -actin was amplified as an internal control. Each assay was repeated three times in independent experiments.

and considered negative in 39/71 (54.9%) tumor samples. Decrease of RhoGDI2 expression significantly correlated with lymph node metastasis ($P=0.009$). Of 32 patients whose tumors were rated positive for RhoGDI2 expression, 26 (81.25%) were node-negative, node metastasis was observed in 19/39 (48.72%) cases considered negative. Concerning the number of involved axillary lymph nodes, patients were classified into four groups: none, 1-3, 4-10, 9 and ≥ 10 involved nodes (15). RhoGDI2 expression negatively correlated with the number of involved axillary lymph nodes ($P=0.019$; Table II). However, there was no statistical significance between RhoGDI2 expression and age ($P=0.68$), histological type ($P=0.311$), clinical stage ($P=0.058$), tumor size ($P=0.679$) or ER ($P=0.197$), PR ($P=0.088$) and c-erbB-2 ($P=0.197$).

In general liner model analysis, lymph node involvement and clinical stages showed a significant negative correlation with the RhoGDI2 expression ($P=0.008$ and $P=0.017$, respectively), whereas none of the remaining tumor characteristics was significantly related to the RhoGDI2 status.

The qualitative analysis of RhoGDI2 status among normal epithelium, hyperplasia, *in situ* and invasive lesions was made by comparison with each other and the results were correlated with the clinical parameters, but no relationship was found (data not shown).

RhoGDI2 expression in human breast cancer cell lines. In order to evaluate the importance of RhoGDI2 expression in the progression of human breast cancer metastasis, we compared the RhoGDI2 expression levels in four established breast cancer cell lines that differ in *in vivo* metastasis. MCF7, MDA-MB-468, MDA-MB-231 and MDA-MB-435 reflected a stepwise increase in malignant biological behavior (i.e. invasion and metastasis) on the basis of xenografted studies ($MCF7 < MDA-MB-468 < MDA-MB-231 < MDA-MB-435$) (19,20).

Western blot analysis was performed by using a specific monoclonal antibody (97A1015) against RhoGDI2. The molecular weight of RhoGDI2 protein detected in these cells was approximately 28 kDa (Fig. 2A). In repeated experiments,

MDA-MB-435 cells, which are highly metastatic (21,22), barely expressed RhoGDI2. MDA-MB-231 cells, which were reported to be highly invasive but modestly metastatic in athymic nude mice (21,22), had a comparatively high expression level of RhoGDI2. A nonmetastatic breast cancer cell line (21), MDA-MB-468, also showed abundant RhoGDI2 expression. However, RhoGDI2 was undetectable in MCF7 cells, which have been found to be very low invasive and essentially non-metastatic (19). The measurements of RhoGDI2 expression at RNA level by semi-quantitative RT-PCR were consistent with the Western blotting results (Fig. 2B). Thus, similar to our findings in breast cancer tissues, RhoGDI2 expression showed biphasic pattern in the examined cell lines that differ in *in vivo* metastasis.

Discussion

The involvement of Rho molecules and their regulators in human cancer has been explored in recent years. Over-expression or reduced expression of either Rho GTPase itself or its signaling has been detected in many human tumors including breast cancer, prostate cancer, and ovarian cancer (8,23). RhoGDIs, which include three members in mammals, are known to inhibit the activation of Rho GTPases (10). Increasing numbers of reports on the role of RhoGDI2 in tumorigenesis are appearing; however, the results are still controversial at present.

Tapper *et al* demonstrated that upregulation of RhoGDI2 was associated with the malignancy of ovarian carcinoma by cDNA array analysis (27). Interestingly, a high-frequency occurrence of autoantibody against RhoGDI2 was detected in the sera from acute leukemia patients (28). In addition, increased motility of murine cancer cells by overexpression of autocrine motility factor was related to increased expression of *RhoGDI2* (29). All these findings indicated that RhoGDI2 was upregulated in the progression of tumorigenesis. On the contrary, Theodorescu *et al* reported that RhoGDI2 is an invasion and metastasis suppressor in bladder cancer (26). Furthermore, the suppressant function of RhoGDI2 in cancer metastasis was associated with anchoring Rho proteins to the cell membrane by the C-terminal of RhoGDI2 (30). In the present study, we assessed the expression of RhoGDI2 in human breast cancer tissues and identified the possible relation between expression levels and clinicopathological parameters. Starting with normal epithelia, then hyperplasia, progressing into *in situ*, and then invasive lesions, the expression pattern of RhoGDI2 was biphasic. The phase from normal epithelia to hyperplasia showed the most markedly increasing pattern. While histological entity progressed from *in situ* to invasive, a significantly decreased pattern was observed. This pattern was also observed in four examined breast cancer cells that differ in *in vivo* metastasis (Fig. 2). These phenomena might imply that increased expression of RhoGDI2 could inhibit progression of breast cancer at the early stage, and then reduced expression of RhoGDI2 might contribute to the malignant progression. So it is not surprising that expression of RhoGDI2 in most malignancies decreased compared to normal epithelia in the same section. Statistical analysis showed the correlation between the reduced expression of RhoGDI2 and lymph node metastases. Meanwhile, RhoGDI2 expression negatively

correlated with the number of involved axillary lymph nodes. Although there is a possibility of recurrence after resection even in patients with node negative breast cancer, the histopathological presence of lymph node metastases is considered to be the most informative parameter to predict the occurrence of relapses and the prognosis in breast cancer patients, and clinical consensus has long held that the absolute number of positive axillary lymph nodes is one of the most important prognostic factors in breast cancer (15,32,33). So the increased expression of RhoGDI2 may be a potential marker which positively correlates with the prognosis of breast cancer.

In breast cancer, Jiang *et al* found that the transcription level of *RhoGDI2* remained similar between normal and tumor tissues. In their study, frozen tissues, a mixture of the cells at distinct states, were mixed for RNA extraction and subsequent analyses, and then *in situ* analysis and the examination of protein level was not included (13). Breast cancer is heterogeneous and dynamically changes (31), so *in situ* analysis is necessary to observe the expression of genes in the breast. Moreover, the level of transcription and translation of genes is not always consistent. Thus, it is not strange to find the biphasic pattern of RhoGDI2 expression in different lesions of the same breast cancer tissue in our study. Our data also suggested RhoGDI2 may have a positive effect on the metastasis suppression of breast cancer. Although recent studies have demonstrated that RhoGDI2 promoted MDA-MB-231 and BT549 cell invasiveness *in vitro* (14), the role of RhoGDI2 in the *in vivo* metastasis process of breast cancer was still not clear. Besides invasiveness, several steps are also involved in the metastatic process. Each step is critical and inability to complete any step of the metastatic cascade renders a cell nonmetastatic (3,26). Furthermore, we think clinical research is quite important to elucidate the true role of RhoGDI2 in breast cancer. Our data and that of Zhang and Zhang (14) regarding alternative RhoGDI2 expression in breast cancer may reflect the diversity and complexity of its role in the course of malignancy.

In summary, a biphasic pattern of RhoGDI2 expression was observed during the extent of mammary cell proliferation and carcinogenesis. Meanwhile, the reduction of the RhoGDI2 expression level may be used as a potential marker for metastasis in human breast cancer. Although the biologic function of RhoGDI2 has not been identified yet, a marker can be clinically useful (2). Further research should uncover the role of RhoGDI2 in breast cancer.

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