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).

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...
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.
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.

<table>
<thead>
<tr>
<th>Number of involved lymph nodes</th>
<th>RhoGDI2 (n=71)</th>
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<tbody>
<tr>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>1-3</td>
<td>7</td>
</tr>
<tr>
<td>4-9</td>
<td>4</td>
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<tr>
<td>≥10</td>
<td>8</td>
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*P was computed using χ² test.

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%, respectively. These data showed a biphasic (increase and then decrease) pattern of RhoGDI2 expression throughout the breast cancer progression.

Association of RhoGDI2 expression status with clinicopathological parameters. RhoGDI2 expression was observed in 71 breast cancer samples by immunohistochemistry. The correlation of RhoGDI2 expression levels with the clinicopathological features and other molecular marker data of the patients are shown in Table 1. By comparing the staining intensity between the most malignant and the normal epithelia in the same section, RhoGDI2 expression was down-regulated...
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). How-

ever, 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 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.

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-ß-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.

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). RhoGDI2, which includes 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 heterogenous 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.

Acknowledgments

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References