RESEARCH ARTICLE

Differentially Expressed Proteins in ER+ MCF7 and ER- MDA-MB-231 Human Breast Cancer Cells by RhoGDI-α Silencing and Overexpression

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Abstract

Background: The consequence of Rho GDP dissociation inhibitor alpha (RhoGDIα) activity on migration and invasion of estrogen receptor positive (ER+) and negative (ER-) breast cancer cells has not been studied using the proteomic approach. Changes in expression of RhoGDIα and other proteins interacting directly or indirectly with RhoGDIα in MCF7 and MDA-MB-231, with different metastatic potentials is of particular interest. Materials and Methods: ER+ MCF7 and ER- MDA-MB-231 cell lines were subjected to two-dimensional electrophoresis (2-DE) and spots of interest were identified by matrix-assisted laser desorption/ionization time of- flight/time-of-flight (MALDI-TOF/TOF) mass spectrometry (MS) analysis after downregulation of RhoGDIα using short interfering RNA (siRNA) and upregulated using GFP-tagged ORF clone of RhoGDIα. Results: The results showed a total of 35 proteins that were either up- or down-regulated in these cells. Here we identifed 9 and 15 proteins differentially expressed with silencing of RhoGDIα in MCF-7 and the MDA-MB-231 cells, respectively. In addition, 10 proteins were differentially expressed in the upregulation of RhoGDIα in MCF7, while only one protein was identified in the upregulation of RhoGDIα in MDA-MB-231. Based on the biological functions of these proteins, the results revealed that proteins involved in cell migration are more strongly altered with RhoGDI-α activity. Although several of these proteins have been previously indicated in tumorigenesis and invasiveness of breast cancer cells, some ohave not been previously reported to be involved in breast cancer migration. Hence, these proteins may serve as useful candidate biomarkers for tumorigenesis and invasiveness of breast cancer cells. Conclusions: Future studies are needed to determine the mechanisms by which these proteins regulate cell migration. The combination of RhoGDIα with other potential biomarkers may be a more promising approach in the inhibition of breast cancer cell migration.

Keywords: Proteomics - biomarkers - RhoGDIα - ER+ MCF7 - ERT- MDA-MB-231 - breast cancer cells

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Introduction

Breast cancer is one of the leading causes of cancer-related deaths in women worldwide. It is complex and heterogeneous. The disease arises from changes in gene and protein expressions of a normal cell (Reis-Filho and Lakhani, 2003; Nagaraja et al., 2006). These changes have been correlated with a number of cellular processes, including growth control, apoptosis, tumorigenesis and metastasis (Mommers et al., 1999). Consequently, researchers use different approaches for genome and proteome analyses to characterize the molecular mechanisms associated with tumorigenesis and metastasis among normal breast cells, non-invasive and invasive breast cancer cells. Proteomic strategies have been used to analyze the changes in different proteins and peptides in cancer cells. In recent years, proteomics has successfully identified novel biomarkers for diagnostic, prognostic and therapeutic purposes in a variety of cancer types, including breast cancer. By comparing the proteomes of drug resistant cancers with sensitive ones or invasive cancers with non-invasive ones, many potential proteins involved in drug resistance or cancer invasiveness have been identified (Wilkins et al., 1996; Brenton et al., 2005; Gast et al., 2009). In a study by Nagaraja et al. (2006) analysis by using two-dimensional gel electrophoresis (2-DE) identified 26 proteins as the potential biomarkers involved in tumorigenesis and invasiveness in the

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proteomes of normal breast cells, non-invasive breast cancer cells, and invasive breast cancer cells. Lai et al. (2010) compared the proteomic profiles of normal breast cells (MCF-10A) from non-invasive breast cancer cells (MCF-7) and invasive breast cancer cells (MB-MDA-231) to identify a set of potential candidate biomarkers in the tumorigenesis of breast cancer using 2D-DIGE and MALDI-TOF MS. Sarvaiya et al. (2006) identified more than 2000 proteins, of which approximately 200 proteins were involved in cancer-relevant cellular processes, and over 25 proteins could be used as a cancer biomarkers. The largest proteome database of the highly invasive MDA-MB-231 breast cancer cell line was conducted by Stande et al. (2009). They identified a total of 3481 proteins and classified them according to their cellular distribution and molecular functions such as cancer initiation and progression. Recent proteomic profiling study by Lee et al. (2011) identified nine differentially regulated proteins after MMP-26 expression knockdown in the human breast cancer cell line MDA-MB-231. They also reported that MMP-26 downregulation in MDA-MB-231 cells increased invasion commensurate with changes in invasion-associated protein expression.

Another protein which is identified in several proteomics studies is RhoGDIα protein which has been involved in tumour cell apoptosis, invasion and metastases (Barone et al., 2011). However, there have been no proteomic studies on the consequence of RhoGDIα activity on migration and invasion of ER^+ and ER^- cancer cell lines. Changes in expression of RhoGDIα and other proteins which interacting directly or indirectly with RhoGDIα in MCF7 and MDA-MB-231, with different metastasis potentials could be of particular interest. Our previous study examined silencing of RhoGDIα in MCF7 and MDA-MB-231 cells and concluded that it could increase the in vitro migration and invasion of these two cells regardless of their ER status (Hooshmand et al., 2013). In this study, we performed comparative proteome analysis of the RhoGDIα function in ER^+ and ER^- breast cancer cell lines in order to identify the protein expression changes potentially involved in invasion and migration.

Materials and Methods

Cell culture

The human breast cancer cell lines MDA-MB-231 and MCF7 were purchased from National Cell Bank, Institute Pasteur of Iran. They were cultured in phenol red-containing RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 100 unit/ml penicillin and 100 μg/ml streptomycin at 37°C in humidified incubator (>95%) with 5% CO_2.

Transient gene transfection

Gene silencing and overexpression were performed as described previously (Hooshmand et al., 2013). Briefly, MDA-MB-231 and MCF7 cells were transfected with either duplexes of RhoGDIα -specific siRNA oligos (00058983 and 00337331, Sigma), scrambled control siRNA (Sigma), Green Fluorescent Protein (GFP)-tagged clone of homo sapience RhoGDI α plasmid (Origene), or control plasmid pCMV-AC-GFP (Origene) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

The maximum transfected cell rates were given by 40-50% cell confluence for gene silencing experiments and 80-90% cell confluence for overexpression experiments. Western blotting and quantitative real-time PCR were performed to confirm gene silencing. In addition, fluorescence microscopy, flow cytometry, and western blotting were performed for confirmation of overexpression experiments as described previously (Hooshmand et al., 2013). To obtain the large-scale transfected cells for proteomics experiment, 21 μL of Lipofectamine 2000 and 17 μL of 20 μM siRNA stock solutions of either test or control siRNA for gene silencing and 21 μL of Lipofectamine 2000 and 12 μg of either test or control plasmid for gene overexpression was applied in transfection of MCF-7 and MDA-MB-231 cells in each of T-75 flask.

Sample preparation for 2-DE

Cells lysates were prepared from MCF7 and MDA-MB-231 cells following knockdown and overexpression of RhoGDIα. Briefly, the samples were cultured under standard conditions, harvested by trypsinization, and then they were resuspended in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2% immobilized pH gradient (IPG) buffer (pH 3-10) (GE Healthcare, Uppsala, Sweden)), aliquoted and stored at -70°C until use. The protein concentration was determined by the Bradford protein method and bovine serum albumin (Sigma, Germany) as standard.

Two-dimensional electrophoresis (2DE)

The lysates of MCF7 and MDA-MB-231 cells silencing and overexpressed RhoGDIα with their control cells were subjected to 2DE. Approximately 250 μg of protein extract was applied per IGP strips (18-cm: pH 3-10 nonlinear) for isoelectric focusing (IEF). The loaded IPG strips were focused at 70000 Vh. After IEF, the focused strips were equilibrated, and run on the top of a second dimension SDS-PAGE (12%) until the tracking dye reached the bottom of the gels. The resulting 2-D gels were visualized by a modified silver staining protocols which is the standard protocol compatible with MS analysis, scanned using a GS-800 scanner (Bio-Rad, USA) at 300 dpi resolution and analyzed using the Prodigy SameSpots version 1.0 software (Nonlinear Dynamic, UK), according to manufacturer's instructions. Protein spots that showed >2-fold and $p<0.05$ in the average normalized volume between MDA-MB-231 cell before and after overexpression of RhoGDIα treatment (3 gels in each group), were considered as differentially expressed proteins and were removed from the gels stained with the MS-compatible method. Spots were then sent for matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) MS analysis to Department of Biochemistry, School of Medicine, National University of Singapore.

Results

In order to identify proteins showing changes in expression of downregulation and upregulation of RhoGDIα in ER+ MCF7 and ER- MDA-MB-231 breast cancer cells, the cells were transiently downregulated using lipofectamine and RhoGDIα siRNA, or upregulated using lipofectamine and RhoGDIα plasmid. Real time PCR and western blot revealed that more than 90% RhoGDIα expression decreased in both cell lines compared to the controls. In addition, fluorescent microscopy and flow cytometry confirmed the highest transfection efficiency of RhoGDIα upregulation as described previously (Hooshmand et al., 2013). Then proteomics analysis was performed to compare the differences in protein expression of these cells. We identified 35 protein spots with two-fold increase or decrease changes in the downregulation and upregulation of RhoGDIα among these two cell lines.

Protein expression in downregulated RhoGDIα MCF7

11 differentially expressed protein spots from 2DE gels derived from downregulation of RhoGDIα in ER+ MCF7 cells and nine proteins were identified by mass spectrometry. These proteins are listed in Table 1 and their position on gels is shown in Figure 1.

In our study, Profilin1 which is a ubiquitously expressed actin-binding protein was shown to be under expressed with RhoGDI-α silencing in ER+ MCF7. This protein is involved in cell migration and breast cancer metastasis (Ding et al., 2013). Interestingly, Profilin1 was identified as a corepressor of estrogen receptor alpha in MCF7 breast cancer cells (Kanaujiya et al., 2013). Consistent with our finding, it has been reported that the loss of profilin-1 expression enhances breast cancer cell motility by Ena/VASP proteins (Bae et al., 2009).

Apolipoprotein E (ApoE) which is shown as a potent inhibitor of angiogenesis and tumor cell growth protein is another downregulated protein in both ER+ and ER- cell lines in our study (Niemi et al., 2000). Increasing ApoE expression can decrease metastatic disease in melanoma (Pola, 2012). Decreased expression of Catechol-O-methyl transferase (COMT) was identified in this study. It has been proposed that as an important estrogen-metabolizing enzyme, common genetic variants in this gene might influence breast cancer risk (Ji et al., 2008). In addition, it was shown that decreased COMT activity might be a risk factor for breast cancer (Dawling et al., 2001). Down-regulation of Smac/DIABLO in our study was in accordance with the in vitro increase migration and invasion of ER+ MCF7 which can be explained by other studies that showed lower Smac/DIABLO levels in patients with metastatic disease than those with localized disease (Yan et al., 2004). Smac/DIABLO expression has also been inversely correlated with breast cancer progression suggesting its important role in the breast cancer development (Pluta et al., 2011). The expression of programmed cell death 6 (PDCD6) which is known by the proapoptotic functions and regulates cell migration and invasion was decreased. Down-regulation of PDCD6 was reported in gastric cancer and HeLa cells (Yamada et al., 2008; Hoj et al., 2009). It was also observed that PDCD6 suppression was associated with tumorigenesis by inhibiting apoptosis in an ovarian cancer system (Rho et al., 2012). The Adenine phosphoribosyltransferase (APRT) enzyme catalyzes the synthesis of adenosine monophosphate (AMP). dCTP pyrophosphatase 1 (DCTPP1) hydrolyzes deoxynucleoside triphosphates (dNTPs) to the corresponding nucleoside monophosphates which has a strong preference for modified dCTP. The human fumarylacetoacetate hydrolase domain-containing protein 1 (FAHD1) was found as a novel mitochondrial enzyme with acylpyruvate hydrolase activity. However, there is currently no information available in the literature regarding the function of these 3 upregulated proteins in breast cancer progression.

Figure 1. Representative of 2D Gels from MCF7 cells Downregulation of RhoGDIα. Arrowheads indicate the differentially expressed proteins

Protein expression in downregulation of RhoGDIα MDA-MB-231

15 protein spots differentially expressed (both decreased and increased levels expression), in downregulation of RhoGDIα MDA-MB-231, in comparison to the control. The proteins and their position are given in Table 2 and Figure 2.

We identified the overexpression of ATP synthases α-subunit, which was frequently associated with

Table 1. Identification of Proteins Which are Differentially Expressed after Downregulation of RhoGDIα in MCF7

Spot Number	Accession	Name	% Cov	Peptides (95%)
1	sp|P52565|GDIR1_HUMAN	Rho GDP-dissociation inhibitor 1	39.2	2
2	sp|P21964|COMT_HUMAN	Catechol O-methyltransferase	38.4	6
3	sp|Q9H773|DCTP1_HUMAN	dCTP pyrophosphatase 1	32.4	4
4	sp|Q9NR28|DBLOH_HUMAN	Diablo homolog, mitochondrial	8.4	2
5	sp|O75340|PDCD6_HUMAN	Programmed cell death protein 6	23.6	1
6	sp|P07741|APT_HUMAN	Adenine phosphoribosyltransferase	66.1	8
7	sp|P02649|APOE_HUMAN	Apolipoprotein E	3.5	1
8	sp|P07737|PROF1_HUMAN	Profilin-1	65.7	23
9	sp|Q6P587|FAHD1_HUMAN	Acylpyruvase FAHD1, mitochondrial	16.1	2

Table 2. Identification of Proteins Which are Differentially Expressed after Downregulation of RhoGDIα in MDA-MB-231

Spot Number	Accession	Name	% Cov	Peptides (95%)
1	sp|P04632|CPNS1_HUMAN	Calpain small subunit 1	34	9
2	sp|P52565|GDIR1_HUMAN	Rho GDP-dissociation inhibitor 1	67.7	4
3	sp|P52566|GDIR2_HUMAN	Rho GDP-dissociation inhibitor 2	10.5	1
4	sp|Q9Y5S9|RBM8A_HUMAN	RNA-binding protein 8A	10.9	1
5	sp|O75947|ATP5H_HUMAN	ATP synthase subunit d, mitochondrial	46.6	8
6	sp|Q53FT3|HIKES_HUMAN	Protein Hikeshi	14.7	2
7	sp|P07741|APT_HUMAN	Adenine phosphoribosyltransferase	82.2	17
8	sp|P32119|PRDX2_HUMAN	Peroxiredoxin-2 OS=Homo sapiens	72.7	22
9	sp|O75489|NDUS3_HUMAN	NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, mitochondrial	29.2	3
10	sp|P02649|APOE_HUMAN	Apolipoprotein E	6.9	1
11	sp|P28070|PSB4_HUMAN	Proteasome subunit beta type-4	71.6	8
12	sp|Q9NR31|SAR1A_HUMAN	GTP-binding protein SAR1a	11.6	2
13	sp|P06730|IF4E_HUMAN	Eukaryotic translation initiation factor 4E	19.4	1
14	sp|P49411|EFTU_HUMAN	Elongation factor Tu, mitochondrial	5.1	1
15	sp|P11215|ITAM_HUMAN	Integrin alpha-M	4.3	1

Figure 2. Representative of 2D Gels from MDA-MB-231 Cells Downregulation of RhoGDIα. Arrowheads indicate the differentially expressed proteins

increase migration and invasion of MDA-MB-231 cells. Overexpression of this protein was also reported in high metastatic breast cancer cells compared with low metastatic cells (Pan et al., 2011). We also demonstrate NDUFS3 upregulation that is supported by the study which identified the higher NDUFS3 expression level as a robust indicator of breast cancer aggressiveness (Suhane et al., 2011).

Another upregulated protein found in our study is peroxiredoxin 2 (PRDX2). Stresing et al. (2013) showed a higher level of the protein PRDX2 in breast cancer cells that metastasize to the lung. The expression level of EF-Tu is altered in different human cancers. Similar to up-regulation of EF-Tu in human pancreatic adenocarcinoma (Grant et al., 1992), we found increased levels of EF-Tu expression, whereas, down-regulation of EF-Tu expression was found in hepatocellular carcinoma cell line HepG2 (Srisomsap et al., 2004). Eukaryotic translation initiation factor 4E (EIF4E) was overexpressed in our proteomic result. Current studies suggested that EIF4E knockdown would inhibit MDA-MB-231 breast cancer cell growth (Graff et al., 2007).

Silencing of RhoGDI-α led to a decrease in expression of RhoGDI2 which has been identified as a potential regulator of invasive and metastasis cancer. In addition, the reduced expression of RhoGDI2 demonstrated in breast cancer and gastric carcinoma. Consistent with our result, Hu et al. (2007) demonstrated that the reduced expression of RhoGDI2 in breast cancer was associated with lymph node metastasis. The reduced expression of RhoGDI2 mRNA in gastric carcinoma is associated with venous system invasion and lymph node metastasis (Shida et al., 2013).

Calpain small subunit 1 which may have a role in the regulation of several tumor-associated phenomena as migration, adhesion, cellular spreading, membrane protrusion, and invasion was downregulated in our proteomic results (Undyala et al., 2008). We indicated low expression of the RNA-binding protein 8a (RBM8A). Salicioni et al. (2000) showed that the biological relevance of RBM8A interaction with the ovarian cancer 1 gene (OVCA1) a candidate tumor suppressor for the breast and ovarian tumor. ApoE, proteasome subunit beta type-4, GTP-binding protein SAR1a, adenine phosphoribosyltransferase, Protein Hikeshi and integrin alpha-M were downregulated in our study, however, little is known about their role in breast cancer progression.

Protein expression in upregulation of RhoGDIα MDA-MB-231 and MCF7

Only 2 spots were reproducibly differentially expressed between 2DE gels from overexpressed RhoGDIα MDA-MB-231 cells and controls. One of the proteins was identified as RhoGDIα while the other one was unable to be identified. In contrast, comparison of MCF7 RhoGDIα upregulation with the control identified 10 spots (Table 3 and Figure 3).

Our analyses of MCF7 RhoGDIα upregulation identified higher expression of calpain small subunit. Hyperactivation of calpain is reported in tumor cell (Carragher et al., 2004; Libertini et al., 2005). We observed higher expression level of protein L-isoaspartyl O-methyltransferase (PIMT) which plays a major role in cell adhesion in various cancer cell lines. However, the level of PIMT in our result was in contrast to Ryu et al. (2011) study, which suggested that the development of specific inhibitors of PIMT may lead to a novel route of inhibition of the epithelial mesenchymal transition (EMT) and metastasis in EMT of MDA-MB-231 cells. RBM8A was also overexpressed here.

Interestingly, in contrast to decreased expression of catechol O-methyltransferase (COMT) and adenine phosphoribosyltransferase in silencing of RhoGDIα

Table 3. Identification of Proteins Which are Differentially Expressed after Overexpression of RhoGDIα in MCF7

Spot Number	Accession	Name	% Cov	Peptides (95%)
1	sp|P04632|CPNS1_HUMAN	Calpain small subunit 1	16	2
2	sp|P52565|GDIR1_HUMAN	Rho GDP-dissociation inhibitor 1	44.1	11
3	sp|P21964|COMT_HUMAN	Catechol O-methyltransferase	38.4	7
4	sp|Q9Y5S9|RBM8A_HUMAN	RNA-binding protein 8A	41.4	4
5	sp|P07741|APT_HUMAN	Adenine phosphoribosyltransferase	71.7	9
6	sp|P28070|PSB4_HUMAN	Proteasome subunit beta type-4	39.4	7
7	sp|P62993|GRB2_HUMAN	Growth factor receptor-bound protein 2	53.5	7
8	sp|P22061|PIMT_HUMAN	Protein-L-isoaspartate(D-aspartate) O-methyltransferase	30	5
9	sp|P30048|PRDX3_HUMAN	Thioredoxin-dependent peroxide reductase, mitochondrial	36.7	1
10	sp|Q9HAV7|GRPE1_HUMAN	GrpE protein homolog 1, mitochondrial	29	2

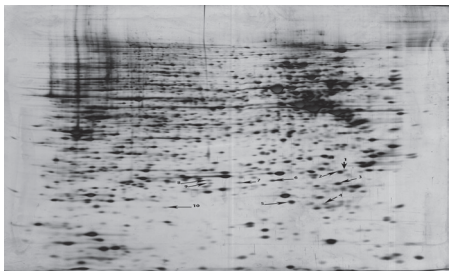

Figure 3. Representative of 2D Gels from MCF7 Cells Overexpressed of RhoGDIα. Arrowheads indicate the differentially expressed proteins

in MCF7, higher expression of these two proteins were identified in upregulated RhoGDIα MCF7. The functional roles of GrpE protein homolog 1 mitochondrial (HMGE) and Proteasome subunit beta type-4 which were overexpressed in our results have not been clearly elucidated in cancer cell migration.

We demonstrated lower level Grb2 expression. Similar to our finding, downregulation of Grb2 protein has been associated with inhibition of breast cancer cell growth (Tari et al., 1999). In addition, decreased expression level of the Peroxiredoxin III (PRDX3) gene, an antioxidant protein that plays a significant role in cell cycle regulation was observed suggesting its potential as a proliferation marker in breast cancer. It has been reported that cell proliferation in breast cancer is decreased by silencing the PRDX3 gene (Chua et al., 2010).

Discussion

The advances of proteomic technologies to explore the protein profiles would be helpful to obtain potential biomarkers involved in the development and progression of cancer cells. It is well established that ER expression is linked to prognosis (Sofi et al., 2012; Zhang et al., 2013). Previous studies reported the proteomic profiles of ER+ MCF7 and ER- MDA-MB-231 breast cancer cells. Identifying proteins up and down regulated in ER-positive vs. ER-negative patients may help to identify biomarkers and targets in human breast tumors. However, to the best of our knowledge the proteomic profiles of ER+ MCF7 and ER- MDA-MB-231 breast cancer cells, with RhoGDIα silencing or overexpression by a proteomic approach using mass spectrometry have not been established. RhoGDI-α is a member of Rho GDP dissociation inhibitors (RhoGDIs) which is involved in cancer progression invasion, and metastasis by inactivating the RhoGTPases (Lin and van Golen, 2004).

Our previous data demonstrated that downregulation of RhoGDIα in ER+ MCF7 and ER- MDA-MB-231 cells similarly affected the in vitro migration and invasion of these cell lines and significantly increased their migration and invasion into the lower surface of porous membrane of the chambers. However, differential expression of ER between these two cell lines (ER+ and ER-) does not critically affect the activity of RhoGDIα on migration (Hooshmand et al., 2013). Herein, our proteomics result identified a total of 35 proteins were up- or down-regulated in these cell lines. We reported 9 and 15 proteins differentially expressed in silencing of RhoGDIα MCF-7 and the MDA-MB-231 cells, respectively. Compared to 10 identified proteins involved in the upregulation of RhoGDIα in MCF7, only the RhoGDIα protein was overexpressed in MDA-MB-231. However, the differentially expressed proteins were not the same among these cell lines by RhoGDI-α treatment. A common argument for this is the unique phenotypes of these cells, compared to the ER-positive and non-invasive MCF-7, the MDA-MB-231 is well known as an invasive estrogen-independent human breast cancer cells. In addition, further analysis is needed to provide information whether these differences are due to the interaction of RhoGDI-α and ER in MCF7. Although several of these proteins have been previously shown in various types of cancer metastases, some other proteins have never been implicated in cancer cell migration, therefore, such proteins may be regarded as key candidate markers in cancer migration and further investigation is required to identify their function in cancer.

In summary our proteomics analyses demonstrated multiple changes in protein expression when RhoGDI-α was up or downregulated. Interestingly, a large proportion of these proteins have been previously indicated in tumorigenesis and invasiveness of breast cancer cells. The three upregulated proteins include APRT, DCTPP1, FAHD1 and six downregulated proteins include Apolipoprotein E, Proteasome subunit beta type-4, GTP-binding protein SAR1a, Adenine phosphoribosyltransferase, Protein Hikeshi and Integrin alpha-M in silencing RhoGDIα of MCF7 and MDA-MB-231, respectively and two overexpressed GrpE protein homolog 1 mitochondrial and Proteasome subunit beta type-4 in RhoGDIα upregulation of MCF7 have not been observed in certain types of cancers before. This study can be used to determine the set of proteins that differ between ER+ and ER- which may serve as biomarkers for tumorigenesis and invasiveness

among these cell lines. The combination of RhoGDI-α with other potential biomarkers might be a more promising approach in inhibition of cancer migration.

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