ROLE OF RhoGDIα ON MIGRATION AND INVASION OF ESTROGEN RECEPTOR POSITIVE MCF7 AND ESTROGEN RECEPTOR NEGATIVE MDA-MB-231 BREAST CANCER CELLS

By

SOMAYEH HOOSHMAND

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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DEDICATION

To

Improving the health of women with breast cancer and

for a future without breast cancer.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirements for the degree of Doctor of Philosophy

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Chairperson: **Professor Rozita Rosli, PhD**
Faculty : **Medicine and Health Sciences**

Breast cancer arises from changes in gene and protein expression of a normal cell. These changes have been correlated with a number of cellular processes, including growth control, apoptosis, tumorigenesis and metastasis. Rho GDP dissociation inhibitor (RhoGDI) family of proteins can inhibit cell motility, invasion, and metastasis in cancer cells by inactivating the RhoGTPases. Rho GDP dissociation inhibitor α (RhoGDIα) in particular, a member of RhoGDI family, has been consistently shown to interact with the estrogen receptor (ER) resulting in a change to its transcriptional activity which is inversely correlated with cell motility and invasion in breast cancer. The consequence of RhoGDIα activity on migration and invasion of estrogen receptor positive (ER+) and negative (ER-) breast cancer cells is not clear.

The main objective of this study was to investigate the consequence of RhoGDIα activity on migration and invasion of ER^+ and ER^- breast cancers cells. The first specific objective is to observe the likely possible opposing effect of RhoGDIα on migration and invasion of ER^+ (MCF7) and ER^- (MDA-MB-231) breast cancer cells with or without 17β-estradiol (E2), since the interaction of E2 with ER has been shown to induce cell proliferation. These cells were treated with E2 to assess whether exposure of these cells to E2 affected the level of RhoGDIα. The RhoGDIα was silenced by short interfering RNA (siRNA) and overexpressed using GFP-tagged ORF clone of RhoGDIα and cell transfection was performed with Lipofectamine. More than 90% RhoGDIα gene silencing in these cells was confirmed both at mRNA and protein levels by qRT-PCR and Western blot. Successful RhoGDIα overexpression was also confirmed by flow cytometry and Western blot in both cell lines. There was no significant difference in the RhoGDIα mRNA expression with or without E2 in these cell lines. However, using migration and transwell invasion assays, it was found that silencing of RhoGDIα in MCF7 and MDA-MB-231 cells significantly increased migration and invasion of these cells. Overexpression of RhoGDIα in MCF7 cells suppressed their migration and invasion, but this was not significant on MDA-MB-231 cells. These results indicate that the silencing of RhoGDIα similarly affects *in vitro* migration and invasion of ER^+ MCF7

and ER$^-$ MDA-MB-231 cells. However, *in vitro* migration and invasion assays are differently affected by the overexpression of RhoGDIα in these two cell lines and this may be due to the differences in ER expression, primary invasive ability and/or other molecules between these two cell line models, which warrant further investigation.

In the second specific objective comparative proteome analysis of the RhoGDIα function in MCF7 and MDA-MB-231 breast cancer cells was performed in order to identify the protein expression changes potentially involved in invasion and migration. These cells were subjected to two-dimensional electrophoresis after RhoGDIα silencing and overexpression and spots of interest identified by matrix-assisted laser desorption/ionization time of- flight/time-of-flight mass spectrometry. The results showed a total of 35 proteins that were either up- or down-regulated in these cells. Here, 9 and 15 differentially expressed proteins were identified in silencing of RhoGDIα MCF7 and the MDA-MB-231 cells, respectively. In addition, 10 proteins were differentially expressed in the overexpression of RhoGDIα in MCF7, while only one protein was identified in the overexpression of RhoGDIα MDA-MB-231. A large proportion of the identified proteins in this study have been previously indicated in tumorigenesis and invasiveness of breast cancer cells such as Profilin1, Apolipoprotein E, Catechol-O-methyl transferase, Smac/DIABLO, programmed cell death 6, ATP synthases α-subunit, NADH dehydrogenase (ubiquinone) Fe-S protein, peroxiredoxin 2, EF-Tu, Eukaryotic translation initiation factor 4E, Rho GDP-dissociation inhibitor 2, Calpain small subunit 1, RNA-binding protein 8a, protein L-isoaspartyl O-methyltransferase, Growth factor receptor-bound protein 2 and Peroxiredoxin III. However, not much is known on the function of adenine phosphoribosyl transferase, dCTP pyrophosphatase 1, fumaryl acetoacetate hydrolase domain-containing protein 1, Proteasome subunit beta type-4, GTP-binding protein SAR1a, Protein Hikeshi, Integrin alpha-M, GrpE protein homolog 1 mitochondrial in breast cancer. Hence, these proteins may serve as useful candidate biomarkers for tumorigenesis and invasiveness of breast cancer cells. Future studies are needed to determine the mechanisms by which these proteins regulate cell migration to explain the observed differences of the invasion and migration of MCF7 and MDA-MB-231 cells in response to RhoGDIα overexpression. The combination of RhoGDIα with these or other biomarkers might be a more promising approach in inhibition of cancer migration.

Abstrak tesis dibentangkan kepada Senat Universiti Putra Malaysia bagi perlaksanaan syarat untuk Ijazah Doktor Falsafah

REGULASIRhoGDIα TERHADAP PENGHIJRAHAN DAN SERANGAN PENCEROBOHAN SEL-SEL KANSER PAYUDARA RESEPTOR ESTROGEN POSITIFMCF7 DAN RESEPTOR ESTROGEN NEGATIF MDA MB231

Oleh

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Kanser payudara terhasil daripada perubahan pada ekspresi gen dan protein dalam satu sel normal. Perubahan ini telah dihubungkaitkan dengan pelbagai proses selular, termasuk kawalan pertumbuhan, apoptosis, tumorigenesis dan metastasis. Protein-protein keluarga perencat penceraian Rho GDP (RhoGDI) mampu menghalang motiliti sel, pencerobohan, dan metastasis dalam sel kanser melalui penyahaktifan RhoGTPase. Perencat penceraian, khususnyaRho GDP α (RhoGDIα) yang merupakan salah satu ahli keluarga RhoGDI, menunjukkan interaksi secara konsisten dengan reseptor estrogen (ER) yang mengakibatkan perubahan terhadap aktiviti transkripsi yang berhubung kait dengan motiliti sel dan serangan pencerobohan dalam kanser payudara.

Objektif utama kajian ini ialah untuk mengkaji selidik kesan kegiatan RhoGDIα terhadap migrasi dan pencerobohan sel-sel kanser payudara ER^+ dan ER^-.Objektif spesifik pertama kajian ini ialah untuk mengkaji selidik kesan RhoGDIα terhadap migrasi dan pencerobohan sel kanser payudara ER^+(MCF7) dan ER^- (MDA-MB-231) denganatau tanpa 17β estradiol (E2), oleh kerana interaksi E2 dengan ER telah menunjukkan pencetusan pembiakan sel. Sel-sel MCF7 dan MDA-MB-231 telah dirawat dengan E2 untuk menilai sama ada pendedahan sel ini kepada E2 menjejaskan tahap RhoGDIα. Regulasi RhoGDIα telah diturunkan dengan gangguan RNA singkat (siRNA) dan regulasi ditingkatkan regulasi menggunakan klon GFP-tagged ORF RhoGDIα dan transfeksi sel dilakukan dengan Lipofectamine. Keupayaan sel-sel MCF7 dan MDA-MB-231 untuk penghijrahan dan pencerobohan telah dicerakin dengan menggunakan kamar transwell. Lebih daripada 90% gen gen RhoGDIα dalamsel-sel MCF7 dan MDA-MB-231 dapat dihentikan dan telah disahkan dengan aras mRNA dan protein melalui qRT-PCR dan western blot. Ekspresi lebihan RhoGDIα telah berjaya disahkan juga melalui sitometri aliran dan western blotting dalam kedua-dua jenis sel. Tiada perbezaan ketara dalam ekspresi mRNA RhoGDIα dengan atau tanpa E2 dalam titisan-titisan sel-sel. Walaubagaimanapun, denganmenggunakan ujian penghijrahan dan serangan pencerobohan transwell, penghentian RhoGDIα dalam sel-sel MCF7 dan MDA-MB-231 didapati meningkatkan penghijrahan dan serangan

pencerobohan sel-sel inidengan nyata sekali. Ekspresi lebihan RhoGDIα dalamsel-selMCF7 menyekat penghijrahan dan serangan pencerobohan mereka, tetapi initidak ketara pada sel-sel MDA-MB-231. Keputusanini menunjukkan bahawa penurunan regulasi RhoGDIα menjejaskan penghijrahan danserangan pencerobohan *in vitro* sel-sel ER^+ MCF7 dan ER^-MDA-MB-231. Walau bagaimanapun, ujian penghijrahan dan serangan pencerobohan *in vitro* terjejas secara berbeza oleh peningkatan regulasi RhoGDIα dalam kedua-dua jenis sel ini dan ini mungkin adalah disebabkan oleh perbezaan pada ekspresi ER, Keupayaan serangan primer dan / atau molekul-molekul lain antara dua model titisan-titisan sel-sel ini, yang memerlukan siasatan lanjut.

Matlamat spesifik kedua kajian ini ialah untuk menjalankan analisis komparatif proteome bagi fungsi RhoGDIα dalamtitisan-titisan sel kanser payudara ER^+ dan ER^- untuk mengenal pasti perubahan ekspresi protein yang berpotensi untuk terlibat dalam serangan pencerobohan danpenghijrahan.Dalam hal ini, elektroforesisdua-dimensi(2-DE) dijalankan keatas titisan-titisan sel MCF7 danMDA-MB-231 setelah penurunan dan peningkatan regulasi RhoGDIα serta bintik yang sesuai telah dikenal pasti melalui analisis *Matrix-Assisted Laser Desorption/Ionization Time Of-Flight/Time-Of-Flight* (MALDI-TOF/TOF) *Mass spectrometry* (MS). Keputusannya menunjukkan sejumlah 35 protein telah mengalami samaada penurunan atau peningkatan regulasi dalamtitisan-titisansel-sel ini. Sebanyak sembilan dan 15 ekspresi protein yang berbezatelah dikenalpasti dalam masing-masing penyahaktifanRhoGDIα sel-sel MCF 7 dan MDA-MB 231. Tambahan pula, 10 proteintelah diekspreskan secara berbeza dalampeningkatan regulasi RhoGDIα dalam MCF7, manakala hanya satu protein telah dikenalpasti dalam peningkatan regulasiRhoGDIα MDA-MB-231. Sebahagian besar daripada protein yang telah dikenalpasti dalam kajian ini, sebelum ini telah menunjukkan penghasilan tumor dan penyerangan sel kanser payudara seperti Profilin1, Apolipoprotein E, Catechol-O-methyl transferase, Smac/DIABLO, program sel mati 6, ATP synthases α-subunit, NADH dehydrogenase (ubiquinone) protein Fe-S, peroxiredoxin 2, EF-Tu, faktor terjemahan permulaan eukariotik 4E, Rho KDNK-pengasingan rencatan 2, subunit kecil Calpain 1, protein pengikat RNA 8a, protein L-isoaspartyl O-Methyltransferase, protein faktor pertumbuhan reseptor-terikat 2 dan Peroxiredoxin III. Walau bagaimanapun, masih banyak yang belum diketahui mengenai fungsi adenine phosphoribosyltransferase, pirofosfatase dCTP 1, protein mengandungi domain fumarylacetoacetate hidrolase1, beta subunitProteasome jenis-4, GTP-binding protein SAR1a, Protein Hikeshi, Integrin alpha-M, homolog protein mitokondria GrpE 1 `dalamkanser payudara. Maka, protein-protein ini mungkin berguna sebagaipenanda-bio untuk penghasilan tumor kanser dan serangan sel-sel kanser payudara.Kajian masa hadapan diperlukan untuk menentukan mekanisme di manaproteininimeregulasi migrasisel yang menjelaskan perbezaan serangan pencerobohan dan migrasi MCF7 dan MDA-MB-231 yang telah diperhatikan sebagai respons kepada ekspresi lebihan RhoGDIα. Potensinya, gabungan RhoGDIα dengan penanda-bio lainmungkin memberi jaminan dalam perencatan migrasi kanser.

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I certify that a Thesis Examination Committee has met on 23 September 214 to conduct the final examination of Somayeh Hooshmand on her thesis entitled "Role of RhoGDIα on Migration and Invasion of Estrogen Receptor Positive MCF7 and Estrogen Receptor Negative MDA-MB-231 Breast Cancer Cells" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirements of the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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AMP	Adenosine monophosphate
ApoE	Apolipoprotein E
APRT	Adenine phosphoribosyltransferase
APS	Ammonium persulfate
BME	Basement membrane extract
BSA	Bovine serum albumin
CBB	Coomassie Brilliant Blue
CDCS	Charcoaldextran treated calf serum
CIS	Carcinoma in situ
COMT	Catechol-O-methyl transferase
DCTPP1	dCTP pyrophosphatase 1
2DE	Two-dimensional electrophoresis
DFS	Disease-free survival
DIGE	Differential in-gel electrophoresis
dNTPs	deoxynucleoside triphosphates
dsRNAs	double-stranded RNAs
ECL	Enhanced chemiluminescence
ECM	Extra Cellular Matrix
EIF4E	Eukaryotic translation initiation factor 4E
EMT	Epithelial Mesenchymal Transition
ERα	Estrogen Receptor α
ER	Estrogen
ER+	Estrogen receptor positive
EGFR	Epidermal growth factor receptor
ERM	Ezrin/radixin/moesin
ESR1	Estrogen Receptor 1
ESR2	Estrogen Receptor 2
ESI MS	Electrospray ionization mass spectrometry
FAHD1	Fumarylacetoacetate hydrolase domain-containing protein 1
FBS	Fetal bovine serum
FFE	Free flow electrophoresis
FT-MS	Fourier transform ion cyclotron
GFP	Green Fluorescent Protein
HER-2	Human epidermal growth factor receptor-2
HMGE	GrpE protein homolog 1 mitochondrial
Hsp27	Heat shock protein 27
IPG strips	Immobilized pH gradient strips
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
MS	Mass spectrometry
MW	Molecular Weight
Mr	Molecular mass

NR3C3	Nuclear receptor subfamily 3,group C, member 3
OS	Overall survival
OVCA1	Ovarian cancer 1 gene
PDCD6	Programmed cell death 6
pI	Isoelectric Points
PIMT	protein L-isoaspartyl O-methyltransferase
PKC	protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PRDX2	Peroxiredoxin 2
PRDX3	Peroxiredoxin III
PR+	Progesterone-receptor-positive
PTM	Post-translational modifications
PVDF	Polyvinylidene fluoride
RBM8A	RNA-binding protein 8a
RhoGDI	Rho GDP dissociation inhibitor
RhoGDI α	Rho GDP dissociation inhibitor alpha
RISCs	RNA-induced silencing complexes
SERMs	Selective estrogen receptor modulators
SERDs	Selective estrogen receptor downregulators
siRNA	Small interfering RNA
SDS-PAGE	SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis
TNBC	Triple Negative Breast Cancer
TNM	Tumor Node Metastasis

CHAPTER 1

INTRODUCTION

Cancer is formed when normal cells divide rapidly out of control and a genetic transformation takes place from normal cells to cancer cells. This genetic transformation alters the function of the resulting protein and disrupts the cell's communication network (Balmain, 2001; Futreal *et al.*, 2004). Cancer is the leading cause of the death in many parts of the world and has claimed several million lives over years. Among different cancers, breast cancer is the most common cancer among women worldwide (Pramanik *et al.*, 2008).

Breast cancer can be formed from changes in gene and protein expression of a normal cell (Nagaraja *et al.*, 2006; Reis-Filho and Lakhani, 2003). These changes have been correlated with a number of cellular processes, including growth control, apoptosis, tumorigenesis and metastasis (Mommers *et al.*, 1999). Metastasis, or the spread of cancer cells from the primary site to a distant organ accounts for the majority of cancer-related deaths. However, the course and determinants for tumor progression and metastasis are not fully understood. The ability of cancer cells to migrate and invade into surrounding tissues are prerequisites for cancer spread and metastasis, and studies have thus been directed to identify molecules that contribute to cancer cell motility, migration, and invasion (Palmer *et al.*, 2011). Agents that specifically target the motility of tumor cells are not only potentially more effective at treating metastasis but also eliminate the side effects of the current generally acting therapies.

Consequently, 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 have been used. Proteomic strategies have been used to analyze the changes in various 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 (Brenton *et al.*, 2005; Gast, Schellens*, et al.*, 2009; Wilkins *et al.*, 1996).

In a study by Nagaraja *et al*. (2006), analysis by using two-dimensional gel electrophoresis (2-DE) identified 26 proteins as potential biomarkers involved in tumorigenesis and invasiveness in the 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 MS7. 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 cancer biomarkers. The largest proteome database of the highly invasive MDA-MB-231 breast cancer cell line was established 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 silencing 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 the RhoGDIα protein. This protein is involved in tumour cell apoptosis, invasion and metastases(Barone et al., 2011). RhoGDIα is one of the Rho GDP dissociation inhibitor (RhoGDI) family mammalian members. RhoGDIα has been identified as a regulator of Rho GTPases, which binds to most of the Rho GTPases including RhoA, Cdc42, and Rac1(Bielek *et al.*, 2009).The expression of RhoGDIs is altered in a variety of cancers but its role in cancer remains controversial.

There are now two models of ER^+ and ER^- breast cancers, MCF7 and MDA-MB-231 cell lines, which are being used, respectively (Yokotsuka *et al.*, 2011). The RhoGDIα has been detected in cytosolic fractions of both cell lines using western blotting with no significant different levels of expression(El Marzouk *et al.*, 2007). In contrast to MDA-MB-231, which is a highly migratory and invasive cell line, MCF7 has a low migratory and invasive activity as assessed using transwell chambers(Yokotsuka *et al.*, 2011).

Problem Statement

Considering the impact of ER on the invasiveness of breast cancer, and direct binding of ER and RhoGDIα (Barone *et al.*, 2011; El Marzouk *et al.*, 2007; Su *et al.*, 2001), a partly different role for RhoGDIα in invasion and migration of ER^+ and ER^-breast cancers is not unlikely. In this study, it is hypothesized that RhoGDIα plays different roles in invasion and migration of ER+ and ER- breast cancers.

The importance of this hypothesis is underscored by the opinion that effects of RhoGDIs on tumors are evidently multifaceted, and even a single Rho family member can have opposite effects in different types of a tumor(Garcia-Mata *et al.*, 2011). In addition, there have been no proteomic studies on the consequence of RhoGDIα activity on migration and invasion of ER^+ and ER^- cancer cell lines.

Therefore, in this study, the consequence of RhoGDIα silencing and overexpression on migration and invasion of MCF7 (ER^+) and MDA-MB-231 (ER^-) breast cancer cell lines with or without 17β -estradiol (E2) using transwell chambers was investigated. The interaction of E2 with ER has been shown to induce cell proliferation. Therefore, MCF7 and MDA-MB-231 cells were treated with E2 to assess whether exposure of these cells to E2 affected the level of RhoGDIα. In addition, two dimensional gel electrophoresis (2DGE) coupled with mass spectrometric analysis was applied in order to identify proteins that show changes in expression of silencing and overexpression of RhoGDIα in

ER^+ MCF7 and ER^- MDA-MB-231 breast cancer cells.

Research Objectives

Main Objective

The main objective of this study was to investigate the role of RhoGDIα expression on migration and invasion of ER^+ and ER^- breast cancers cells.

Specific Objectives

The specific objectives of this study were:

1. To investigate the role of RhoGDI-α silencing and overexpression on migration and invasion of ER+ MCF7 breast cancer cell line (with and without 17-β-estradiol (E2)).

2. To investigate the role of RhoGDI-α silencing and overexpression on migration and invasion of ER– MDA-MB-231 breast cancer cell line (with and without 17-β-estradiol (E2)).

3. To identify differentially expressed proteins in ER^+ MCF7and ER^- MDA-MB-231 breast cancer cell lines, with RhoGDI-α silencing or overexpression.

4. To identify the potential molecular targets associated with RhoGDI-α in ER^+ MCF7and ER^- MDA-MB-231 breast cancer cell lines.

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