UNIVERSITI PUTRA MALAYSIA

APPLICATION OF PROTEOMICS APPROACHES IN THE IDENTIFICATION OF NEW MARKERS AND THERAPEUTIC TARGETS FOR BREAST CANCER

LAMA ABDEL QADER MOH'D HAMADNEH

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BY

LAMA ABDEL QADER MOH'D HAMADNEH

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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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Chair: **Associate Professor Rozita Rosli, PhD**

Faculty: **Medicine and Health Sciences**

Breast cancer is the most common cancer in most parts of the world and is a leading cause of death among women. Even though the incidence of the disease is increasing each year, early detection and improved treatments have increased the survival rates. Currently, only a few markers are used for either early diagnosis, treatment response or for survival of breast cancer. In this study, two-dimensional gel electrophoresis (2DGE) was used in the quest for new potential biomarkers for the disease. Breast cancer cell lines and normal breast cell line were used to optimize the conditions to produce the respective proteome maps. Fresh frozen samples representing tumor and adjacent normal tissues were then collected from patients who underwent breast surgery at HUKM, HKL and Hospital Putrajaya. A total of sixty samples representing tumor and adjacent normal tissues were collected from June 2005 to December 2006 and were screened using 2DGE. Subsequently, 24 samples representing the different stages of infiltrating ductal carcinoma were used for further analysis using 17 cm IPG strips with 2 pH ranges 3-10

and 4-7, and the gels were analyzed using PDQuest 7.3 software. Several protein spots of interest were then excised and analyzed using MALDI-TOF spectrometer. Tumor rejection antigen (gp96), heat shock protein 90α, nucleosome assembly protein 1-like 1 and opioid-binding cell adhesion molecule precursor were identified and found to be up-regulated in breast cancer cell lines when compared to the normal breast cell line. Calreticulin, tumor rejection antigen (gp96), heat shock protein 60 and cytokine induced apoptosis inhibitor 1 were found to be up-regulated by 2 folds or more in tumor tissues when compared to the adjacent normal tissues. On the other hand, actin γ 2 and protein tyrosine phosphatase were found to be down-regulated in tumor tissues. Since Calreticulin, a calcium binding protein was more intense at different stages of the disease with its expression confirmed by Western blotting, it was chosen for further investigations. Quantitative RT-PCR with GAPDH as a house keeping gene was used to monitor the level of gene expression and to correlate the mRNA levels with calreticulin levels. In the samples that represent later stages of the disease, mRNA levels were found to be highly expressed in tumor tissues when compared to the adjacent normal tissues where in average more than 18 folds increase was observed. The mRNA level was also found to be decreased in stage IV sample where 12 folds increase was observed, indicating a possible role of calreticulin in the progression of the disease. In conclusion, the proteomics approaches were utilized in this study and was found to be valuable in the search for potential new biomarkers for breast cancer.

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Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah Sains

PENGGUNAAN PENDEKATAN PROTEMIKS DALAM PENEMUAN PENANDA DAN SASARAN TERAPI BARU UNTUK KANSER PAYUDARA

Oleh

LAMA ABDEL QADER MOH'D HAMADNEH

Jun 2008

Pengerusi: **Profesor Madya Rozita Rosli, PhD**

Fakulti: **Perubatan dan Sains Kesihatan**

Kanser payudara merupakan penyakit kanser yang paling banyak dihidapi sebahagian besar penduduk dunia dan menjadi penyebab utama kematian di kalangan wanita. Walaupun insiden penyakit ini meningkat setiap tahun, namun pengesanan awal dan peningkatan dalam bidang pengubatan telah berjaya meningkatkan kekal hidup pesakit. Buat masa ini, hanya beberapa penanda yang digunakan pada penyakit kanser samada untuk diagnosis awal, mengesan tindak balas rawatan ataupun untuk menyelamatkan pesakit kanser payudara. Dalam kajian ini, elektroforesis gel dua-dimensi (2DGE) telah digunakan dalam usaha mencari penanda baru yang berpotensi untuk penyakit ini. Kultur sel kanser payudara dan sel normal telah digunakan untuk mengoptimumkan keadaan bagi menghasilkan peta proteom masing-masing kultur sel tersebut. Sampel tisu segar beku dari tumor serta tisu normal yang berdekatan telah dikumpulkan dari pesakit-pesakit yang menjalani pembedahan di HUKM, HKL dan Hospital Putrajaya. Sejumlah enam puluh sampel telah berjaya dikumpulkan dari bulan Jun 2005 sehingga

Disember 2006, dan telah disaringkan dengan 2DGE. Seterusnya, 24 sampel mewakili peringkat yang berbeza merupakan jenis karsinoma duktal infiltrasi (IDC) telah dianalisis dengan lebih lanjut menggunakan 17cm lajur IPG pada dua tahap pH yang berlainan iaitu lingkungan 3-10 dan 4-7. Kesemua gel kemudian dianalisis dengan menggunakan perisian PDQuest 7.3. Beberapa bintik protein penting yang berjaya dicerap telah dikeluarkan dari gel dan dianalisis dengan menggunakan spektrometer MALDI-TOF. Antigen penghambat tumor (gp96), protein kejutan haba 90 alfa (HSP 90α), himpunan nukleosom-1 menyerupai protein-1(NAP1L1) dan molekul pemula pelekat sel penambat opioid (OBCAM) telah dikenalpasti dan diekspresikan secara berlebihan dalam kultur sel kanser payudara, berbanding dengan kultur sel payudara normal. Kalretikulin, antigen penghambat tumor (gp96), protein kejutan haba 60 (HSP 60), dan sitokin penghambatan aruhan apoptosis-1 (CIAPIN1) telah didapati juga diekspresikan secara dua kali ganda atau lebih berlebihan dalam tisu tumor berbanding dengan tisu normal yang berdekatan. Sebaliknya, aktin γ 2 dan enzim pemfosfatan tirosin protein (PTPs) pula telah didapati ekspresinya ditekan dalam tisu tumor. Memandangkan ekspresi kalretikulin, sejenis protein pengikat kalsium, adalah lebih ketara pada peringkat penyakit yang berbeza yang mana ekspresinya disahkan dengan western blotting dan ianya telah dipilih untuk analisis yang lebih mendalam. Kuantitatif RT-PCR dengan GAPDH sebagai gen kekal perumah telah digunakan sebagai kawalan aras ekspresi gen dan hubung kait antara aras mRNA dan aras protein. Pada sampel yang mewakili peringkat lewat penyakit ini, aras mRNA didapati diekspresikan amat tinggi pada tisu tumor berbanding tisu normal yang berdekatan di mana secara purata lebih daripada 18 kali ganda peningkatan didapati. Aras mRNA juga didapati menurun di

dalam sampel peringkat ke IV di mana peningkatan sebanyak 12 kali ganda didapati, menandakan adanya peranan yang munasabah bagi protein ini di dalam perkembangan penyakit tersebut. Sebagai kesimpulan, pendekatan proteomik telah digunakan dalam kajian ini dan didapati berpotensi tinggi sebagai teknik dalam usaha mencari penanda yang baru untuk kanser payudara.

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I certify that an Examination Committee has met on to conduct the final examination of Lama Abdel Qader Moh'd Hamadneh on her Doctor of Philosophy thesis entitled "Application of Proteomics Approaches in the Identification of New Markers and Therapeutic Targets for Breast Cancer" in accordance Universiti Pertanian Malaysia (Higher Degree) act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The committee recommends that candidate be awarded the Doctor of Philosophy. Members of the Examination Committee are as follows:

Fauziah Othman, Ph.D.
Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Abdul Rahman Omar Ph.D.
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Internal Examiner)

Noorjahan Banu bt Mohamad Alitheen , Ph.D.
Lecturer
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Sheila Nathan, Ph.D.
Professor
Faculty of Science and Technology
Universiti Kebangsaan Malaysia
(External Examiner)

_____

HASANAH MOHD. GHAZALI, Ph.D.
Professor and Deputy Dean,
School of Graduate Studies,
Universiti Putra Malaysia

Date:

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

Rozita Rosli, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Sabariah Abdul Rahman, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

Chong Pei Pei, PhD
Lecturer
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

_____
AINI IDERIS, PhD
Professor and Dean,
School of Graduate Studies,
Universiti Putra Malaysia

Date:

DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously and is not concurrently submitted for any other degree at Universiti Putra Malaysia or at any other institutions.

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LAMA ABDEL QADER MOH'D HAMADNEH

Date: 7th August 2008

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LIST OF ABBREVIATIONS

2D	two-dimensional
2DGE	two dimensional gel electrophoresis
ACTB	Actin β
ACTG2	Actin γ 2
AJCC	American Joint Committee on Cancer
ASR	age standardized incidence rate
ATCC	American Type Culture Collection
CBB	Coomassie Brilliant Blue
cDNA	complementary deoxyribonucleic acid
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate
CIAPIN1	cytokine induced apoptosis inhibitor 1
DCIS	Ductal carcinoma *in situ*
DIGE	differential in-gel electrophoresis
DMEM	Dulbecco's modified Eagle's medium
DTE	dithioerythreitol
DTT	dithiothreitol
ECL	Enhanced chemiluminescence
ER	estrogen receptor
ER	endoplasmic reticulum
FNA	fine needle aspiration
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
gp96	tumor rejection antigen
H&E	hematoxylin and eosin
HER2	human growth factor receptor 2
HKL	Hospital Kuala Lumpur
HSP	heat shock protein
HSP60	Chaperonin / heat shock protein 60
HUKM	Hospital Universiti Kebangsaan Malaysia
IDC	infiltrating ductal carcinoma
IHC	immunohistochemistry
ILC	Invasive lobular carcinoma
IPG	immobilized pH gradient
LCIS	Lobular carcinoma *in situ*
LCM	laser capture microdissection
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometer
MHC	major histocompatibility complex
MMP-2	matrix metalloprotease-2
mRNA	messenger ribonucleic acid
M_r	molecular mass
NAP1L1	Nucleosome assembly protein 1-like 1
NOS	not otherwise specified

OPCAML	opioid-binding cell adhesion molecule precursor
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PgR	progesterone receptor
pI	isoelectric points
PTK	protein tyrosine kinase
PTP	protein tyrosine phosphatase
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodocyl sulphate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SELDI-TOF	surface-enhanced laser desorption/ionization time-offlight
SERM	selective estrogen receptor modulator
TCP1	T-complex protein 1
TEMED	Tetramethylethylenediamine
TMA	tissue microarray

CHAPTER 1

INTRODUCTION

Breast cancer is the most frequent cancer in most parts of the world and is a leading cause of death among women. In the year 2002, 1.15 million new cases were estimated to have occurred globally (Parkin *et al*., 2005). Since more than half of the cases were reported from developed countries, it suggests that screening programs adopted in the these countries led to the detection of early invasive tumors (Jemal *et al*., 2007), which on the other hand would have been missed or detected at later stages in developing countries (Parkin *et al*., 2005).

In 2008, a total of 184, 450 new cases from both sexes is estimated to be reported in the United States of America. Among them, 182, 460 new cases are expected be reported in females with 40,930 deaths (40,480 women and 450 men) (Jemal *et al*, 2008). It is also noted that breast cancer death rates have been decreasing gradually since 1990 due to early detection and improved treatments (ACS, 2007).

In Malaysia, the national cancer registry in 2003 reported breast cancer as the most common cancer in females from all age groups above 15, and in all ethnic groups with 3738 new cases. The overall age standardized incidence rate (ASR) was 46.2 per 100,000 of population and 64.1 % of the cases were diagnosed in women between 40

and 60 years old. Chinese had the highest incidence followed by Indians and Malays with ASR of 59.7, 55.8 and 33.9 per 100,000 of population, respectively (NCR, 2004).

However, the cultural and social beliefs of breast cancer in Malaysia are the most important contributors to the advanced stage of disease presentation (Hisham and Yip, 2003). Also, in a study performed on patients from 1998 to 2001, the average size of tumor was 5.4 cm in diameter and Malay women had larger tumors and a later stage of disease at presentation than other ethnic groups. This was due to a strong confidence in traditional medicine, poverty and poor education, the negative perception of the disease together with fear and denial (Hisham and Yip, 2004).

Current detection methods for breast cancer depend on mammography, but even though tumors detected by screening are significantly smaller than those non-screened ones, only 90% of tumors can be detected (Celis *et al*., 2005) and a tumor should be at least a few millimeters in size to be discovered, causing an important limitation to mammography (Hondermarck, 2003).

After diagnosis, patients with primary breast tumors are often offered surgery followed by adjuvant therapy. Nevertheless, factors like tumor size, auxiliary lymph node involvement, steroid receptor status and metastasis to other organs affect the 5 year survival rates (Celis *et al*., 2005), in which lymph node negative patients have approximately 25 % recurrence rate while around 40% with lymph node positive will experience a relapse and they often die from metastatic tumor. Also, in the quest to

increase the survival rates among patients, systemic adjuvant therapy has led to the improvement in the prognosis of the disease but carried an important side effect of over-treatment (Bergh and Holmquist, 2001).

Consequently, there is a critical need to identify specific predictive and prognostic factors to determine the groups that can benefit from individual treatment and also diagnostic markers that will aid in detecting the disease at very early stages and thus increase the survival rates.

Therefore, a better understanding of the changes that occur at the molecular and protein levels in breast cancer will facilitate the disease early detection and intervention by identifying new drug targets. The completion of human genome project has led to the rapid development in the technologies for the rapid and efficient analysis of the genes and their products, in which gene expression arrays and proteomics research particularly, are expected to provide important information to identify and characterize regulatory and functional networks of genes and proteins within cells (Celis *et al*., 2003) that are expected to accelerate the translation of basic research findings into clinical applications.

Even though studies of protein properties and their relation with diseases started in the 1970s, the field regained attention in the 1990s and a new name was applied "Proteomics" in 1995, that was defined as the large-scale characterization of the entire protein complement of a cell line, tissue, or organism (Kellner, 2000). A more inclusive definition was then introduced to combine the protein and genetic analyses (Pandey and

Mann, 2000) which has led to the emerging of different areas of research under proteomics, including protein-protein interaction studies, protein function, protein modifications, and protein location studies (Graves and Haystead, 2002). Accordingly, the involvement of different research disciplines such as biochemistry and molecular biology together with advances in mass spectrometry and bioinformatics resulted in the growth of proteomics as a powerful field towards the characterization of new markers and therapeutic targets (Hondermarck *et al*., 2002).

In breast cancer research, cell lines are widely used experimental models to obtain a better understanding of the disease. There are a number of established cell lines such as MCF-7 and MDA-MB-231 that are well characterized and commonly used (Clarke *et al*., 1996). Even though breast cancer cell lines are easy to handle and have a high degree of homogeneity, they are mostly obtained from pleural effusions (Burdall *et al*., 2003). Consequently, the results obtained from *in vitro* studies represent the aggressive tumors rather than primary lesions (Burdall *et al*., 2003). Thus, studying primary lesions of different tumor grades is more clinically relevant because most therapies are directed to these tumors.

In this study, two dimensional gel electrophoresis (2DGE) coupled with mass spectrometric analysis was applied in search of new prognostic markers and therapeutic targets for breast cancer. Breast cancer cell lines, tumors and adjacent normal tissues surgically obtained from Malaysian patients diagnosed with primary breast cancer between 2005 and 2006 were used.

Thus, the objectives of this study are:

1. To optimize the 2DGE conditions using breast cancer cell lines (MCF-7 and MDA-MB-231) and normal breast cell line (MCF-10A).
2. To screen and select protein samples extracted from surgically obtained tumor and their adjacent normal tissues.
3. To perform comparative differential analysis between tumor and normal tissues from infiltrating ductal carcinoma (IDC) samples.
4. To confirm the expression of potential markers in IDC samples using Western blotting.
5. To correlate the potential marker levels with their gene expression levels using real-time PCR.