OVER-EXPRESSION OF BIOMOLECULES IN PHOSPHATIDYLINOSITOL-3-KINASE/AKT SIGNALING PATHWAY IN BREAST CANCER

By

LOH HUI WOON

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

November 2005

Specially dedicated to,

The one who had given me the strength to complete this course…..

For their invaluable love, understanding, tolerance, sacrifice and moral support.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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Chairman : Professor Seow Heng Fong, PhD

Faculty: Medicine and Health Science

Breast cancer is the leading cancer among women in Malaysia. Genetics, experimental and epidemiological data suggest that breast cancer develops from complex interaction between inherited susceptibility and environmental factors. Accumulating evidence suggests that the PI3K/Akt signaling pathways play a causative role in tumorigenesis of breast cancer.

By employing the immunohistochemical method, the expression of several key regulators or related biomolecules of the PI3K/Akt signaling pathways in 43 archived formalin fixed, paraffin embedded tissues of surgically resected breast carcinoma specimens from 1999 to 2002, were studied. A functional assay was performed to determine the expression of Akt related molecules when treated with SDF-1α recombinant protein.

The results showed that: 1) The expression rates in tumour tissue of ERα, ERβ, c-erbB2, p-Akt^{T308}, p- Akt^{S473}, p-BAD^{S136}, SDF-1 and Ki67 were 25.6%, 4.7%, 51.2%, 81.4%, 48.8%, 67.4%, 93.0% and 26.8%, respectively. In contrast, in the apparently normal adjacent tissue, the expression rates of these molecules were 23.1%, 53.8%, 0%, 7.7%, 7.7%, 53.8%, 92.3% and 15.4%, respectively. 2) Correlation of biomolecules with tumour tissues and apparently normal adjacent tissues was seen in the following biomolecules: ERβ ($p=0.001$), c-erbB2 ($p<0.001$), p-Akt^{T308} ($p=<0.001$) and Ki67 ($p=<0.001$). 3) In tumour tissue, significant correlation was found between ERβ with p-BAD^{S136} ($p=0.004$), p-Akt^{S473} with p-BAD^{S136} ($p=0.006$), c-erbB2 with p-Akt^{T308} and Ki67 ($p=0.014$ and $p=0.000$ respectively) and c-erbB2 with SDF-1 ($p=0.047$). In the apparently normal adjacent tissue, a significant correlation was found between ERα with p-BAD^{S136} ($p=0.042$), ERβ with p-Akt^{S473} and p-BAD^{S136} ($p=0.009$ and $p=0.001$ respectively), and c-erbB2 with p-Akt^{T308} ($p=0.042$).

Our study also showed that SDF-1α protein had a different effect on the expression of biomolecules, namely p-Akt^{T308}, p- Akt^{S473} and p-BAD^{S136}. In this functional assay, we found that SDF-1α could possible induce cell survival by inducing phosphorylation of Akt at Thr308 and Ser473 as well as phosphorylation of BAD at Ser136 which are anti-apoptotic signals. Similar patterns were observed with all three cell lines, namely MCF-7, MDA-MB-231 and MCF10A but the level of expression differed from each other.

This study had provided three important information for researchers and clinicians in terms of: (1) evidence of the involvement of the SDF-1α in the PI3K/Akt signaling pathway in breast carcinoma tumourigenesis with detection of p-Akt. 2) For the first

time, we found c-erbB2 was inversely correlated with SDF-1α expression. 3) Identification of potential targets for therapeutic intervention of breast carcinoma.

On the basis of our data, we conclude that PI3K/Akt signalling pathway is involved in tumorigenesis of breast cancer. To the best of our knowledge, this is the first report from Malaysia. PI3K/Akt pathway might act with upstream molecules such as estradiol, SDF-1α, c-erbB2 independently in promoting tumour growth and inhibition of apoptosis. This study has also provided useful information for the search or design of antitumour interventions.

Abstrak thesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

EKSPRESI BERLEBIHAN BIOMOLEKUL-BIOMOLEKUL YANG TERDAPAT DALAM LALUAN ISYARAT PHOSPHATIDYLINOSITOL-3-KINASE/AKT PADA BARAH PAYU DARA

Oleh

LOH HUI WOON

November 2005

Pengerusi: **Profesor Seow Heng Fong, PhD**

Fakulti: **Perubatan dan Sains Kesihatan**

Barah payudara adalah barah yang paling biasa di Malaysia. Data dari segi genetik, eksperimen dan epidemiologi menunjukkan bahawa barah payudara berlaku apabila terdapat komunikasi yang kompleks antara faktor kejadian dan alam sekitar. Terdapat bukti yang mengatakan bahawa laluan isyarat PI3K/Akt memain peranan dalam kejadian barah payudara.

Hubungan antara beberapa pengawal-atur atau biomolekul yang berkaitan dengan lintasan isyarat PI3K/Akt telah dikaji dalam 43 tisu yang diperolehi daripada pesakit barah payudara dari tahun 1999 hingga 2002 telah diblokkan di dalam paraffin. Eksperimen fungsian telah dijalankan untuk menentukan ekspresi biomolekul Akt yang berhubungan dengan protin SDF-1α.

Keputusan kajian nenunjukkan bahawa: 1) ekspresi biomolekul ERα, ERβ, c-erbB2, p-Akt^{T308}, p- Akt^{S473}, p-BAD^{S136}, SDF-1 and Ki67 terdapat di tisu barah dalam peratus 25.6%, 4.7%, 51.2%, 81.4%, 48.8%, 67.4%, 93.0% and 26.8% masing-masing. Manakala ekspresi biomolekul tersebut pada tisu selkeliling yang kelihatan biasa adalah 23.1%, 53.8%, 0%, 7.7%, 7.7%, 53.8%, 92.3% and 15.4% masing-masing. 2) Kolorasi biomolekul dengan tisu selkeliling yang kelihatan biasa adalah seperti berikut: ERβ ($p=0.001$), c-erbB2 ($p<0.001$), p-Akt^{T308} ($p=<0.001$) dan Ki67 ($p=<0.001$). 3) Di tisu barah, kolerasi yang mencukupi terdapat di antara ERβ dengan p-BAD ^{S136} ($p=0.004$), di antara p-Akt ^{S473} dengan p-BAD ^{S136} ($p=0.006$), di antara c-erbB2 dengan p-Akt ^{T308} dan Ki67 ($p=0.014$ dan $p=0.000$ masing-masing), di antara c-erbB2 dengan SDF-1 ($p=0.047$). Pada tisu selkeliling yang kelihatan biasa, kolerasi yang mencukupi terdapat di antara ERα dengan p-BAD ^{S136} ($p=0.042$), di antara ERβ dengan p-Akt ^{S473} dan p-BAD ^{S136} ($p=0.009$ dan $p=0.001$ masing-masing), di antara c-erbB2 dengan p-Akt ^{T308} ($p=0.042$).

Kajian ini juga menunjukkan bahawa protin SDF-1α menpunyai rangsangan yang berbeza terhadap exkpresi pelbagai biomolekul seperti p-Akt^{T308}, p- Akt^{S473} and p-BAD^{S136}. Dalam kajian fungsi ini, kami mendapati SDF-1α boleh merangsang kehidupan sel melalui penfosforilasi Akt pada Thr 308 dan Ser473 yang akan menfosforilasi BAD pada Ser136 dan lalu menutup laluan apoptotic. Terdapat kesamaan antara eksresi biomolekul pada tiga jenis sel yang lain, MCF-7, MDA-MB-231 dan MCF10A dengan perbezaan tahap ekspresi yang berlainan.

Kajian kami menghasilkan sekurang-kurangnya tiga maklumat yang penting kepada para penyelidik dan perubatan dari segi: 1) bukti pembabitan SDF-1α dalam laluan

isyarat PI3K/Akt dalam barah payudara. 2) Buat kali pertama, kami menunjukan bahawa c-erbB2 berkolerasi terbalik dengan ekspresi SDF-1. 3) Pengenalan sasaran-potensi bagi intervensi barah payudara terapi maju. Berdasarkan keputusan ini, kami mencadangkan bahawa SDF-1α boleh dianggap sebagai sasaran terapeutik yang berpotensi untuk rawatan immuno barah payudara.

Berdasarkan keputusan yang diperolehi, kami membuat kesimpulan bahawa lintasan isyarat PI3K/Akt adalah berkait dengan tumorigenesis barah payudara di Malaysia. Lintasan isyarat ini adalah saling berhubungan dengan molekul awalan seperti estradiol, SDF-1α dan c-erbB2 walaupun mereka juga boleh bertindak secara berasingan untuk menggalakkan pertumbuhan barah dan perencatan apoptosis. Kajian ini telah memberi maklumat yang berguna kepada para penyelidik dan perubatan dalam penemuan dan perekaan cara anti-barah yang lebih baik.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my main supervisor, Prof. Dr. Seow Heng Fong for giving me the chance to work on this most interesting project. Her generous guidance, endless help and encouragement throughout the period of this project have been the base and driving force for this thesis. Her careful review and constructive criticism have been crucially important for this thesis.

I would also like to acknowledge and thank my co-supervisors, Assoc. Prof. Dr. Hairuszah Ithnin and Dr. Thuaibah Hashim, for their endless help, advice and comments throughout the entire progress of this project.

I am grateful to the staff of Pathology and Surgery, Hospital Universiti Kebangsaan Malaysia, especially Mr. Rohaizak and Ms. Nakyiah, for generously providing tissue specimens and collection of the clinical information. Not forgetting all the staff of Histopathology Unit, Dept of Clinical Laboratory Science, UPM, especially Mrs. Juita and Pn. Normah for kindly allowing me access to their laboratory facilities.

I am indebted to my labmates, especially Dr. Maha Abdullah, Mr. Khor Tin Oo, Mr. Yip Wai Kien, Ms. Lim Pei Ching, Ms. Leong Pooi Pooi, Ms. Ong Hooi Tin, Ms. Cheah Hwen-Yee, Ms. Masriana Hassan and Mr. Anthonysamy for their collaboration and sharing everyday joys and miseries in the making of science.

My family, Mom, Dad, Sisters (Hui Ling and Hui Yee) who had given me endless support in completion of this thesis.

I certify that an Examination Committee met on 29th November 2005 to conduct the final examination of Loh Hui Woon on her Master of Science thesis entitled "Over-Expression of Biomolecules in Phosphatidylinositol-3-Kinase/Akt Signaling Pathway in Breast Cancer" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee are as follows:

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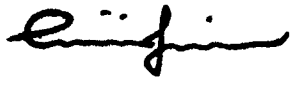

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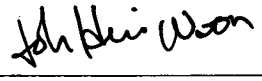
Date: 07 FEB 2006

DECLARATION

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



LOH HUI WOON

Date: 10/04/2006

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APES	3-Aminopropyltrimethoxysilane
BSA	Bovine serum albumin
°C	Celsius degree
$CaCl_2$	Calcium chloride
DAB	3,3'-Diminobenzidine
dH_2O	Distilled water
DNA	Deoxyribonuleic acid
DTT	1,4-Dithiothreitol
EDTA	Ethylenediaminetetracetic acid
g	Gram
h	Hour(s)
HCl	Hydrochloric acid
mg	Millligram
$MgCl_2$	Magnesium chloride
min(s)	Minute(s)
ml	Milliliter
mM	Millimolar
n	Nano
NaCl	Sodium chloride
NaOH	Sodium hydroxide
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol-3 Kinase
PMSF	Phenylmethylsulfonyl fluoride

RNA	Ribonucleic acid
rpm	Revolutions per minute
s	Second(s)
TAE	Tris acetate EDTA buffer
Taq	Thermus aquaticus
µl	Microlitre
µg	Microgram
v/v	Volume per unit volume

CHAPTER 1

INTRODUCTION

Cancer is a disease that involves the dysfunction of the immune system. Transformation of normal cells to abnormal cells usually leads to apoptosis of that transformed cells. Cancer occurs when the immune system lost its ability to do surveillance to destroy those abnormal cells. The cancer cells could then proliferate uncontrollably into a mass. Loss of their normal function may interfere with the other body systems.

Breast cancer is the commonest cancer among women all around the world and it is a significant global disease burden. In Malaysia, there were 4337 cases reported by the National Cancer Registry Malaysia 2002. Worldwide, the ratio of mortality to incidence is about 36% which, compared to other cancer types, represents a relatively good prognosis. However, it remains the leading cause of cancer mortality in women and its treatment is often associated with toxicity and unfavourable cosmetic outcome that impacts greatly on quality of life.

After several decades of cancer research focusing only on the tumour cell itself, we are just realizing that cancer is not only a group of abnormally growing cells, but it is an abnormal mass with multiple cell types communicating with each other (Polyak, 2001).

Many methods of early detection and treatment of breast cancer had been developed, but they are still not enough to fully and successfully treat all breast cancer patients. Intensive research efforts have been conducted to find the cause of this disease, but unfortunately, the causative factor of the disease has still not been found.

Oestrogen receptor α (ERα) belongs to the superfamily of steroid nuclear receptor transcriptional factors. It regulates the proliferation and differentiation of many tissues, especially reproductive tissues. On binding to specific DNA sequences such as estrogen responsive elements (EREs), oestrogen-ERα complexes activate or repress target gene transcription. The biological activity of oestrogen is now realized to be more complex than initially thought, with the discovery of a second oestrogen receptor (ER) named ERβ (Girault *et al.*, 2003). ERs utilize the membrane epidermal growth factor receptor (EGFR) to rapidly signal through various kinase cascades that influence both transcriptional and non-transcriptional actions of estrogen in breast cancer cells (Levin *et al.*, 2003). Recent evidence suggests that common adaptations which occur during resistance to both tamoxifen and oestrogen deprivation use various signal transduction pathways, often involving cross-talk with a retained and functional ER protein (Johnston *et al.*, 2003). Oh and colleagues (2001) found that hyperactivation of mitogen-activated protein kinase (MAPK) could induce loss of ERα expression in breast cancer cells. This might be one of the causes of resistance to antioestrogen drugs in ERα positive cells. Studies of forced c-erbB2 overexpression in animals and cell lines have demonstrated the oncogenic potential of c-erbB2, and spontaneous homodimerization leading to tyrosine kinase activation is most likely an important mechanism for the oncogenicity of c-erbB2 overexpression (Siegel and Muller, 1996). Lindberg and colleagues identified the