

**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-1 α recombinant protein.

The results showed that: 1) The expression rates in tumour tissue of ER α , ER β , 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: ER β (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 ER β 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 ER α with p-BAD^{S136} (p=0.042), ER β 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-1 α 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-1 α could possibly 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-1 α 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-1 α 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-1 α , 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 DARAH

Oleh

LOH HUI WOON

November 2005

Pengerusi: Profesor Seow Heng Fong, PhD

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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 memainkan 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-1 α .



Keputusan kajian menunjukkan bahawa: 1) ekspresi biomolekul ER α , ER β , 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: ER β (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 ER β 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 ER α dengan p-BAD^{S136} (p=0.042), di antara ER β 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-1 α mempunyai rangsangan yang berbeza terhadap ekspresi pelbagai biomolekul seperti p-Akt^{T308}, p-Akt^{S473} and p-BAD^{S136}. Dalam kajian fungsi ini, kami mendapati SDF-1 α 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 ekspresi 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-1 α dalam laluan

isyarat PI3K/Akt dalam barah payudara. 2) Buat kali pertama, kami menunjukkan 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-1 α 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-1 α 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.

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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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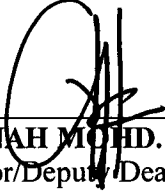
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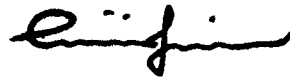
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DECLARATION

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

LOH HUI WOON

Date: *10/01/2008*

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LIST OF ABBREVIATIONS

| | |
|-------------------|--------------------------------|
| APES | 3-Aminopropyltrimethoxysilane |
| BSA | Bovine serum albumin |
| °C | Celsius degree |
| CaCl ₂ | Calcium chloride |
| DAB | 3,3'-Diminobenzidine |
| dH ₂ O | Distilled water |
| DNA | Deoxyribonucleic acid |
| DTT | 1,4-Dithiothreitol |
| EDTA | Ethylenediaminetetracetic acid |
| g | Gram |
| h | Hour(s) |
| HCl | Hydrochloric acid |
| mg | Milligram |
| 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 |
| μ l | Microlitre |
| μ g | 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 (ER α) 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-ER α 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 ER β (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 ER α expression in breast cancer cells. This might be one of the causes of resistance to antioestrogen drugs in ER α 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