UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF COMPLEMENT C5A AND ITS RECEPTOR IN MALIGNANT TUMOUR CELLS AS MAMMARY TUMOUR BIOMARKER

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By

NORHAIFA BINTI GANTI

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

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DEDICATION

This thesis is specially dedicated to my beloved parents,
Ganti bin Hakem and Napsiah binti Mohd Aris,
for their never ending love and whom always pray for
my success and guided me through life

To my dear husband, Mohd Reduzuan bin Mahfod
and my lovely daughter, Siti Nuraqilah binti Mohd Reduzuan,
also not forgetting my sisters, Noramiza, Norazila,
Noraidila, Norliana, Siti Aisah and Siti Asiah,
Thank you for supporting my aspirations
with love, encouragement, patience and belief in me.

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

DEVELOPMENT OF COMPLEMENT C5A AND ITS RECEPTOR IN MALIGNANT TUMOUR CELLS AS MAMMARY TUMOUR BIOMARKER

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Chairman: Mohd Hezmee bin Mohd Noor, PhD
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Mammary cancer is the most common disease among women and second cause of female mortality related to cancer. The relationship between immune system and mammary cancer is still questionable. C5a is an important chemotactic protein that is recognized as an anaphylatoxin and chemoattractant that exerts proinflammatory actions in many pathological states. The complement C5a and its receptor are believed to be involved in development of mammary tumour due to its inflammatory properties.

This study investigates the role of complement 5a (C5a) on mouse mammary cancer development by using malignant mouse mammary tumour cell line; 4T1. The expression of C5a/C5aR was determined by using Immunofluorescence staining and Reverse-Transcriptase PCR (RT PCR) and subsequently with Real Time PCR (qPCR) to measure the magnitude of C5a/C5aR expression. 40 specific pathogen free (SPF) Balb/c mice were randomly divided into four treatment groups. Each mouse was injected subcutaneously with $1x10^6$ cells/ml into the mammary fat pad and treatments were given in the same manner. The liver samples were used for further analysis to validate the use of C5a and its receptor as mammary tumour biomarker by using Enzyme-linked Immunosorbent Assay (ELISA) and qPCR.

The Immunofluorescence staining showed the green colour surrounding the blue colour of nucleus. The green colour indicates the presence of C5a receptor in the membrane of 4T1 cell line. Meanwhile, the Reverse Transcriptase PCR technique gave an intense single band in the agarose gel when viewed under gel documentation machine. These collective results

proved that there are expression of C5a and its receptor in the malignant mammary tumour cell line; 4T1.

The result for *in-vitro* studies showed that the PMX205 peptide gave lower percentage of cell viability than EP54 peptide when compared to the control group for each time line. The trend of the cell viability values was consistent and showed a significant result. This indicates that this peptide is capable to regress and kill these tumour cells.

Meanwhile, qPCR technique showed that the magnitude of C5a and its receptor in PMX 205 treated group was lower than EP54 treated group and the magnitude of C5a and its receptor in normal tissue were significantly lower compared to its magnitude in tumour tissue. The consistency of *in-vitro* and *in-vivo* results strengthens the arguments regarding the involvement of C5a and its receptor in the development of malignant mammary cancer.

The findings in this study showed that there is a functional relationship between C5a and the development of mammary tumour which suggested that C5a and its receptor can be used as mammary tumour biomarkers and C5a antagonist peptide (PMX205) has a potential in treating and preventing development of mammary tumour by blocking the receptor of C5a and inhibits the binding of C5a to its receptor. However, further studies are required to validate these findings including the exact role of C5a and its mechanism in malignant mammary tumour development and the potential of C5a as mammary tumour biomarkers.

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

PERKEMBANGAN PENGGUNAAN KOMPLIMEN C5A DAN RESEPTOR DI DALAM SEL MALIKNAN KETUMBUHAN SEBAGAI PENUNJUK BIOLOGI KETUMBUHAN PAYUDARA

Oleh

NORHAIFA BINTI GANTI

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Kanser payudara adalah penyakit yang selalu dialami dikalangan wanita dan menjadi penyebab kedua kematian dikalangan wanita. Hubungan diantara sistem keimunan dan kanser payudara masih menjadi persoalan. C5a adalah protein kemotaktik yang penting di dalam membantu perekrutan sel-sel radang. Ia juga dikenal pasti sebagai anafilotoksi dan agen penarik kimia yang mencetuskan tindakan keradangan awal di dalam banyak jenis kondisi patologi. Komplimen C5a dan reseptornya dipercayai terlibat di dalam pembentukan ketumbuhan payudara kerana ia mempunyai ciri-ciri keradangan.

Kajian ini menyiasat peranan komplimen C5a ke atas kanser payudara pada mencit dengan menggunakan sel maliknan ketumbuhan payudara mencit; 4T1. Ekspresi C5a/C5aR ditentukan dengan menggunakan teknik Pewarnaan Imunoflorasi, dan Transkrip Berbalik PCR (RT-PCR) dan seterusnya, teknik Realiti Masa PCR (qPCR) telah digunakan untuk mengukur maknitud ekspesi C5a/C5aR. 40 ekor mencit jenis balb/c yang bebas daripada spesifik patogen telah dibahagikan secara rawak kepada 4 kumpulan perawatan. Setiap mencit telah disuntik melalui bawah kulit dengan $1x10^6$ sel/ml di tisu lemak payudara dan rawatan telah diberikan pada masa yang sama. Sampel tisu hati telah digunakan untuk analisis seterusnya untuk mengesahkan penggunaan C5a dan pemegangnya sebagai penunjuk biologi untuk ketumbuhan payudara dengan menggunakan Realiti Masa PCR (qPCR) dan Asai Penghubung Enzim dan Imun (ELISA).

Kaedah Pewarnaan Imunoflorasi telah menunjukkan bahawa sekeliling nukleus yang berwarna biru telah diwarnai dengan warna hijau. Ini menunjukkan reseptor C5a terletak di membran sel 4T1. Sementara itu, kaedah Transkrip Berbalik PCR telah menunjukkan satu jalur yang pekat pada gel agaros apabila

diperhatikan di bawah mesin dokumentasi gel. Keseluruhan keputusan ini membuktikan bahawa terdapat ekspresi C5a dan reseptornya di dalam sel maliknan ketumbuhan payudara; 4T1.

Keputusan dari kaedah *in-vitro* pula menunjukkan peptida PMX205 memberi peratus keupayaan maju sel yang rendah daripada peptida EP54 apabila dibandingkan dengan kumpulan kawalan. Manakala maknitud C5a dan reseptornya di dalam tisu normal adalah signifikasi rendah berbanding dengan tisu ketumbuhan. Keputusan yang konsisten di dalam kaedah *in-vitro* dan *in-vivo* menguatkan lagi teori bahawa C5a dan reseptornya terlibat dalam pembentukan kanser maliknan payudara.

Hasil kajian ini menunjukkan bahawa terdapat hubungan berfungsi di antara C5a dan pembentukan ketumbuhan payudara di mana ianya mempunyai potensi untuk dijadikan sebagai penunjuk biologi untuk ketumbuhan payudara dan juga peptida PMX205 mempunyai potensi di dalam merawat dan menghalang pembentukan ketumbuhan payudara kerana sifatnya yang boleh menghalang percantuman di antara C5a dan reseptornya. Walaubagaimanapun, kesinambungan kajian diperlukan termasuk fungsi sebenar C5a dan mekanismanya untuk mengesahkan penggunaan C5a dan reseptornya sebagai penunjuk biologi untuk ketumbuhan payudara.

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I certify that a Thesis Examination Committee has met on 3 April 2015 to conduct the final examination of Norhaifa binti Ganti on her thesis entitled "Development of Complement C5A And its Receptor in Malignant Tumour Cells As Mammary Tumour Biomarker" 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 Master of Science.

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This thesis was 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 were as follows:

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Declaration by graduate student

I hereby confirmed that:

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LIST OF ABBREVIATIONS

ACUC	Animal Care and Use Committee
ASCO	American Society of Clinical Oncology
ASR	Age Standardized Incidence Rate
ATCC	American Type Culture Collection
BC	Before Century
Bp	Base pair
BRCA	Breast Cancer Gene
BSA	Bovine Serum Albumin
CAP	College of American Pathologists
cDNA	Complementary Deoxyribonucleic Acid
CO_2	Carbon dioxide
C3	Complement 3
C3a	Complement 3a
C3aR	Complement 3a Receptor
C3b	Complement 3b
C5	Complement 5
C5a	Complement 5a
C5aR	Complement 5a Receptor
C5b	Complement 5b
CT	Cycle Threshold
ddH2O	Double distilled water
dsDNA	Double stranded Deoxyribonucleic Acid
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-linked immunosorbent assay
EP54	C5a Agonist
ER	Estrogen Receptor
fg	Femtogram
FITC	fluorescein isothiocyanate
g	Gram
GAPDH	Glyceraldehyde 3—phosphate dehydrogenase
HER-2	Human Epidermal Growth Factor Receptor 2
IFN-γ	Gamma Interferon
kDa	Kilo Dalton
LAFAM	Laboratory Animal Facility and Management
MAC	membrane attack complex
mg	Miligram
MgSO4	Magnesium Sulphate
min	Minute
mins	Minutes
ml	Mililiter
mM	Milimolar
mol	Molar
mRNA	Messenger Ribonucleic Acid
MTT	Methylthiazol Tetrozolium
NCR	National Cancer Registry
ng	Nanogram

OD	Optical Density
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
pg	Picogram
PMX 205	C5a Antagonist
PR	Prongesterone Receptor
qPCR	Real Time Polymerase Chain Reaction
RLT	RNeasy lysis buffer
RNA	Ribonucleic Acid
rpm	Rotation per minute
RPMI	Roswell Park Memorial Institute
RQ	Relative quantification
RT	Reverse Transcriptase
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
Secs	Seconds
SERMs	Selective estrogen receptor modulators
SPF	Specific Pathogen Free
TAE	Tris-Acetate-EDTA
Tm	Melting Temperature
TRITC	tetramethyl rhodamine isothiocyanate
UV	Ultraviolet
WHI	Women's Health Initiative
^{o}C	Degree Celcius
µg	Microgram
µl	Microlitre
µmol	Micromolar

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Cancer is a worldwide disease that leads to thousands of deaths every year. In developing country like the United States, breast cancer is the most common type of cancer and is a second leading cause of death after lung cancer. It is estimated that around 232,670 new cases of breast cancer with an estimation of 40,000 deaths (National Cancer Institute, 2014) were reported in the year 2014. In this country according to the Malaysian National Cancer Registry Report (2005), about one in 20 women are at risk of getting breast cancer compared to other developing countries.

There are many factors that can contribute to breast cancer occurrence. One of the factors is the body's immune system. Even though the immune system is a biological system that help protects the body from infectious organisms and biological invaders, it also can cause harmful effects to the host itself in form of autoimmune diseases and acute and chronic inflammation.

Previous studies indicate that there are functional relationship between cancer and inflammation in which the chro6nic inflammation promotes several types of cancer (Fitzpatrick, 2001; Hong et al. 2010). This is because an improper cell proliferation resulting from chronic inflammation can rendered the cells or tissues undergone a severe inflammation to change its morphological, biological and physiological characteristics thus transforming these cells or tissues to become cancerous.

C5a is one of the complement activation products in immune system that involves in the recruitment of inflammatory cells to the site of infection or targeted region in the body. The exact role of C5a in the body is still unclear due to lack of information about this type of complement. However, previous study showed that C5a is the most potent inflammatory peptides and thus involves in several inflammatory diseases (Dondorp et al., 2005).

1.2 Problem Statement

The early detection of breast cancer is essential because it may give higher chances for the patients to live longer and requiring less extensive treatment (Hortobagyi et al., 2005). However, the early detection of breast cancer is difficult due to its asymptomatic trait when the tumour is still small and undetectable.

The mortality rate for breast cancer patients is high due to its late detection and diagnosis in some cases. Usually, breast cancer is detected during the third stage in which the cancer has overwhelmed the hosts and had already metastasized to other site in the body. Even though there are many treatments for breast cancer such as radiation therapy, chemotherapy, hormone therapy, targeted therapy and surgery but all these treatments have substantial amount of negative side effects to the patients.

1.3 Significance of Study

Breast cancer is the most common type of cancer in women worldwide. The rate of deaths due to breast cancer is reaching an alarming rate. It is due to late stage detection of breast cancer resulting from the difficulties in early detection of these cancers. Obviously, breast cancer has a potential to be cured if it can be detected early (Etzioni et al., 2003).

Nowadays, scientists are continuously discovering and establishing biomarkers for early detection of the breast cancer stages. The examples of established breast cancer biomarkers are estrogen receptor (ER), progesterone receptor (PR) and HER-2/neu. However, until now, the suitable biomarker to detect an early stage of breast cancer occurrence is still unidentified (Weigel & Dowsett, 2010). Previous studies showed that C5a may involve in the tumour development and appear very early in the pathological states (Dondorp et al. 2005; Markiewski et al. 2008; Patel et al. 2008). Thus, the main objective of this study is to develop C5a and its receptor as a potential mammary tumour biomarker for early stage detection.

1.4 Objectives of Study

1) To determine the expression of C5a and its receptor in malignant mammary tumour cell line;4T1;
2) To measure the magnitude of the expression and up-regulation of C5a and its receptor in mouse mammary cancer;
3) To validate the use of C5a and its receptor as potential biomarkers forthe screening of mammary cancer in animals.

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