



***EXPRESSION AND IDENTIFICATION OF C5a AND ITS RECEPTOR
(C5aR) AS A DIAGNOSTIC MARKER FOR BENIGN MAMMARY
TUMOUR***

NOOR FARHANA BINTI BACHEK

FPV 2016 11



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By

NOOR FARHANA BINTI BACHEK



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

February 2016

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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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Chair: Mohd Hezmee Bin Mohd Noor, PhD

Faculty: Veterinary Medicine

Complement system is a part of innate and adaptive immune system that act as a 'first line of defense' against infections and immune complex diseases. There are three main pathways activate the complement system; classical pathway, alternative pathway and lectin pathway. Although these three pathways are activated by different triggers and stimuli, these step-wise activations eventually lead to the cleavage of the C5 molecule and generating C5a and C5b. C5a is a 74 amino acids sequence protein fragment derived from the cleavage of complement component by protease C5-convertase. C5a was first described as a classical anaphylatoxin and a highly inflammatory peptide and have been found in the serum of patient with inflammatory disorder such as cancer. However, the possible involvement between C5a anaphylatoxin and tumour progression is not yet well understood. In this study, we assessed the expression of C5a/C5aR in the EMT6 cells and measured the expression and up-regulation of C5a/C5aR in both mRNA and protein levels. We demonstrate that the expression of C5a/C5aR is up-regulated in EMT6 cells and can be potentially use as a screening biomarker for mammary cancer.

The first part of study was the determination of the expression of C5a/C5aR mouse mammary cancer cells by Immunofluorescence staining using compatible antibodies followed by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) of EMT6 cell. The study was further analyzed by treating the EMT6 cells using three types of treatment; EP54 (C5a agonist peptide),

PMX205 (C5a antagonist peptide) and control drug of tamoxifen. Thus, the magnitude of the C5a/C5aR expression of the treated EMT6 cell was measured by quantitative Real-Time Polymerase Chain Reaction (qPCR). The validation assessment of C5a/C5aR as a screening biomarker was determined using cell cytotoxicity assay of MTT and enzyme-linked immunosorbent assay (ELISA) using C5a antibodies.

The immunofluorescence staining of EMT6 cells indicate the presence of C5aR receptors located on the membrane of EMT6 cells. This finding is further justified the C5a receptor expression of EMT6 cells in mRNA level with detection of a single band using RT-PCR. The relative quantification of C5a/C5aR magnitude of treated EMT6 cell showed the reduction C5a/C5aR level in PMX205 as compared to EP54 with the fold change of 0.307 ± 0.2885 and 0.625 ± 1.2109 , respectively. This findings showed similar pattern in validation assessment of C5a/C5aR where PMX205 reduced the level of C5a in MTT and ELISA as compared to EP54. Overall, the viability of EMT6 cells treated with PMX205 showed a greater cell inhibition as compared to EP54 throughout the time points. The value recorded for PMX205 on 24, 48 and 72 hours treatment were (0.1069 ± 0.016) , (0.1066 ± 0.008) and (0.0543 ± 0.001) , respectively. As for EP54, the value recorded for 24, 48 and 72 hours treatment were (0.1137 ± 0.03) , (0.1164 ± 0.04) and (0.0561 ± 0.02) , respectively. While on ELISA, the PMX205 exhibited a reduced C5a protein level (0.1713 ± 0.08) in comparison to EP54 (0.2511 ± 0.183) .

In conclusion, these data suggest that there EMT6 cells indeed exhibit the C5a/C5aR expression and have the capacity to generate C5a due to magnitude and up-regulation of C5a concentration detected in both mRNA and protein levels. This study also exhibit the potential pharmacological blockage using C5a antagonist decreased the level of C5a in the mammary cancer and hence, C5a/C5aR has a potential utility as a screening biomarker for mammary cancer.

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

**EKSPRESI DAN IDENTIFIKASI KOMPLIMEN C5A DAN RESEPTOR
C5A (C5aR) SEBAGAI PENANDA DIAGNOSTIK BAGI TUMOR
PAYUDARA BENIGNA**

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Sistem komplimen adalah sebahagian daripada sistem pertahanan inat dan penyesuaian yang bertindak sebagai barisan pertama pertahanan terhadap jangkitan dan penyakit imun kompleks. Terdapat tiga laluan utama pengaktifan sistem komplemen, laluan klasikal, laluan alternatif dan laluan lektin. Walaupun ketiga – tiga laluan ini diaktifkan dengan pencetus dan rangsangan yang berbeza, langkah pengaktifan ini menjurus kepada pembahagian molekul C5 and penghasilan C5a dan C5b. C5a adalah serpihan 74 asid amino rantaian protein terbitan daripada pembahagian komponen komplemen oleh protease C5-konvertase. C5a telah pertama kali digambarkan sebagai anafilatoksin klasikal dan peptida tinggi radangan dan telah dijumpai di dalam serum pesakit yang mempunyai gangguan radangan seperti kanser. Walaubagaimanapun, kemungkinan penglibatan antara anafilatoksin C5a dan perkembangan tumor masih belum difahami dengan jelas. Dalam kajian ini, kami menaksir ekspresi C5a/C5aR dalam sel EMT6 dan mengukur ekspresi dan peningkatan regulasi C5a/C5aR di dalam kedua – dua tahap mRNA dan protein. Kami membuktikan bahawa peningkatan regulasi ekspresi C5a/C5aR dalam sel EMT6 dan kedapatan ini berpotensi digunakan sebagai penanda bio saringan bagi kanser payudara.

Bahagian pertama kajian ini adalah penentuan ekspresi C5a/C5aR sel kanser payudara tikus melalui pewarnaan Imunopendarflour menggunakan antibodi serasi diikuti dengan Transkriptase Berbalik Reaksi Rantaian Polimerase (RT-PCR) sel EMT6. Kajian ini dianalisis secara lanjut dengan merawat sel EMT6 dengan menggunakan tiga jenis rawatan; EP54 (peptida agonis C5a), PMX205 (peptida antagonis C5a) dan tamoxifen. Oleh demikian, magnitud ekspresi C5a/C5aR sel EMT6 telah diukur dengan kuantitatif Waktu Nyata Reaksi Rantaian Polimerase (qPCR). Penaksiran pengesahan C5a/C5aR sebagai saringan penanda bio ditentukan dengan

asai kesitotoksikan sel MTT dan asai imunoserap terangkai ensim (ELISA) menggunakan antibodi C5a.

Pewarnaan Imunopendarflour sel EMT6 menunjukkan kewujudan reseptor C5aR yang terletak di membran sel EMT6. Ini memberi justifikasi lanjutan tentang ekspresi C5a sel EMT6 di tahap mRNA dengan pengesahan jalur tunggal menggunakan RT-PCR. Kuantifikasi relatif magnitud C5a/C5aR sel EMT6 terawat menunjukkan penurunan aras C5a/C5aR dalam PMX205 berbanding EP54 dengan perubahan kali ganda masing – masing 0.307 ± 0.2885 dan 0.625 ± 1.2109 . Penemuan ini menunjukkan pola yang sama dalam penaksiran pengesahan C5a/C5aR dimana PMX205 mengurangkan tahap C5a dalam MTT dan ELISA berbanding EP54. Secara keseluruhan, kebolehhdidan sel EMT6 yang dirawat dengan PMX205 menunjukkan perencatan sel yang lebih berbanding EP54 sepanjang titik masa. Nilai yg dicatatkan bagi PMX205 pada 24, 48 and 72 jam rawatan masing – masing adalah (0.1069 ± 0.016) , (0.1066 ± 0.008) dan (0.0543 ± 0.001) . Bagi EP54, nilai yang dicatatkan pada 24, 48 and 72 jam rawatan masing – masing adalah (0.1137 ± 0.03) , (0.1164 ± 0.04) dan (0.0561 ± 0.02) . Manakala pada ELISA, PMX205 memperlihatkan penurunan tahap protein C5a (0.1713 ± 0.08) berbanding dengan EP54 (0.2511 ± 0.183) .

Secara kesimpulannya, data ini mencadangkan bahawa sel EMT6 sememangnya mempamerkan ekspresi C5a/C5aR dan mempunyai kebolehan untuk menjana C5a berdasarkan magnitud and peningkatan regulasi kepekatan C5a yang dikesan dalam kedua – dua tahap mRNA dan protein. Kajian ini juga mempamerkan potensi sekatan farmakologikal menggunakan antagonis C5a dalam penurunan tahap C5a dalam kanser payudara dan dengan demikian, C5a/C5aR mempunyai utiliti yang berpotensi sebagai penanda bio saringan untuk kanser payudara. pemahaman mekanisma ketoksikan dadah.

ACKNOWLEDGEMENTS

“In the name of Allah, Most Gracious, Most Merciful”

I would like to express my appreciation to my supervisor, Dr. Mohd Hezmee Bin Mohd Noor for his guidance throughout the whole period my project. I also want to thank him for his support, constructive comments and valuable suggestion during the project. Not to forget, special gratitude to my co-supervisors, Prof. Dr. Zuki bin Abu Bakar, Dr. Intan Shameha binti Abdul Razak and Dr. Rozaini binti Mohd Zohdi and also Pre-Clinical science lecturers, Dr. Hafandi bin Ahmad and Dr. Mokrish bin Ajat.

Secondly, I would like to express my gratitude to all my friends and especially my project group members; Nurul Hazwani Binti Kamarudin, Norhaifa Binti Ganti and Nurneqman Nashreq Bin Kosni for all the support and their precious co-operation and helps through my thick and thin.

Last but not least, I would like to express my gratitude and appreciation to my family members; especially my parents who always be there for me for all time and my friends. Their moral supports had always encouraged and inspire me to complete my Master of Science Degree.

Without them, nothing would have been possible.

I certify that a Thesis Examination Committee has met on 2nd February 2016 to conduct the final examination of Noor Farhana Binti Bachek on her thesis entitled “Expression and Identification Of C5a and Its Receptor (C5aR) as a Diagnostic Marker for Benign Mammary Tumour” 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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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
AI	Aromatase Inhibitor
AMD	Age-related Macular Degeneration
AMV	Avian Myeloblastosis Virus
BCA	Bicinchoninic Acid
BCS	Breast-Conserving Surgery
Bp	Base pair
BRCA	Breast Cancer Gene
BSA	Bovine Serum Albumin
CHEK2	Checkpoint Kinase 2
C-terminus	Carbon-Terminus
CO ₂	Carbon dioxide
C1INH	C1 Inhibitor
C3	Complement 3
C3b	Complement 3b
C4bp	C4-binding Protein
C5	Complement 5
C5a	Complement 5a
C5aR	Complement 5a Receptor
C5a-des arg	C5a derivative
C5b	Complement 5b
C5L2	C5a receptor 2
C6	Complement 6
C7	Complement 7
C8	Complement 8

C9	Complement 9
CD35	Complement receptor type 1
CD46	Complement regulatory protein CD46
CD59	Complement regulatory protein CD59
CD55	Complement decay-accelerating factor
CD88	C5a receptor 1
cT	Cycle Threshold
DCIS	Ductal Carcinoma In Situ
DAPI	4',6-Diamidino-2-Phenylindole
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide
DSS	Dextran Sulphide Sodium
EBCTCG	Early Breast Cancer Trialists' Collaborative Group
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-linked immunosorbent assay
EP54	C5a Agonist
ER	Estrogen Receptor
FDA	Food and Drug Administration
FBS	Fetal Bovine Serum
fH	Complement Regulatory Protein Factor H
fI	Complement Regulatory Protein Factor I
FITC	fluorescein isothiocyanate
g	Gram
G	Gauze
GAPDH	Glyceraldehyde 3—phosphate dehydrogenase

HER-2	Human Epidermal Growth Factor Receptor 2
HRT	Hormone-Replacement Therapy
IARC	International Agency for Research on Cancer
IDC	Invasive Ductal Carcinoma
IgG	Immunoglobulin G
ILC	Invasive Lobular Carcinoma
IgM	Immunoglobulin M
KCl	Potassium Chloride
kDa	Kilo Dalton
LCIS	Lobular Carcinoma In Situ
M	Molar
MAC	Membrane Attack Complex
MBL	Mannan Binding Lectin
mg	Miligram
MgSO ₄	Magnesium Sulphate
ml	Milliliter
mM	Millimolar
mRNA	Messenger Ribonucleic Acid
MTT	Methylthiazol Tetrozolium
N-terminus	Nitrogen Terminus
NaCl	Sodium Chloride
ng	Nanogram
nm	Absorbance unit
OD	Optical Density
p53	Tumour protein p53
PAHS ²	Polycyclic Aromatic Hydrocarbon
PALB2	Partner and Localizer of BRCA2

PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
pg	Picogram
PMX 205	C5a Antagonist
PR	Progesterone Receptor
Psi	Pounds per square inch
qPCR	Real Time Polymerase Chain Reaction
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic Acid
rpm	Rotation per minute
RQ	Relative quantification
RT-PCR Reaction	Reverse Transcriptase Polymerase Chain
SDS-HCL	MTT solvent of Sodium Dodecyl Sulfate-Hydrochloric Acid
SERMs	Selective estrogen receptor modulators
TAE	Tris-Acetate-EDTA
<i>Tfl</i>	<i>Thermus Flavus</i> DNA polymerase
Tm	Melting Temperature
u	unit
UV	Ultraviolet
°C	Degree Celcius
µg	Microgram
µl	Microlitre
µmol	Micromolar

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Cancer is normally described as an abnormal growth of cell in the body and has become a major health problem that causing huge numbers of mortality worldwide. There are three major phases of cancer progression: initiation, promotion and progression. Among the existing cancer-related death known today, breast cancer has become major occurrence death among women in the United States and it is expected 40,290 deaths occurred in 2014 (National Cancer Society, 2015).

Variable treatment options are available. For example surgery, hormonal therapy, or chemotherapy (tamoxifen and aromatase inhibitors, radiation and/or immunotherapy (Bonneterre et al., 2001; Clarke et al., 2005; Florescu et al., 2011). In the case of hormone-related breast cancer, it is possible to treat them in the way of inhibiting the effects of these hormones such as estrogen and progesterone. The magnitude of these hormones in body can be an indicative for the presence of cancer, which can be used as biomarkers. To date, the existing serum biomarkers for breast cancer which are estrogen, HER-2/neu, and progesterone receptors are totally insufficient for an early detection, as it does not possess the required sensitivity and specificity. However, those biomarker could have implementation among the other established protein biomarkers (Misek and Kim, 2011).

When body is introduced to foreign organism and subsequently react to its stimuli, inflammation will takes place as it is a natural eradication process. The theory of cancer cells arise from the inflammation process as it has been long discussed by previous studies (Balkwill and Mantovani, 2001; Coussens and Werb, 2002). While neoplastic progression can be halt by acute inflammation (Markiewski & Lambris, 2009; Loveland & Cebon, 2008; Ostrand-Rosenberg, 2008), chronic inflammation on the other hand is believed to be the key contributor to cancer development and progression.

As a part of body defence system, the complement system is crucial mechanisms against exogenous threat such as microbial attack and also endogenous threat including altered-self molecules and apoptotic cells (Klos et al., 2009). There are over 30 proteins that involved in complement system and one of the key player is C5a. C5a is a 74 amino acid polypeptide and its anaphylatoxin properties plays a pivotal role as causative agent of several inflammation-related diseases such as sepsis (Guo and Ward, 2006; Markiewski et al., 2008b; Ward, 2004), allergy and asthma (Gerard and Gerard, 2002), Alzheimer (Fonseca et al., 2009), rheumatoid arthritis and periodontitis (Chai et al., 2010; Chang et al., 2008). Immunomodulatory properties of C5a and its receptor have been extensively

characterized in the immunological literature, suggesting that C5a promotes and perpetuates inflammation that provides the basis of their involvement in cancer models. In breast cancer cases, immediate detection is imperative to improve survival for a number of patients.

However, the established serum biomarkers are inadequate for early identification of breast cancer (Misek and Kim, 2011). Besides that, well established of breast cancer biomarkers such as ER, PR and HER2 status are unstable throughout tumour progression in contrast to the primary tumour (Lindström et al., 2012). There is an increasing body of evidence to suggest that modulation of complement, particularly the C5a component, has significant therapeutic potential. Therefore, a discovery of a new and much improved biomarker for breast cancer is warranted and hence the potential of C5aR as a candidate for the new protein biomarker for breast cancer.

1.2 Objectives of Study

The objectives of the studies are as follow:

1. To assess the expression of C5a/C5aR in the mouse mammary cancer cells;
2. To measure the magnitude of the expression and up-regulation of C5a/C5aR in mammary cancer cell line;
3. To correlate the amount of proteins and their respective mRNAs in mouse expressing C5a/C5aR.

1.3 Hypothesis of Study

The hypotheses of the studies are as follow:

1. Complement C5a and its receptor C5aR is up-regulated in mouse mammary cancer cells.
2. C5a/C5aR up-regulation can be used as a screening biomarker for mammary cancer in both human and animal.

CHAPTER 2

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APPENDICES

Appendix A1

Preparation of Cell culture's Reagents

Reagent	Preparation
Waymouth's complete medium	To make 50 ml of complete medium, 5% (2.5 ml) of fetal bovine serum (FBS) and 1 % (0.5 ml) of penicillin streptomycin are mix with 47 ml of Waymouth's media
Freezing medium	To make 50 ml of freezing medium, 5% (2.5 ml) of DMSO is mix with 47.5 ml of Waymouth's complete medium
1X Phosphate buffer saline (PBS)	One tablet of PBS (10X)(10 mM sodium phosphates, 2.68 mM KCl, 140 mM NaCl) is dissolved in 100 ml of distilled water and autoclaved at 121 ⁰ C, 15 psi for two hours to make 1X PBS.
Fetal bovine serum (FBS)	FBS is heat deactivated in water bath at 56 ⁰ for 30 minutes and gently swirl the FBS in every 10 minutes internal.

Appendix A2

Preparation of Immunofluorescence staining's Reagents

Reagent	Preparation
4% paraformaldehyde	Mix 4.3 ml of 37% formaldehyde with 35.7 ml of 1X PBS to total volume of 40 ml.
0.1% Triton-X	To make 100 ml solution, 0.1% (0.1 ml) of Triton X-100 is mixed with 99.9ml of 1X PBS solution.
Blocking solution	To make 10 ml blocking solution, 1% (1 g) bovine serum albumin (BSA) and 5% (5 ml) horse serum are mixed with 5 ml of 1X PBS solution.
Hoechst dye	To make 1:500 dilution, 1µl of Hoechst dye is mixed with 499 µl of 1X PBS solution.

Appendix A3

Bicinchoninic acid assay (BCA assay)

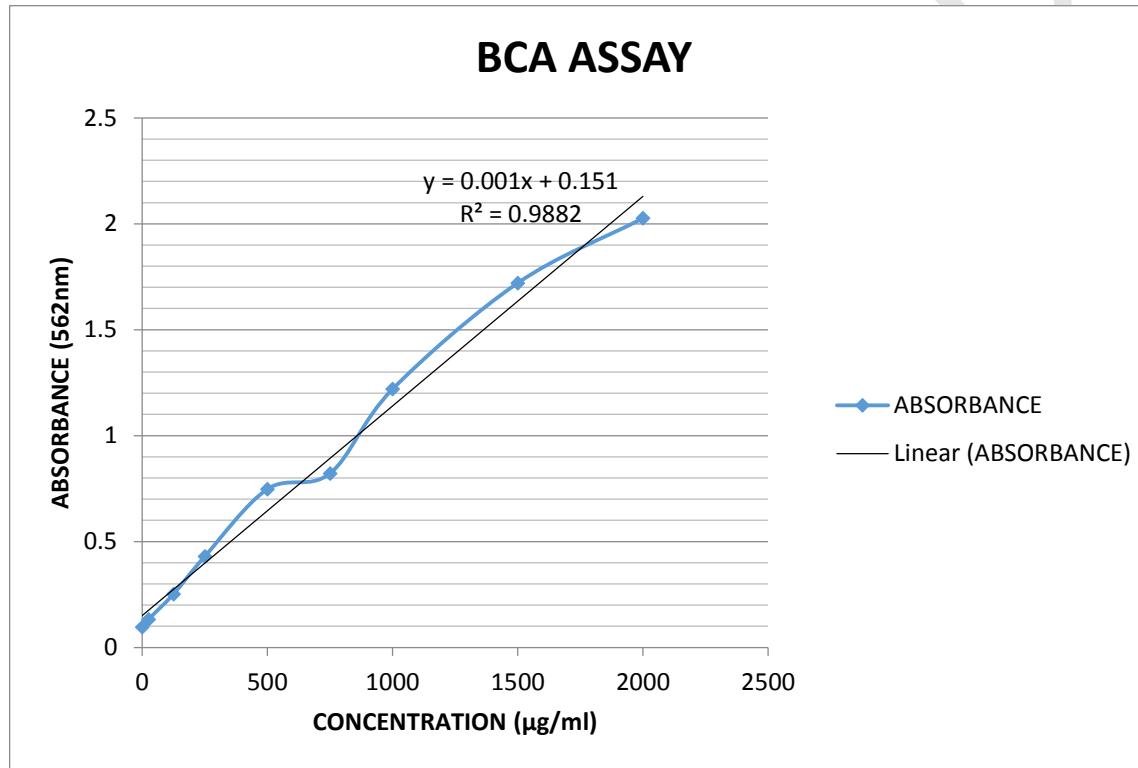
Preparation 10 mg/ml bovine serum albumin (BSA) stock solution

1. Weigh 0.1 g BSA powder and mix with 10 ml of 1X PBS.
2. Roll the mixture on the roller until the BSA is dissolved (do not vortex to prevent protein denaturation).
3. Aliquot the BSA stock solution into 2 mg/ml by adding 0.2 ml BSA stock solution into 0.8 ml of 1X PBS solution

Preparation of diluted albumin (BSA) standard

Vial	Volume of diluent (µl)	Volume and source of BSA (µl)	Final BSA concentration (µg/ml)
A	0	300 of stock	2000
B	125	375 of stock	1500
C	325	325 of stock	1000
D	175	175 of vial B dilution	750
E	325	325 of vial C dilution	500
F	325	325 of vial E dilution	250
G	325	325 of vial F solution	125
H	400	100 of vial G dilution	25
I	400	0	0 = blank

BCA standard curve



Appendix A4

Reverse Transcriptase – Polymerase Chain Reaction

Preparation of the reaction mix

Component	Volume per 50 µl reaction	Final concentration
Nuclease-Free water (to a final volume of 50 µl)	X µl	-
AMV/ <i>Tfl</i> 5X reaction buffer	10 µl	1 X
dNTP mix (10 mM each dNTP)	1 µl	0.2 mM
Forward primer	1 µl	1 µM
Reverse primer	1 µl	1 µM
25 mM MgSO ₄	2 µl	1 mM
AMV reverse transcriptase (5 u/µl)	1 µl	0.1 u/µl
<i>Tfl</i> DNA polymerase (5 u/µl)	1 µl	0.1 u/µl
RNasin inhibitor	1 µl	40 u/ µl
RNA sample or control	2 µl	10 ³ – 10 ⁶ copies

Appendix A5

Real – Time Polymerase Chain Reaction (qPCR)

Preparation of the reaction mix

Component	Volume per 50 µl reaction	Final concentration
GoTaq® qPCR master mix, 2X	25 µl	1X
Forward primer	1 µl	1 µM
Reverse primer	1 µl	1 µM
RNasin inhibitor	1 µl	40 u/ µl
RNA sample or control	2 µl	10 ³ – 10 ⁶ copies
Nuclease-Free water (to a final volume of 50 µl)	X µl	-

BIODATA OF STUDENT

Noor Farhana Binti Bachek was born in Kuala Lumpur on the 5th of October 1990. Her primary education was obtained from Sekolah Kebangsaan Bangsar, Kuala Lumpur from 1997 to 2002. She carried on with her secondary education from 2002 to 2007 at Sekolah Menengah Kebangsaan Bangsar, Kuala Lumpur where she sat for her Penilaian Menengah Rendah (PMR) and Sijil Pelajaran Malaysia (SPM) examination in 2005 and 2007 respectively.

In 2008, the author furthered her study in matriculation program in field of Biology in Kolej Matrikulasi Negeri Sembilan for one year. After completion of matriculation, the author furthered her study in Bachelor Science (Hons.) of Microbiology in Faculty of Biotechnology and Biomolecule Sciences, Universiti Putra Malaysia for three years. Upon graduation, the author enrolled as a full-time candidate pursuing her study in Master of Science program in field of Pharmacology under supervision of Dr. Mohd Hezmee Bin Mohd Noor in Faculty of Veterinary Medicine, Universiti Putra Malaysia.

LIST OF PUBLICATION

PROCEEDINGS

Hezmee, M. N. M., Intan-Shameha, A. R., Mokrish, A., Nashreq, K. N., Hazwani, K. N., Norhaifa, G., **Farhana, B. N.** (2013). Establishment of mechanism of tumour regression through complement mediated inflammatory reaction of C5a-C5aR axis. Poster presented at National Biotechnology Seminar 2013, Penang, Malaysia.

JOURNALS

Farhana, B. N., Hazwani, K. N., Zuki, A. B., Intan-Shameha, A.R., Ajat, M., and Hezmee, M.N.M (2015). The expression of C5a receptor and in vitro viability studies with C5aR agonist and antagonist on EMT6 cells. Chinese Journal of Cancer Research. (Submitted)

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