



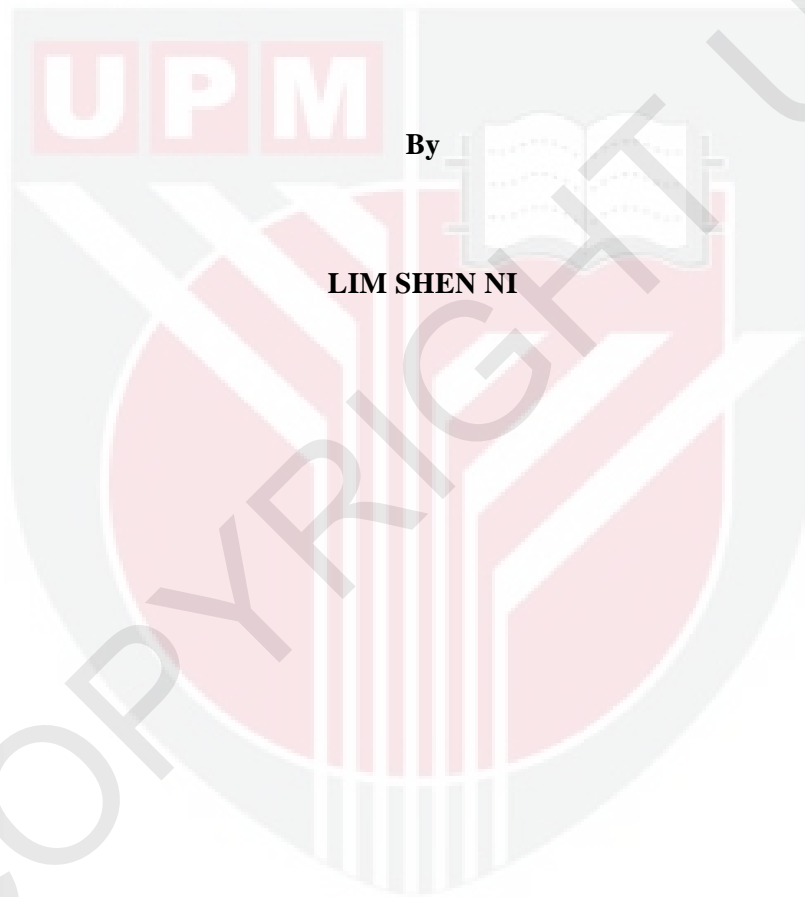
**UNIVERSITI PUTRA MALAYSIA**

**TRUNCATED CHICKEN ANEMIA VIRUS-VP3  
AS A POTENTIAL ANTITUMOUR VACCINE**

**LIM SHEN NI**

**IB 2012 9**

**TRUNCATED CHICKEN ANEMIA VIRUS-VP3 AS A POTENTIAL ANTI-  
TUMOUR VACCINE**



By

**LIM SHEN NI**

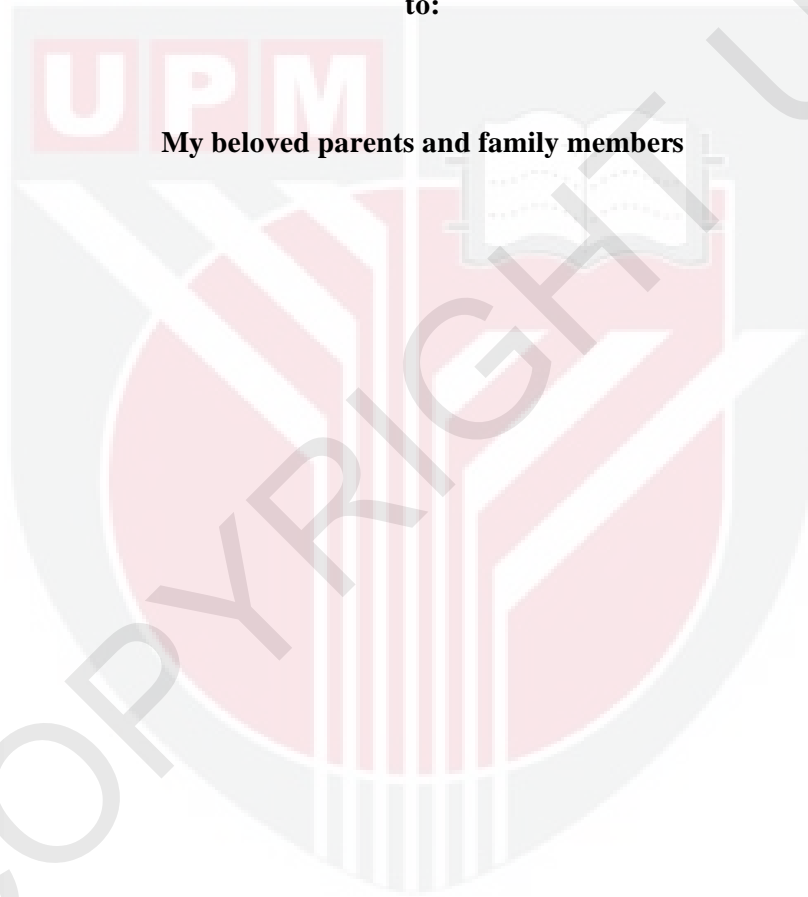
**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia  
in Fulfillment of the Requirement for the Degree of Master of Science**

**April 2012**

**Dedicated with love and gratitude**

**to:**

**My beloved parents and family members**



© COPYRIGHT UPM

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

**TRUNCATED CHICKEN ANEMIA VIRUS -VP3 AS A POTENTIAL ANTI-TUMOUR VACCINE**

By

**LIM SHEN NI**

**April 2012**

**Chairperson : Zeenathul Nazariah Allaudin, PhD**

**Faculty : Institute of Bioscience**

Chicken Anemia Virus (CAV) VP3 protein (also known as Apoptin), a basic and proline-rich protein has been shown to act as an anticancer tool. Its special feature in inducing apoptosis in cancer cells but not in normal cells enables it to be widely studied for gene and protein therapies. When produced by *in vitro* protein synthesis system as a recombinant fusion with maltose-binding protein, the recombinant protein was able to migrate into the nucleus of tumour cells and caused apoptosis in the cells. In principle, minimizing the antigenic regions of a protein could reduce unnecessary side effects of the protein to a cell. The aim of this study was determining the minimal selective domain of Apoptin for apoptotic effect. The full length of Apoptin had been mutated by segmental deletion at the N' terminal and

linking it with nuclear localization sites (NLS1 and NLS2) to develop five truncated constructs. An *in vitro* protein expression system, RTS was used to express Apoptin and truncated Apoptin proteins. Microinjection method was applied to deliver all the purified proteins into human breast cancer cells, MCF7 and normal human liver cells, Chang cells. The cells were demonstrated for efficient protein delivery when using 2 mg/ml of purified proteins at 60 hPa of injection pressure for 0.5 s. Cellular microinjections of truncated Apoptin proteins, PrVP3A1-69N1N2, PrVP3A1-46N1N2 and PrVP3A1-31N1N2, retained the characteristics of ectopically expressed wild-type full length Apoptin in cancerous cells. Annexin V was used to detect the presence of early apoptosis in the cells and the result showed occurrence of apoptosis in the cell nucleus. All the three truncated complexes were successfully translocated to the nucleus of human breast cancer cells (MCF cells) and induced apoptosis, whereas in normal Chang cells they remained in the cytoplasm with no apparent toxicity. However, two other mutagens, PrVP3A32-69N1N2 and PrVP3A32-62N1N2, induced apoptosis in both cancerous and noncancerous cells, thus lost their specific selectivity to cancerous cells. Compared to the other three truncated Apoptin proteins, both latter proteins had deletion spanning a.a. 1-31, the upper region of leucine-rich sequence (LRS). LRS at the a.a. 33-46 can act as nuclear retention signals. Therefore, the findings suggested that the minimal selective domain for Apoptin resides in the N-terminal specifically within the deletions spanning a.a. 1-33, which could be the leucine-rich sequence (LRS) enhancer and the contributing factor to the non-selectivity of truncated Apoptin proteins, PrVP3A32-69N1N2 and PrVP3A32-62N1N2. This new finding associated to the selectivity of LRS upstream probably could play a role in cancer cells selectivity for apoptosis induction by Apoptin, In conclusion, out of the five truncated Apoptin proteins, three

of them (PrVP3A1-69N1N2, PrVP3A1-46N1N2 and PrVP3A1-31N1N2) were functioning similar to the full length wild type Apoptin, in their ability to induce apoptosis selectively to the targeted cancer cells.



Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia  
sebagai memenuhi keperluan Ijazah Saujana Sains

**PEMENGALAN VIRUS ANEMIA AYAM-VP3 SEBAGAI VAKSIN ANTI-  
TUMUR YANG BERPOTENSI**

Oleh

**LIM SHEN NI**

April 2012

**Pengerusi : Zeenathul Nazariah Allaudin, PhD**

**Fakulti : Institut Biosains**

Protin virus yang ketiga (VP3) virus anemia ayam (CAV) (juga dikenali sebagai Apoptin) merupakan protin alkali yang kaya dengan prolin telah menunjukkan keupayaannya bertindak sebagai alat anti-kanser. Ciri khas Apoptin dalam mendorong apoptosis dalam sel-sel kanser tetapi tidak pada sel-sel normal telah mendorong protin ini dikaji secara meluas dalam kajian terapi gen dan protin. Apabila protin Apoptin yang terpotong digabungkan bersama protin pengikat maltosa dan dihasilkan melalui sistem sintesis protin secara *in vitro*, protin rekombinan ini berupaya beralih ke nukleus sel-sel tumour dan mengakibatkan berlakunya apoptosis dalam sel. Secara prinsip, meminimumkan kawasan antigen protin boleh mengurangkan kesan sampingan yang tidak perlu kepada sel. Tujuan kajian ini dijalankan adalah untuk menentukan domain selektif minimum Apoptin

bagi tujuan apoptotik. Apoptin asal telah bermutasi dengan penghapusan segmen di terminal N dan menghubungkan segmen-segmen yang tinggal dengan tapak penyetempatan nuklear (NLS1 dan NLS2) untuk membina lima kompleks yang baru. Sistem pengekspresan protin secara *in vitro*, RTS telah digunakan untuk mengesksespres Apoptin dan Apoptin yang dipenggal. Kaedah mikro-suntikan telah digunakan untuk menyuntikkan semua protin yang dituliskan ke dalam sel-sel kanser payudara manusia, sel MCF7 dan sel hati manusia, sel Chang. Kepekatan protin yang dituliskan sebanyak 2 mg/ml telah digunakan untuk dimikro-suntik ke dalam sel pada tekanan suntikan 60 hPa selama 0.5 s. Apoptin protin yang dipenggal, PrVP3A1-69N1N2, PrVP3A1-46N1N2 dan PrVP3A1-31N1N2 yang telah dimikro-suntik ke dalam sel kanser mengekalkan ciri-ciri asal Apoptin. Annexin V telah digunakan untuk mengesan kehadiran apoptosis awal dalam sel dan hasilnya menunjukkan berlakunya apoptosis dalam nukleus sel. Kesemua tiga kompleks Apoptin yang terpotong telah berjaya dipindahkan ke nukleus sel kanser payudara manusia (sel MCF) dan mendorong berlakunya apoptosis dalam sel, sedangkan dalam sel Chang, protin ini kekal di dalam sitoplasma tanpa kesan toksik pada sel. Walau bagaimanapun, dua mutagens lain, PrVP3A32-69N1N2 dan PrVP3A32-62N1N2 telah mendorong berlakunya apoptosis dalam kedua-dua sel kanser dan sel normal, dengan itu ia telah kehilangan selektiviti khususnya pada sel kanser sahaja. Berbanding dengan tiga protin Apoptin lain yang terpotong, kedua-dua protin ini kekurangan a.a. 1-31, rantau atas jujukan-kaya dengan leusin (LRS). LRS yang berkedudukan pada a.a. 33-46 bertindak sebagai isyarat pengekaln nuklear. Oleh itu, kajian ini mencadangkan bahawa domain selektif minimum untuk Apoptin terdapat di terminal N, khususnya pada a.a. 1-33, yang bertindak sebagai pendorong jujukan-kaya leusin berkemungkinan mengakibatkan kehilangan keupayaan selektif pada sel



kanser bagi protin Apoptin terpotong, PrVP3A32-69N1N2 dan PrVP3A32-62N1N2. Penemuan baru ini dikaitkan dengan kepilihan rantau atas LRS mungkin memainkan peranan dalam pemilihan sel kanser untuk induksi apoptosis oleh Apoptin, Kesimpulannya, tiga daripada lima kompleks protin Apoptin terpotong (PrVP3A1-69N1N2, PrVP3A1-46N1N2 dan PrVP3A1-31N1N2) telah memberi kesan apoptosis hanya pada sel kanser sahaja yang mana setanding dengan Apoptin asal.



## ACKNOWLEDGEMENTS

First of all, I would like to express my thanks and deepest gratitude to Associate Professor Dr Zeenathul Nazariah Allaudin, for her guidance and patience throughout the course of this study. With her kind motivation, encouragement, and constant support, this project has finally achieved a breakthrough and brought forth a novel and safer potential therapeutic agent for anti-cancer therapy. My appreciation also goes to my co-supervisors, Professor Dr. Mohd Azmi Mohd Lila and Professor Dr. Fauziah Othman, for their critical comments, and constructive suggestions throughout my study.

Additionally, I would like to thank Mr Kamaruddin, Dr Sandy Loh Hwei San, Mr Tam Yew Joon, Mr Mohd Nik Afizan, and Ms Lo Sewn Cen from Virology laboratory of Faculty of Veterinary Medicine, for their guidance and friendship. Not to forget Dr Abas Mazni Othman, Dr Noorjahan Banu and others who helped directly or indirectly along the progress of my project.

I would like to express my deepest gratitude to my parents and my family members for their encouragement, which had helped me to undertake and complete these studies.

Finally, I would like to acknowledge my financial sponsors, Majlis Kanser Nasional (MAKNA) for the generous research funding. Without the support, this study would not have been possible.

I certify that a Thesis Examination Committee has met on 19<sup>th</sup> April 2012 to conduct the final examination of LIM SHEN NI on her thesis entitled "Truncated Chicken Anemia Virus -VP3 Protein – A Potential Candidate for Anti-Tumour Vaccine" in accordance with the Universities and University Colleges Act 1971 and the Constitution of Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the relevant degree.

Members of the Thesis Examination Committee are as follows:

**Prof. Dato' Dr. Abdul Rani bin Bahaman**  
Department of Veterinary Pathology and Microbiology  
Faculty of Veterinary Medicine, UPM  
(Chairman of the Thesis Examination Committee)

**Prof. Dr. Rozita bt Rosli**  
UPM-MAKNA Cancer Research Laboratory  
Institute of Bioscience, UPM  
(Internal Examiner)

**Prof. Dr. Tengku Azmi bin Tengku Ibrahim**  
Department of Veterinary Preclinical Sciences  
Faculty of Veterinary Medicine, UPM  
(Internal Examiner)

**Assoc. Prof. Dr. Zuridah Hassan**  
Faculty of Health Sciences  
UiTM Puncak Alam  
42300 Puncak Alam  
Selangor  
(External Examiner)

---

**Prof. Dr. Seow Heng Fong**  
Deputy Dean  
School of Graduate Studies  
UPM

This thesis was submitted to the Senat 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:

**Zeenathul Nazariah binti Allaudin, PhD**

Associate Professor  
Faculty of Veterinary Medicine  
Universiti Putra Malaysia  
(Chairman)

**Mohd Azmi bin Mohd Lila, PhD**

Professor  
Faculty of Veterinary Medicine  
Universiti Putra Malaysia  
(Member)

**Fauziah binti Othman, PhD**

Professor  
Faculty of Medicine & Health Sciences  
Universiti Putra Malaysia  
(Member)

---

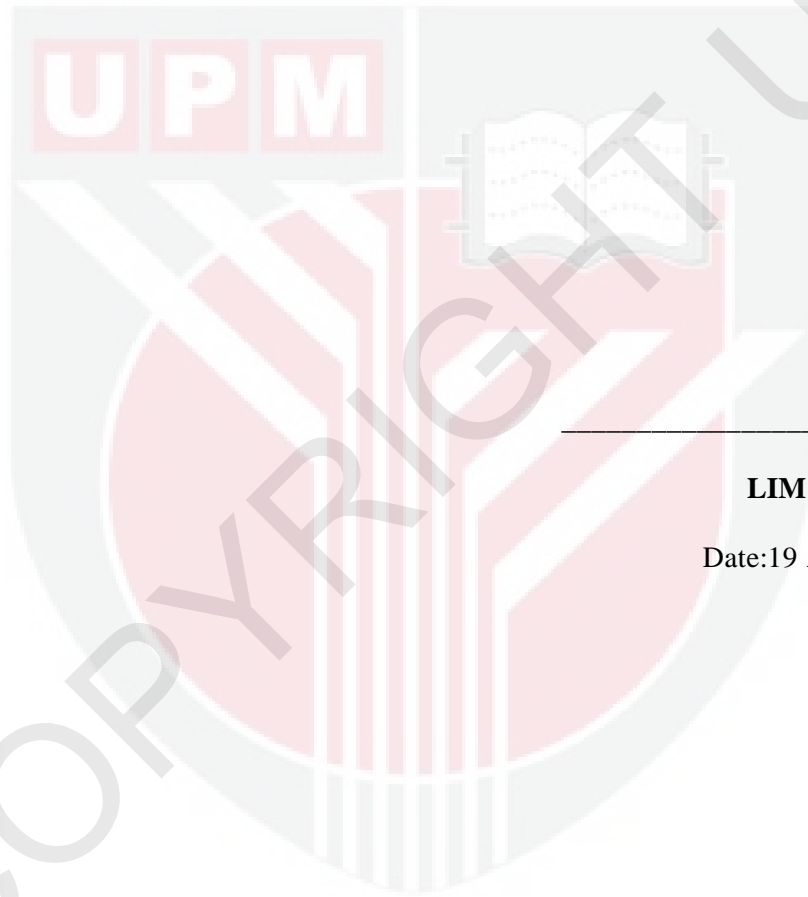
**BUJANG BIN KIM HUAT, PhD**

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 other institutions.



---

**LIM SHEN NI**

Date: 19 April 2012



## TABLE OF CONTENTS

	<b>Page</b>
<b>DEDICATION</b>	ii
<b>ABSTRACT</b>	iii
<b>ABSTRAK</b>	vi
<b>ACKNOWLEDGEMENTS</b>	ix
<b>APPROVAL</b>	x
<b>DECLARATION</b>	xii
<b>LIST OF TABLES</b>	xvi
<b>LIST OF FIGURES</b>	xvii
<b>LIST OF ABBREVIATIONS</b>	xx
 <b>CHAPTER</b>	
<b>1 INTRODUCTION</b>	<b>1</b>
<b>2 LITERATURE REVIEW</b>	
2.1 VP3 Protein/ Apoptin	4
2.1.1 Apoptin Protein Structure and Function	4
2.1.2 Transformed versus Non-Transformed Cells	7
2.1.3 Phosphorylation of Apoptin Protein	8
2.1.4 Mechanism of Cell Death	9
2.2 Protein Expression System	10
2.3 Protein Delivery	13
2.4 Cancer Therapy	14
2.5 Apoptosis	15
2.6 Apoptosis Screening Assay	17
<b>3 CONSTRUCTION OF VECTORS HARBORING TRUNCATED CAV-VP3 GENE</b>	
3.1 Introduction	18
3.2 Materials and Methods	20
3.2.1 Plasmid Vector	20
3.2.2 Primers for Polymerase Chain Reaction (PCR)	21
3.2.3 Amplification of Truncated Apoptin Gene by Polymerase Chain Reaction (PCR)	25
3.2.4 Purification of PCR Products	25
3.2.5 Double Digestion of PCR Products	26
3.2.6 Purification of Double Digested Product	26
3.2.7 Ligation of Digested PCR Products with Plasmid Vector	26
3.2.8 Bacterial Cell Transformation	27
3.2.9 Miniprep Plasmid Extraction	27
3.2.10 Restriction Enzyme Analysis	28
3.2.11 Sequencing of Truncated Apoptin Genes	28
3.2.12 Computer Analysis	29
3.3 Results	29
3.3.1 Amplification of Truncated Apoptin Gene by PCR	29
3.3.2 Cloning of Apoptin Gene and Truncated Apoptin Genes Into pIVEX-MBP Plasmid	42

3.3.3	Restriction Enzyme Analysis	43
3.3.4	DNA Sequencing of Apoptin Gene and Truncated Apoptin Genes	44
3.4	Discussion	50

#### **4 EXPRESSION AND PURIFICATION OF TRUNCATED APOPTIN PROTEIN**

4.1	Introduction	54
4.2	Materials and Methods	57
4.2.1	Protein Expression of Truncated Apoptin Genes	57
4.2.2	Analysis of Expressed Proteins	57
4.2.3	Protein Purification	58
4.2.4	Protein Concentration	59
4.2.5	Determination of Protein Concentration	59
4.2.6	Analysis of Purified and Concentrated Proteins	59
4.2.7	Computer Analysis	60
4.3	Results	60
4.3.1	Expression and Analysis of Truncated Apoptin Protein by Rapid Translation System (RTS)	60
4.3.2	Analysis of Purified and Concentrated Proteins	62
4.3.3	Measurement of Protein Concentration Using BSA Standard Curve	63
4.3.4	The Amino Acid Sequence of Apoptin Protein	64
4.3.5	Homology Search of The Expressed Proteins	66
4.4	Discussion	69

#### **5 APOPTOSIS EFFECT OF TUNCATED APOPTIN PROTEINS IN CANCEROUS AND NORMAL CELLS**

5.1	Introduction	73
5.2	Materials And Methods	75
5.2.1	Cell Culture and Maintenance	75
5.2.2	Pre-Microinjection Preparation	76
5.2.3	Apoptin Protein Preparation	76
5.2.4	Microinjection	77
5.2.5	Viability Observation	78
5.2.6	Indirect Immunofluorescence	78
5.2.7	Detection of Apoptosis Effect in Injected Cells	79
5.3	Results	80
5.3.1	Optimization for Delivery of Recombinant Protein into Cells	80
5.3.1.1	Delivery of Protein into Cells	80
5.3.1.2	Survival Test of Microinjected Cells	82
5.3.1.3	Fluorescent Tracking of MBP Protein in the Injected MCF7 Cells	86
5.3.2	Detection of Injected Apoptin Protein and Truncated Apoptin Proteins in Cancerous Cells and Normal Cells	92

5.3.3	Apoptotic Effect of Truncated Apoptin Protein in MCF Cells and Chang Cells	96
5.4	Discussion	99
<b>6</b>	<b>GENERAL DISCUSSION AND CONCLUSION</b>	<b>104</b>
	<b>REFERENCES</b>	<b>112</b>
	<b>APPENDICES</b>	<b>120</b>
	<b>BIODATA OF STUDENT</b>	<b>123</b>
	<b>PUBLICATIONS AND PATENT</b>	<b>123</b>

