COPYRIGHT© INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA

SITE-DIRECTED MUTAGENESIS FOR THE PRODUCTION OF MUTANT *TP53* GENE AND ANALYSIS OF ITS TUMOR SUPPRESSOR ACTIVITY

BY

ZAFIRAH LIYANA ABDULLAH

A thesis submitted in fulfilment of the requirement for the degree of Master of Pharmaceutical Sciences (Pharmaceutical Technology)

Kulliyyah of Pharmacy
International Islamic University
Malaysia

OCTOBER 2011

ABSTRACT

As a cellular gatekeeper, p53 plays a major role in maintaining the cellular homeostasis. The existence of mutations may change the gene conformation and may affect its major function as the regulator of the cell proliferation. The p53 therapy based approach is a good candidate for treatment of *TP53*-defect cancers. However, this treatment is unsuitable for some cancer cases, especially those caused by dominant-negative activity of the mutant p53 protein. Dominant-negative occur in the presence of a mutated allele which results in the formation of heterotetramer of endogenous wild-type/mutant p53 that are unable to transactivate certain p53 downstream target genes, which are important for the cells regulation. Thus, more effective treatment is needed to overcome this problem and DNA vaccination may be the suitable candidate. A recombinant mutant p53 designated as R248Q has been put forward as a potential antigen for the DNA vaccination strategy. Therefore, the current research aims to produce mutant *TP53R248Q* through PCR site–directed mutagenesis and to confirm the tumor suppression ability of *TP53R248Q*. The mutation of R248Q was generated via Polymerase Chain Reaction (PCR) site-directed mutagenesis to pCMVp53 plasmid by using a set of specifically constructed primers**.** Subsequently, these constructed *TP53R248Q* and *TP53* gene was transfected into the *TP53*-null H1299 cell lines. Following that, phenotype and genotype expression studies on the cell lines were performed by colony formation assay and quantification of a *TP53* downstream target gene $p21^{waf1}$, respectively, in order to investigate the tumor suppression ability of the mutated *TP53*. In phenotype study, the transfection with exogenous *TP53* suppressed the colony growth while the treatment with *TP53R248Q* confirmed the loss of p53 original function by its inability to restrain cell proliferation. The result of phenotype study was confirmed by the expression analysis of downstream *TP53* gene, $p21^{waf1}$. Based on the analysis, it was found that the expression of $p21^{waf1}$ was upregulated in *TP53* and downregulated in *TP53R248Q* treatments. These data therefore confirmed that the PCR site-directed mutagenesis technique has been successfully carried out to induce the desired mutation in the *TP53* gene. Thus, this technique may become an interesting option to generate novel recombinant proteins, which may be useful for the development of specifically designed DNA vaccine as a gene therapy strategy for cancer prevention in the future.

.

خلاصة البحث

كونها من المسؤولين عن مراقبة البوابات الخلوية، تلعب p53 دوراً أساسياً في المحافظة على التوازن الخلوي. إن وجود الطفرات قد يغير تكوين المورثة وقد يؤثر على وظيفتها الأساسية كمنظم لتكاثر الخلية. إن المعالجة المعتمدة على p53 هي مرشُح جيد لمعالجة السرطانات الناتجة عن عيوب *TP53*. لكن هذه المعالجة غير مناسبة لبعض حالات السرطان، وخاصة تلك الناتجة عن النشاط السلبي المهيمن لبروتينات p53 الطافرة. يحدث النشاط المهيمن السلبي بوجود الأليل الطافر الذي يؤدي إلى تكوين تترامير غير متجانس من p53 الداخلية الطبيعية والطافرة والذي يكون غير قادر على نقل التفعيل لمورثات دنيا محددة مستهدفة من قبل p53 ، وهذه المورثات هامة للتنظيم الخلوي. وبالتالي هناك حاجة لمعالجة أكثر فعالية للتغلب على هذه المشكلة وربما يكون التلقيح بالـDNA مرشحا مناسباً. تم اقتراح المورثة الطافرة p53 المؤشبة والمهيأة بشكل R248Q كأنتيجين محتمل لاستراتيجية التلقيح بالـDNA. لهذا السبب، يهدف هذا البحث لإنتاج *TP53R248Q* طافر من خلال إنتاج الطفرات الموضعي الموجه بالـPCR وأيضاً لتأكيد القدرة المثبطة للورم لـ*TP53R248Q* . تم تشكيل الطفرة R248Q من خلال إنتاج الطفرات الموضعي الموجه بالـPCR للبلاسميد pCMVp53 عن طريق استخدام مجموعة من البرايمرات المشكلة خصيصاً لذلك. بعد ذلك، هذه الـ*TP53R248Q* المشكلة والمورثة TP53 تم إدخالها في النمط الخلوي *TP53-*null H1299. ومن ثم تم إجراء دراسات التعبير المظهرية والوراثية على هذا النمط الخلوي عن طريق المعايرة بتشكيل المستعمرات والمعايرة الكمية لناتج المورثة *TP53* المستهدف وهو $p21^{waf1}$، على التسلسل، وذلك لاستكشاف القدرة المثبطة للورم للمورثة *TP53* الطافرة. في الدراسة المظهرية، إدخال المورثة الخارجية *TP53* أدى إلى تثبيط نمو المستعمرات بينما أكدت المعالجة بـ*TP53R248Q* فقدان الوظيفة الأصلية لـp53 من خلال عدم قدرتها على كبح تكاثر الخلايا. تم تأكيد نتائج الدراسة المظهرية من خلال التحليل التعبيري لناتج المورثة *TP53*، $p21^{waf1}$. بناء على التحليل، وُجد أن تعبير $p21^{waf1}$ كان في المنحى المتزايد في المعالجة بـTP53 وفي المنحي المتناقص في المعالجة بـ*TP53R248Q*. بناء على ما سبق فإن هذه النتائج أكدت أن تقنية إنتاج الطفرات الموضعي الموجه بالـPCR تم تنفيذها بشكل ناجح لتحريض الطفرة المرغوبة في المورثة *TP53*. وهكذا فإن هذه التقنية قد تصبح خياراً ذا أهمية لإنتاج بروتينات مؤشبة جديدة قد تكون مفيدة لتطوير لقاحات DNA مصممة خصيصاً كاستراتيجية للمعالجة الوراثية لمنع السرطان في المستقبل.

ABSTRAK

Sebagai penjaga sel, p53 memainkan peranan yang penting dalam mengekalkan keseimbangan homeostasis sel. Kehadiran mutasi boleh menyebabkan perubahan pada konformasi gen tersebut dan boleh menjejaskan fungsi utamanya sebagai pengawal atur perkembangbiakan sel. Pendekatan berasaskan terapi p53 ialah calon yang sesuai untuk merawat kanser yang diakibatkan oleh kerosakan fungsi gen *TP53*. Walaubagaimanapun, rawatan ini kurang sesuai untuk merawat sesetengah kes kanser, terutamanya yang disebabkan oleh aktiviti dominan-negatif protein p53 yang mutan. Dominan-negatif berlaku apabila terdapat satu alel yang termutasi, yang menghasilkan pembentukan heterotetramer p53 jenis liar/mutan endogen yang tidak berupaya mentransaktifkan gen sasaran hiliran p53 tertentu yang penting dalam mengawal atur sel. Maka, rawatan yang lebih efektif diperlukan untuk mengatasi masalah ini dan vaksinasi DNA dilihat mempunyai potensi yang tinggi. p53 mutan rekombinan yang dinamai R248Q dikenalpasti sebagai antigen yang berpotensi dalam strategi vaksinasi DNA. Oleh yang demikian, penyelidikan yang dijalankan ini bertujuan untuk menghasilkan *TP53R248Q* mutan melalui teknik mutagenesis tapak terarah (SDM) Tindak Balas Pempolimeran Berantai (PCR) dan untuk mengesahkan keupayaan yang ada pada *TP53R248Q* untuk menyekat tumor. Mutasi pada R248Q dihasilkan melalui mutagenesis tapak terarah Tindak Balas Pempolimeran Berantai (PCR) terhadap plasmid pCMVp53 dengan menggunakan satu set primer yang direka khas. Selepas itu, gen *TP53R248Q* dan *TP53* yang terhasil ditransfeksikan ke dalam titisan sel H1299 *TP53*-nol. Seterusnya, kajian ekspresi fenotip dan genotip ke atas titisan sel dilaksanakan melalui asai pembentukan koloni dan pengkuantitian gen sasaran hiliran *TP53*, $p21^{waf1}$, masing-masing, untuk mengkaji keupayaan yang ada pada *TP53* termutasi untuk menyekat tumor. Dalam kajian fenotip yang dijalankan, pentransfeksian dengan *TP53* eksogenus didapati menyekat pertumbuhan koloni, sementara rawatan dengan *TP53R248Q* mensahihkan kelumpuhan fungsi asal p53 berdasarkan ketakmampuannya mengawal perkembangbiakan sel. Keputusan kajian fenotip ini disahkan oleh analisis ekspresi gen *TP53* hiliran, $p21^{waf1}$. Berdasarkan analisa, ekspresi $p21^{waf1}$ adalah lebih terkawal atur apabila dirawat dengan *TP53*, manakala ekspresi $p21^{waf1}$ tidak terkawal atur apabila dirawat dengan *TP53R248Q*. Oleh yang demikian, data ini mengesahkan bahawa teknik mutagenesis tapak terarah (SDM) Tindak Balas Pempolimeran Berantai (PCR) berjaya dilaksanakan untuk mengaruh mutasi yang diingini pada gen *TP53*. Maka, teknik ini boleh menjadi pilihan yang menarik untuk menghasilkan protein rekombinan baharu, yang mungkin berguna dalam penghasilan vaksin DNA yang dibina secara khusus sebagai strategi terapi gen dalam pencegahan kanser pada masa hadapan.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Master of Pharmaceutical Sciences (Pharmaceutical Technology)

....
Muhammad Taher
Supervisor

………………………………..…
Solachuddin Jauhari Arief Ichwan
Co-Supervisor

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Master of Pharmaceutical Sciences (Pharmaceutical Technology)

....
Mohd. Arifin bin Kaderi
Examiner

…………………………………..
Masa-Aki Ikeda
External Examiner

This dissertation was submitted to the Department of Pharmaceutical Technology and is accepted as a fulfilment of the requirement for the degree of Master of Pharmaceutical Sciences (Pharmaceutical Technology)

....
Juliana binti Md. Jaffri
Head, Department of
Pharmaceutical Technology

This dissertation was submitted to the Kulliyyah of Pharmacy and is accepted as a fulfilment of the requirement for the degree of Master of Pharmaceutical Sciences (Pharmaceutical Technology)

....
Tariq bin Abdul Razak
Dean, Kulliyyah of Pharmacy

DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

Zafirah Liyana Abdullah

Signature ……………………………………   Date ……………………..

INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA

DECLARATION OF COPYRIGHT AND AFFIRMATION OF FAIR USE OF UNPUBLISHED RESEARCH

SITE-DIRECTED MUTAGENESIS FOR THE PRODUCTION OF MUTANT *TP53* GENE AND ANALYSIS OF ITS TUMOR SUPPRESSOR ACTIVITY

Copyright © 2011 by International Islamic University Malaysia. All rights reserved.

I hereby affirm that The International Islamic University Malaysia (IIUM) holds all rights in the copyright of this work and henceforth any reproduction or use in any form or by means whatsoever is prohibited without the written consent of IIUM. No part of this unpublished research may be reproduced, stored in a retrieval system, or transmitted, in any form or by means, electronic, mechanical, photocopying, recording or otherwise without prior written permission of the copyright holder

Affirmed by Zafirah Liyana Abdullah.

…………………………….	..……………………..….
Signature	Date

To my beloved parents and sister

ACKNOWLEDGEMENTS

In the Name of Allah, The Most Gracious, The Most Merciful. Praise be upon Him for granting me the strength, patience, and perseverance throughout the completion of this project. The greatest services and achievements will always be presented for Him. The deepest gratitude and pray will always be for Him.

I would like to express my respect and gratitude to my supervisor, Dr. Muhammad Taher, for the chance given to me to work in this project. My respect and gratitude to my supervisor Dr. Solachuddin Jauhari Arief Ichwan, for his guidance and support throughout this project. I am proud and honored to work under them. Their experience, understanding, and kindness are invaluable lesson not only to conduct a research but also to live a meaningful life.

I would like to thank all the lecturers of Kulliyyah of Pharmacy and Kulliyyah of Science, International Islamic University Malaysia for sharing their knowledge especially Dr. Abu Bakar Danjuma, Dr. Phang Ing Chia and Sr. Nurul Wahida Saad. Not to forget, to Prof. Ishak who gave his full commitment and support in introducing me to the molecular research area.

My special thanks to the staff at Department of Pharmaceutical Technology, Kulliyyah of Pharmacy especially Sr. Hazan Haryanti. My appreciation for all lab assistants of Kulliyyah of Pharmacy and Kulliyyah of Science, Br. Haniff, Br. Dzadil, Br. Azmir, Br. Yahya, and Sr. Izyan for assisting me throughout this study.

My deeply thanks to Rahma Fitri Hayati who shares tears and difficulty in conducting the research. Acknowledgment and thanks to my beloved friends, Sr. Nuraniza Azahari, Sr. Wastuti Hidayati Suriyah for the support and understanding. The love and friendship hopefully will be a remembrance memory and lesson for us to move forward.

I would like to specially thank my beloved and respected parents, Abdullah Hussin and Hamiah Ahmad and my lovely special sibling, Farah Iryani Abdullah and last but not least, to my beloved fiancé, Tuan Mohd Faris Tuan Ismail. I am so grateful for their love, patience, encouragement, understanding, pray and full support throughout my study here. Without them, my study here would be impossible. My achievements and accomplishment will always be presented for them.

TABLE OF CONTENTS

Abstract ii
Abstract in Arabic iii
Abstrak iv
Approval Page v
Declaration vi
Dedication viii
Aknowledgements ix
List Of Tables xiii
List Of Figures xiv
List Of Abbreviations xv
List Of Symbols xvii
List Of Appendices xviii

CHAPTER ONE: INTRODUCTION 1
1.1 Background of the Study 1
1.2 Objectives 4

CHAPTER TWO: LITERATURE REVIEW 6
2.1 Incidence of cancer around the world 6
2.1.1 Causes of cancer 7
2.1.2 Lung cancer 7
2.1.3 Types of mutation that leads to cancer development 8
2.1.3.1 Germline mutation 8
2.1.3.2 Somatic mutation 9
2.2 *TP53* gene 11
2.2.1 *TP53* structure 13
2.2.2 *TP53* target genes 15
2.2.3 Regulations and activation of p53 17
2.2.4 Cell cycle regulation of p53 17
2.3 *TP53* gene and cancer 19
2.3.1 Mutations of *TP53* coding region 21
2.3.2 Structural and functional impact of mutations 22
2.3.2.1 Impact on protein structure and transcriptional activities 22
2.3.2.2 Impact of *TP53* mutation on its protein function 23
2.4 The use of human cancer cell lines in cancer research 25
2.4. 1 Study of mutations and gene cloning 26
2.5 *In vitro* mutagenesis 28
2.5.1 PCR site-directed mutagenesis 29
2.6 Alternative treatments of cancer 31
2.6.1 Gene therapy based approaches 31
2.6.2 p53 vaccines 32

CHAPTER THREE: MATERIALS AND METHODS 35
3.1 Materials 35
3.1.1 Reagents and chemicals 35
3.1.2 Cell lines and plasmids 36
3.2 Equipment 36
3.3 Methodology 37
3.3.1 Production of recombinant protein pCMVp53R248Q 39
3.3.1.1 *Dpn*I Digestion of the Amplification Products 38
3.3.1.2 Transformation of XL1-Blue Supercompetent Cells 38
3.3.1.3 Amplification of constructed plasmid 39
3.3.1.4 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis 40
3.3.1.5 Western Blot 41
3.3.2 General procedures for cell culture H1299 Cells 42
3.4.2.1 Cells thawing 42
3.4.2.2 Cells maintainance 43
3.4.2.3 Cells subculture 43
3.4.2.4 Cells freezing 43
3.3.3 Stable transfection of plasmids into H1299 Cells 44
3.3.3.1 Cell culture and plasmids transfection 44
3.3.3.2 Colony formation assay 45
3.3.4 Transient transfection of plasmids into H1299 Cells 45
3.3.4.1 Cell culture and transfection of plasmids into H1299 cells45
3.3.4.2 RNA extraction 46
3.3.4.3 Reverse–transcription PCR 47
3.3.4.4 Quantitative real-time RT-PCR of the *p21*^{waf1} gene 47
3.3.5 Statistical Analysis 48

CHAPTER FOUR: RESULTS 49
4.1 Production of pCMVp53R248Q via PCR Site-Directed Mutagenesis ... 49
4.1.1 Detection of the recombinant protein expression R248Q 50
4.2 Stable transfection analysis 50
4.2.1 Colony Formation Assay 50
4.3 Transient transfection analysis 53
4.3.1 Quantitative real-time RT-PCR analysis of the *p21*^{waf1} gene 53

CHAPTER FIVE: DISCUSSION 55
5.1 Production of recombinant protein pCMVp53R248Q 60
5.2 Genotype and phenotype study of pCMVp53 and pCMVp53R248Q 62
5.2.1 Stable transfection analysis 57
5.2.3 Transient transfection analysis 62

CHAPTER SIX: CONCLUSION AND RECOMMENDATIONS 73
6.1 Conclusion 73
6.2 Recommendations 74

BIBLIOGRAPHY 75

APPENDIX A 84
APPENDIX B 86
APPENDIX C 88
APPENDIX D 89
APPENDIX E 97
APPENDIX F.. 109
APPENDIX G 110

LIST OF TABLES

Table No.		Page No.
4.1	Colony Formation Assay of H1299 cells transfected with pCMV, pCMVp53, and pCMVp53R248Q	52
4.2	Relative fold difference of the *p21*^{waf1} gene between treatments (pCMVp53 and pCMV*p53*R248Q) and control	53
4.3	Relative fold difference of the *p21*^{waf1} gene between treatments (pCMVp53 and pCMVp53R248Q)	54
4.4	The volume of chemicals for control reaction	86
4.5	The volume of chemicals for sample reaction	86
4.6	The volume of chemicals for transformation of XL-1 blue supercompenant cells	87
4.7	*p21*^{waf1} PCR reaction mixture	88
4.8	*β-actin* PCR reaction mixture	88
4.9	*GAPDH* PCR reaction mixture	88
5.0	Number of H1299 colonies of cells transfected with pCMV, pCMVp53 and pCMVp53R248Q plasmids	89

LIST OF FIGURES

Figure No.		Page No.
2.1	Cancer population maps	6
2.2	p53 protein structure and hotspot mutation	11
2.3	Schema of the human *p53* gene structure: alternative splicing (*α, β, γ*).	13
2.4	Putative p53 protein isoforms	14
2.5	Regulations and activation of p53 transcriptional activities	16
2.6	p53 stress response pathways	19
2.7	Types of mutation distribution in cell lines	21
2.8	Effect of *TP53* mutations on the role of p53 in cancer related processes	23
2.9	Formation of homotetramer and heterotetramer of p53	24
2.10	The one day steps of inducing mutagenesis of a plasmid	29
2.11	Molecular route of a DNA vaccine	32
4.1	Codon 248 which has been selected to undergo point mutations.	49
4.2	Western blotting of the R248Q protein	50
4.3	Colony formation assay of H1299 cells	51
5.1	Possible random segregation of exogenous gene in the H1299 daughter cell *in vitro* model	61
5.2	Possible random distribution of *TP53* and *TP53R248Q* inserted plasmid to H1299 *in vitro* model	68

LIST OF ABBREVIATIONS

Bak	Bcl-2 Antagonist/Killer
Bax	Bcl-2 Associated X Protein
CFA	Colony Formation Assay
CMV	Cytomegalovirus
Ct	Threshold cycle
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's Modified Eagle's (Or Essential) Medium
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribonucleic Triphosphates
dsDNA	double stranded DNA
EDTA	Ethylenediaminetetraacetic Acid
EtOH	Ethanol
E. coli	Escherichia Coli
FBS	Fetal Bovine Serum
GADD	Growth Arrest And DNA Damage
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GOF	Gain Of Function
HKG	Housekeeping Gene
IARC	International Agency For Research On Cancer
Kbp	Kilobasepairs
kDa	kiloDalton

LOH	Loss Of Heterozygosity
MDM2	Murine Double Minute Protein
NCBI	National Center For Biotechnology Information
PBS	Phosphate Buffer Saline
TP53	Human protein 53
RNA	Ribonucleic Acid
RPM	Rotation Per Minute
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SD	Standard Deviation
SDS-PAGE	Sodium Dedocyl Sulfate- Polyacrylamide Gel Electrophoresis
SV40	Simian Virus 40
Taq	Thermus Aquaticus
TBS	Tris-Buffered Saline
WHO	World Health Organisation

LIST OF SYMBOLS

Δ	Delta (capital)
α	Alpha
β	Beta
γ	Gamma
δ	Delta (small)
ε	Epsilon
δ	Zeta
ε	Eta
µg	Microgram
µL	Microliter
g	Gram
L	Liter
mg	Milligram
ng	Nanogram
mL	Milliliter
µl	Microliter
µm	Micrometer
°C	Degree celcius

LIST OF APPENDICES

Appendix No.		Page No.
A. 1	DMEM complete medium	84
A. 2	Freezing medium	84
A. 3	PBS (Phosphate Buffer Saline) A	84
A. 4	Preparation of 1.5% agarose gel	84
A. 5	Preparation of IX TAE buffer	85
A. 6	Dilution stock of 1 kb DNA ladder	85
A. 7	Preparation of DEPC (Diethylpyrocarbonate) treated water	85
A. 8	Preparation of LB agar	85
A. 9	Preparation of NYZ+ broth	85
A. 10	Preparation of LB-ampicillin agar	85
B.1	The volume of chemicals for control reaction	86
B. 2	The volume of chemicals for sample reaction	86
B. 3	The volume of chemicals for transformation of XL-1 Blue supercompenant cells	87
C. 1	*p21^{waf1}* PCR Reaction Master Mix	88
C. 2	*β-actin* PCR reaction mixture	88
C. 3	*GAPDH* PCR reaction mixture	88
D. 1	Colony numbers of colony formation assay	89
D. 2	The bar chart of colony formation assay	90
D.3	T-TEST output: Analysis between treatment pCMV and pCMVp53	91
D.4	T-TEST OUTPUT: Analysis between treatment pCMV	93

	and pCMVp53R248Q	
D.5	T-TEST OUTPUT: Analysis between treatment pCMVp53 and pCMVp53248Q	95
E.1	*p21^{waf1}* quantitative real-time PCR output	97
E2	*p21^{waf1}* amplification standard curve	97
E.3	*p21^{waf1}*amplification plot	98
E.4	*p21^{waf1}* melting point curve	98
E.5	The bar chart–relative fold differences of the *p21^{waf1}* gene (normalisation with *β–actin*)	99
E.6	The bar chart – relative fold differences of the *p21^{waf1}* gene (normalisation with *GAPDH*)	100
E.7	*β –actin* quantitative real-time PCR data	101
E.8	*β –actin* amplification standard curve	101
E.9	*β-actin* amplification standard plot	102
E.10	*β-actin* melting point curve	102
E.11	*GAPDH* quantitative real-time PCR data	103
E.12	*GAPDH* standard curve	103
E.13	*GAPDH* amplification standard plot	104
E.14	*GAPDH* amplification melting curve	104
E.15	REST 2009: *p21^{waf1}* relative expression between pCMVp53 treatment group and control group (pCMV)	105
E.16	REST 2009: *p21^{waf1}* relative expression between pCMVp53R248Q treatment group and control group (pCMV)	106
E. 17	REST 2009: *p21^{waf1}* relative expression between pCMVp53 treatment group and pCMVp53R248Q	107
E. 18	Relative fold difference of the *p21^{waf1}* gene between treatments (pCMVp53 and pCMVp53R248Q) and control by Fugene® 6 transfection reagent.	108

E. 19	Relative fold difference of the *p21^{waf1}* gene between treatments (pCMVp53 and pCMVp53R248Q)by Fugene® 6 transfection reagent	108
F. 1	Map of pCMV plasmid by Clontech (Stratagene)	109
G. 1	Restriction map of *p53* full (ATG-TGA)	110
G. 2	Sequence alignment of mutated *p53R248Q* gene	112

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Throughout years, cancer has affected numbers of human population and becoming the number one killer worldwide. As reported by World Health Organization (WHO) (2004), death toll due to cancer was approximately 7.4 million or 13 percent in the respective year. In year 2030, the number of mortality of human population is predicted to increase up to 12 million (WHO).

Generally, cancer is classified as a multifaceted disease which can affect any parts of the body. It starts from a single cell, which undergo uncontrolled growth process that is beyond their normal growth restrain. Once the cancer cells undergo metastases, the mutated cells begin to spread and invading other organs. Thus, this may lead to severe implications to the internal homeostasis of the body and may leads to death.

There are many factors that contribute to the development of cancer cells. Carcinogens and genetic factors, has been identified to be the major cause of the disease. Development of cancer due to genetic factors may expose the family members to the early onset cancer. The accumulation of carcinogens in the body has been proved to alter genetic stability. The widely known tumor suppressor gene, *TP53* was identified to harbor numbers of mutation that contributes to the tumor formation. p53 main function is to regulate cell cycle, DNA repair and cell apoptosis. Loss of function of the gene due to mutation caused by carcinogens and genetic factors, leads to the uncontrolled cells proliferation. Identified more than 30 years ago,

p53 encodes one of the most intensively studied gene, where it accounted more than fifty thousand papers have been published (http://www.ncbi.nlm.nih.gov).

A single normal tumor suppressor gene in a cell is normally adequate to perform the normal function of the *TP53* gene, but loss of function of both alleles, by mutation or deletion or a combination thus, leads to dysregulation of cellular growth (Roth et al., 1999). Numbers of mutations within the gene are found, and several hot-spots such as R175, G245, R243, R248, R273, R282 (Petitjean et al., 2007) have been identified in *p53* structure, mainly at its DNA binding domain (DBD).

Until now, conventional treatments such as surgery, chemotherapy, and radiation, are the common choice for treatment of all cancers. Despite these advancements, cancer morbidity and mortality is still high since some of these treatment approaches are highly invasive and sometimes have only a palliative effect (Haupt et al., 2002).

The introduction of gene therapy using wild-type *TP53* has become a promising treatment for cancer nowadays. Numbers of studies conducted using engineered adenovirus vectors expressing human full length wild-type *TP53* shows that the treatment could infect and inhibit cells proliferation in many different human tumor cells and at the same time, prevent apoptosis or senescence in normal cells (Lane, 2010). However, the occurrence of *TP53* mutation carrying dominant-negative activity in tumor cells shows the difficulties of gene therapy implementation. The *TP53* activity of the treatment may be inhibited by such mutant p53 proteins that are expressed in the tumor cell.

Due to these factors, alternative options to prevent or to treat tumor arise from dominant-negative activity and other tumors are currently under investigation and DNA vaccine is the suitable candidate to be developed. In DNA vaccine, the sequence

of the gene of interest for the specific antigen production is cloned into a bacterial plasmid containing promoter/enhancer elements of choice to activate the antigen expression (Yu and Finn, 2006). The expression of the antigen may induce cellular immune response that can eliminate the specific affected tumor cells. The mechanism of DNA vaccines can produce immune response both humoral and cellular, mediated by both antibodies and cytotoxic T lymphocytes (Yu and Finn, 2006).

Mutations in the *TP53* gene are suitable to be developed as an antigen in this method. As describe earlier, there are several mutations in *TP53* gene that have been identified to contribute to the loss of the gene's function. Mutations in codon 248, shows the highest prevalence in cancer and prone to mutations than others. Mutant R248Q is one of an example of the mutation of *TP*53 found in HSC-4 cancer cell lines. This mutant exhibits dominant-negative manner by the inactivation of the wild-type p53 functions. Compared to carcinogenesis, dominant-negative manner only requires mutation in an allele to results in loss of function of p53. The proposed mechanisms of dominant-negative effect of mutant R248 is due to insufficient participation of the mutant in transactivation of certain p53 target genes, in which heterotetramer formation of wild-type/mutant p53 fails to bind to the DNA (Willis et al., 2004).

Although in some tumor, p53 activation through treatment of exogenous p53 may be suitable to regain the ability of p53 function, however it may not be effective to cancer arise cause by dominant-negative manner. As describe earlier, formation of heterotetramer of endogenous wild-type/mutant p53 unable to transactivate certain p53 downstream target genes although the wild-type p53 is still in normal function (Willis et al., 2004). Therefore, inactivation of the mutant p53 function through siRNA or activation of the immune system through DNA vaccination may be the

better treatment for this kind of cancer manner. The identification of mutant p53 as tumor-specific antigens by the immune system (Novellino et al., 2005) allows the development of DNA vaccination strategy and may become an interesting method to restrain the cells proliferation.

In the laboratory, this specific mutant sequence can be encoded into vector plasmid via PCR based site-directed mutagenesis. This new technique of producing mutant gene makes the cloning process easier compared to the previous conventional cloning process. The induction of mutation can be done with specific oligonucleotides primers, which correspond to the target mutation sequences. Therefore, this technique allows any types of mutant production and suitable technique to develop a tailor-made vaccine.

In this study, genotype and phenotype analyses were conducted on the constructed human recombinant gene that mimics specific hot-spots mutation of codon 248. The mutation was done through Polymerase Chain Reaction (PCR) based site-directed mutagenesis to a p53 plasmid vector using a specific primer. This generated recombinant protein, designated as pCMVp53R248Q was transfected to *TP53*-null H1299 cell lines. Both analyses were conducted to determine the tumor suppression ability of the genetically engineered plasmid pCMVp53R248Q. The results obtained shows the possibilities for the development of other recombinant proteins using PCR based site-directed mutagenesis which in the future, can be used as gene therapy strategy to treat or prevent cancer.

1.2 OBJECTIVES

The objectives of this research project are: i) to produce mutant *TP53* gene (*TP53R248Q*) of plasmid DNA via PCR based site-directed mutagenesis; ii) to