UNIVERSITI PUTRA MALAYSIA

COMBINATION THERAPY USING PLASMID DNA-MEDIATED VP3 AND SHCD147 GENES FOR COLON CANCER IN A MURINE MODEL

RUZILA ISMAIL

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By

RUZILA ISMAIL

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Doctor of Philosophy

December 2014
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Seeking His pleasure...

Dedicated to my father Ismail Mohamed,
My late mother Pathimah Md.Nasir,
My husband Ahmad Mubarak Tajul Arifin

and my beloved family
Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

COMBINATION THERAPY USING PLASMID DNA-MEDIATED VP3 AND SHCD147 GENES FOR COLON CANCER IN A MURINE MODEL

By

RUZILA ISMAIL

December 2014

Chairman : Associate Professor Zeenathul Nazariah Allaudin, PhD

Faculty : Institute of Bioscience

Several strategies have been pursued to develop cancer therapies that selectively act on and kill cancer cells preferentially, leaving normal cells unaffected. The VP3, an avian virus-derived protein, can specifically impulse the death signal to different signal transduction pathways and finally lead to the apoptosis of the host cancer cells. The present work utilized a stress-inducing promoter which is a glucose-regulated protein (GRP) promoter to enhance VP3 expression in solid tumor condition. On the other hand, the over-expression of Basigin/CD147, a transmembrane glycoprotein has association with malignant melanoma invasiveness, metastasis and angiogenesis. Since CD147 has been indicated to be one of the critical cell-surface proteins in promoting chemo-resistance and survivability of cancer stem cells (CSC), down regulation of its expression by RNAi is an attractive way to suppress CD147-dependent cell proliferation, invasion and metastatic activity of cancer cells to eventually induce anoikis. All necessary impact of efficiencies and in vitro evaluation to optimize in this project has been established. In this study, tumor-bearing murine model was established. Investigation was conducted on 175-200 mm³ tumor model receiving highly purified plasmid DNA in single treatment or combination of VP3 and shCD147 via intratumoral route (n=8). An alternate-date dosing approach was practiced whenever 3 doses were needed. Control groups were either a) non-treated, b) received 3 doses of 100 µg of pVIVO1-GFP/LacZ or c) 3 doses of 100 µg of psiRNA-h7SKzeo. Whilst treated mice received either a) 3 doses of 100 µg of pVIVO1-GFP/VP3 or b) 3 doses of 100 µg of psiRNA-CD147. For combinative therapy, mice received either a) 3 doses of 50 µg of pVIVO1-GFP/VP3 with combination of 3 doses of 50 µg of psiRNA-CD147 or b) 3 doses of 100 µg of pVIVO1-GFP/VP3 plus 3 doses of 100 µg of psiRNA-CD147, as representative for low-dose and high-dose respectively. All the 56 mice were subjected to 12 h light/ 12 h dark cycle and kept in individually ventilated cages (IVC) with constant rotation rate of 70 air-changes/ h to ensure sterility. Administration of VP3 alone led to
percentage tumor growth inhibition (TGI) of 40.0% and a 1.3-fold increase in the
tumor growth delay index (TGDI) whilst administration of shCD147 led to TGI of
45.2% and 1.2-fold increase in the TGDI value. Whereas combination of low-dose
treatment led to TGI of 51.1% (p<0.001) and a 2.0-fold increase in the TGDI whilst
high-dose combinative treatment led to higher TGI of 60.3% (p<0.001) and 2.3-fold
increase in the TGDI. These results demonstrated an extensive inhibition of CT26
tumor xenograft growth by VP3 and shCD147 combinative approach. Moreover,
there is no discernible effect observed on histopathology and clinical chemistry
profile of the host. The anti-proliferation and anti-angiogenic activities of the
combinative approach were investigated by the use of the immunohistochemistry
analysis, PCNA and vWF. Apoptotic cells were determined in the treated group by
DNA fragmentation, TUNEL assay and confirmed by AnnV/PI double staining. The
apoptosis percentage in the combinatively (VP3 + shCD14/2) treated tumor was
markedly increased compared to individually treated samples at day-25 post-
treatment. Here, it was found that CD147 silencing induced dual coordinated effects,
resulting in inhibition of tumor cell proliferation and sensitization to VP3-induced
apoptosis. In conclusion, this study showed that the combinative approach was more
promising and effective in controlling tumor growth and inducing apoptosis than
introducing VP3 or shCD147/2 alone. The combinative treatment also offers
potential advantages in control of tumorigenesis, and thus deserves further research
as a preferred approach in cancer gene therapy.
Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai
memenuhi keperluan untuk ijazah Doktor Falsafah

TERAPI GABUNGAN MENGGUNAKAN GEN VP3 DAN SHCD147
BERPERANTARAAN DNA PLASMID UNTUK KANSER KOLON DALAM
MODEL MENCIT

Oleh

RUZILA ISMAIL

Disember 2014

Pengerusi : Profesor Madya Zeanathul Nazariah Allaudin, PhD

Fakulti : Institut Biosains

Beberapa strategi telah dilaksanakan untuk membangunkan terapi kanser yang
bertindak secara terpilih dan membunuh sel-sel kanser secara pilihan, meninggalkan
sel-sel normal tidak terjejas. VP3, protein virus yang diperolehi dari burung, boleh
mendorong isyarat kematian yang berbeza isyarat transduksi dan akhirnya membawa
dapat apoptosis kepada sel-sel kanser perumah secara khusus. Kajian semasa
menggunakan promotor tekanan-mendorong iaitu promotor glukosa dikawal-selia
protein (GRP) untuk meningkatkan ekspresi VP3 dalam keadaan tumor pepejal. Di
sisi lain, lebih-ekspresi Basigin/CD147, glikoprotein transmembran mempunyai
dapat kaitan dengan pencerobohan, metastatik dan angiogenik melanoma malignan.
Memandangkan CD147 telah ditunjukkan untuk menjadi salah satu protein sel
permukaan kritikal dalam menggalakkan rintangan kimo dan kemandirian stem sel
cancer (CSC), menurunkan ekspresinya dengan RNAi adalah cara yang menarik
untuk menindas proliferasi sel, serangan dan aktiviti metastatik sel-sel kanser yang
bergantung kepada CD147 untuk akhirnya mendorong kepada anoikis. Semua kesan
kecekapan dan penilaian dalam vitro yang perlu untuk mengoptimumkan dalam
projek ini telah dilakukan. Dalam kajian ini, model mencit pembawa-tumor telah
ditubuhkan. Siasatan telah dijalankan ke atas model tumor 175-200 mm³ yang
menerima plasmid DNA berketulenan tinggi dalam rawatan tunggal atau kombinasi
VP3 dan shCD147/2 melalui kaedah intratumoral (n=8). Pendekatan dos silih ganti
hari diamalkan apabila 3 dos yang diperlukan. Kumpulan kawalan adalah sama ada
a) tidak dirawat, b) menerima 3 dos 100 μg daripada pVIVO1-GFP/LacZ atau c) 3
dos 100 μg daripada psiRNA-h7SKzeo. Manakala mencit yang dirawat menerima
sama ada a) 3 dos 100 μg daripada pVIVO1-GFP/VP3 atau b) 3 dos 100 μg daripada
psiRNA-CD147/2. Untuk terapi kombinasi, mencit menerima sama ada a) 3 dos 50
μg daripada pVIVO1-GFP/VP3 dengan gabungan 3 dos 50 μg daripada psiRNA-
CD147/2 atau b) 3 dos 100 μg daripada pVIVO1-GFP/VP3 ditambah 3 dos 100 μg
Daripada psiRNA-CD147/2, masing-masing sebagai wakil dos rendah dan dos tinggi. Kesemua 56 mencit telah tertakluk kepada kitaran 12 jam cahaya/12 jam gelap dan disimpan dalam sangkar pengudaraan secara individu (IVC) dengan kadar putaran udara berterusan 70 kali/jam untuk memastikan kesterilan. Pemberian suntikan VP3 membawa kepada perencatan peratusan pertumbuhan tumor (TGI) sebanyak 40.0% dan peningkatan 1.3 kali ganda dalam indeks kelewatan pertumbuhan tumor (TGDI) manakala pemberian suntikan shCD147/2 menyebabkan TGI sebanyak 45.2% dan peningkatan 1.2 kali ganda dalam nilai TGDI. Sedangkan gabungan rawatan dos rendah menyebabkan TGI sebanyak 51.1% (p <0.001) dan peningkatan 2.0 kali ganda dalam TGDI manakala rawatan kombinasi dos tinggi membawa kepada yang lebih tinggi TGI sebanyak 60.3% (p <0.001) dan peningkatan 2.3 kali ganda dalam TGDI. Keputusan ini menunjukkan satu perencatan yang banyak terhadap pertumbuhan tumor xenograft CT26 oleh pendekatan kombinasi VP3 dan shCD147. Tambahan pula, tidak ada kesan yang ketara diperhatikan pada histopatologi dan profil kimia klinikal perumah. Aktiviti anti-proliferasi dan anti-angiogenik daripada pendekatan kombinasi telah disiasat oleh penggunaan analisis immunohistokimia, PCNA dan vWF. Sel-sel apoptotic ditentukan dalam kumpulan yang dirawat menggunakan fragmentasi DNA, asei TUNEL dan disahkan menggunakan pewarnaan berganda AnnV/PI. Peratusan apoptosis dalam tumor dirawat secara kombinasi (VP3 + shCD14/2) telah meningkat dengan ketara berbanding dengan sampel yang dirawat secara individu pada hari-25 selepas rawatan. Di sini, didapati bahawa penyenyap CD147 mendorong kesan penyelarasan dwi, menyebabkan perencatan percambahan sel tumor dan pemekuan untuk apoptosis VP3-teraruh. Kesimpulannya, kajian ini menunjukkan bahawa pendekatan secara kombinasi lebih menjanjikan dan berkesan dalam mengawal pertumbuhan tumor dan mendorong apoptosis daripada memperkenalkan VP3 atau shCD147/2 sahaja. Rawatan secara kombinasi juga menawarkan kelebihan yang berpotensi mengawal tumorigenesis, dan dengan itu layak mendapat penyelidikan lanjut sebagai pendekatan utama dalam terapi gen kanser.
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I certify that a Thesis Examination Committee has met on 8 December 2014 to conduct the final examination of Ruzila Ismail on her thesis entitled “Combination Therapy using Plasmid DNA-Mediated VP3 and shCD147 Genes for Colon Cancer in a Murine Model” in accordance with 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 degree of Doctor of Philosophy.

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<tr>
<td>%</td>
<td>Percentage</td>
</tr>
<tr>
<td>µl</td>
<td>microliter</td>
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<tr>
<td>A</td>
<td>ampere</td>
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<tr>
<td>a.a</td>
<td>Amino acid</td>
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<tr>
<td>AI</td>
<td>Apoptotic Index</td>
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<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
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<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulfate</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate aminotransferase</td>
</tr>
<tr>
<td>ATV</td>
<td>Antibiotic Trypsin Versin</td>
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<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
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<tr>
<td>bp</td>
<td>Base pair</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>BUN</td>
<td>Blood urea nitrogen</td>
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<tr>
<td>CAV</td>
<td>Chicken anaemia virus</td>
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<td>CD147</td>
<td>Cluster of differentiation 147</td>
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<tr>
<td>CMVenh</td>
<td>Cytomegalovirus enhancer</td>
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<tr>
<td>CRC</td>
<td>Colorectal cancer</td>
</tr>
<tr>
<td>CT26</td>
<td>Colon Tumor #26</td>
</tr>
<tr>
<td>Cyp</td>
<td>cyclophilin</td>
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<tr>
<td>DAB</td>
<td>3', 3'-diaminobenzidine</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>deionised distilled water</td>
</tr>
<tr>
<td>dH₂O</td>
<td>distilled water</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<tr>
<td>dNTPs</td>
<td>Dideoxynucleotide triphosphates (dATP, dTTP, dCTP and dGTP)</td>
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<tr>
<td>dsRNA</td>
<td>Double-stranded RNA</td>
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<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
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<td>EMMPRIN</td>
<td>Extracellular matrix metalloproteinase inducer</td>
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<tr>
<td>et al.</td>
<td>et alii</td>
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<td>EtBr</td>
<td>Ethidium bromide</td>
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<tr>
<td>FBS</td>
<td>Fatal bivine serum</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<tr>
<td>g</td>
<td>Gram</td>
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<td>G</td>
<td>Gauge</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GRP</td>
<td>Glucose regulate protein</td>
</tr>
<tr>
<td>h</td>
<td>Hour(s)</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<tr>
<td>HSV</td>
<td>Herpes Simplex Virus</td>
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<tr>
<td>i.e.</td>
<td>“id est”</td>
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xxi
i.m  Intramuscular
i.p  Intraperitoneal
i.t  Intratumoral
i.v  Intravenous
lg   immunoglobulin
IHC  Immunohistochemistry
Kb   Kilo base
kDA  Kilo Dalton
mA   Milliampere
MCT  monocarboxylate transporter
mg   Milligram
MgCl$_2$  Magnesium chloride
min  minute
miRNA micro Ribonucleic Acid
ml   milliliter
mM   millimolar
MMP  Matrix metalloproteinase
mRNA Messenger Ribonucleic Acid
MT1  membrane type 1
MTT  3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
ng   nanogram
nt   Nucleotide(s)
NTC  Non-template control
PBS  Phosphate buffered saline
PBST  Phosphate buffered saline tween-20
PCD  Program cell death
PCNA Proliferating Cell Nuclear Antigen
PCR  Polymerase chain reaction
pol  polymerase
PVDF Polyvinylidene difluoride
RA   rheumatoid arthritis
RE   Restriction endonuclease
RISC RNA Interference Specificity Complex
RNA  Ribonucleic Acid
RNAi Ribonucleic Acid interference
rpm  Revolution per minute
RPMI Roswell Park Memorial Institute medium
RT   Reverse transcription or reverse transcriptase
RTV  Relative tumor volume
s.c. Subcutaneous
SDS  Sodium Dodecyl Sulfate
SDS-PAGE SDS-polyacrylamide gel electrophoresis
sec  Second(s)
<table>
<thead>
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<tr>
<td>shRNA</td>
<td>Short hairpin Ribonucleic Acid</td>
</tr>
<tr>
<td>siRNA</td>
<td>Short interfering Ribonucleic Acid</td>
</tr>
<tr>
<td>ssRNA</td>
<td>Single-stranded Ribonucleic Acid</td>
</tr>
<tr>
<td>SV40enh</td>
<td>Simian virus 40 enhancer</td>
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<tr>
<td>TAE</td>
<td>Tris acetate EDTA buffer</td>
</tr>
<tr>
<td>Taq</td>
<td>Thermus aquaticus thermostable DNA</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris borate EDTA buffer</td>
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<tr>
<td>TCSF</td>
<td>Tumor collagenase stimulatory factor</td>
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<td>TE</td>
<td>Tris-EDTA buffer</td>
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<tr>
<td>TEMED</td>
<td>N, N, N', N'-tetramethylethylenediamine</td>
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<td>TGD</td>
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<td>TGDI</td>
<td>Tumor growth delay index</td>
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<tr>
<td>TGI</td>
<td>Tumor growth inhibition</td>
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<tr>
<td>TIMP</td>
<td>Tissue inhibitor of metalloproteinase</td>
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<td>TUNEL</td>
<td>Terminal deoxynucleotidyltransferse-mediated dUTP Nick End Labeling</td>
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<td>UV</td>
<td>Ultraviolet</td>
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<tr>
<td>V</td>
<td>Volt</td>
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<td>VP3</td>
<td>Virus protein 3</td>
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<td>vWF</td>
<td>von Willebrand factor</td>
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CHAPTER 1

INTRODUCTION

1.1 Background

Cancer remains one of mankind’s most feared diseases worldwide. Malignant cancer has remained as one of the five main causes of mortality for the past 20 years. According to International Agency Research for Cancer, colorectal cancer was the third most commonly diagnosed cancer worldwide with total of 1.36 million incidence in 2012 (IARC 2014a). In Malaysia, cancer incidence was listed as one of the ten leading causes of hospitalization (3.82%) and five leading causes of death (13.63%) in both Ministry of Health (MoH) and private hospitals (MoH, 2013). Colorectal cancer was the second commonest form of cancer in both Malaysian men and women after lung and breast cancer, respectively. According to National Cancer Registry, a total of 1185 (14.6%) of male and 1011 (10.0%) of female incidence per 100,000 population were reported in 2007 and the figure was expected to increase (Omar & Ibrahim Tamin, 2011).

High incidence in colorectal cancer was due to the delay in diagnosis and ineffectiveness in treatment. The current treatment applies begin with surgical procedures to excise colon tumor segments followed by radiotherapy and/or chemotherapy. These conventional treatments may not be effective as it is not only killing the cancer cells, but also the healthy cells surround. Besides, their adverse side-effects are debilitating for patients. Thus, it is crucial to develop new therapeutic methods for cancer so that cancer patients can have more alternative for the best therapy they need.

Gene therapy is an alternative method to cure or slow down the progression of advanced stage of malignant cancer. In somatic gene therapy, therapeutic DNA transgene either integrated in the genome, as an external episomal or plasmid are introduce into cells. Selective therapy for cancer cells, only attacking actively dividing cells while sparing normal cells unaffected is an emerging field in recent years. Several viruses need actively dividing cells during completion of their life cycle, thus viral proteins have gained attention as a feasible cancer selective therapy.

Apoptosis or programmed cell death is an energy-dependent, innate and genetically determined process utilized by multicellular organisms to eliminate unwanted or damaged cells. Cancer is one of the diseases that is related to the loss of control between apoptosis and survival (Bjelaković et al., 2005). The use of apoptosis inducing gene is one of the alternative treatments for cancer therapy. Ideally, the killing cancer cells via apoptosis is a safer way for cancer therapy because the side effects of conventional treatments can be reduced to a minimum and the choice of
targeting therapeutic gene can kill cancer cells without affecting the surrounding cells.

VP3, a product of the third open reading frame (ORF) of the chicken anaemia virus can specifically impulse the apoptosis signals in cancer cells (Backendorf et al., 2008). Not hampered by tumor-suppressor p53 mutations or over-expression of anti-apoptosis proteins makes VP3 the most eligible candidate for various cancers. Therefore, recombinant plasmid expressing the VP3 therapeutic gene will increase chances of cure for cancer patient.

In addition, numerous macromolecules and cellular agents have been attempted to target tumor cells more specifically rather than other factors related to tumor growth. These include cytokines, small molecules antagonists, monoclonal antibodies, oligonucleotides and gene-targeting vector (Wong, 2011). Although apoptosis is a popular goal in treatment strategies, current preclinical and clinical findings have demonstrated the lack of desired expectation in achieving this matter. This is due to evasion of apoptosis by cancer cells.

These disappointing results arise due to multiple factors predominantly the vasculatures that supply nutrients and oxygen in tumors. When the continuous supply of nutrients and oxygen to the tumor cells keeps tumors growing, even well-designed drugs are hindered from acting on the target in an effective manner. One promising solution arisen from the observation that growing tumors are dependent on the multiple vascularisation mechanism, angiogenesis (Döme et al., 2007).

CD147, an integral plasma membrane protein has the capability to promote angiogenesis formation through stimulation of vascular endothelial growth factor (VEGF) expression on cancer cells (Tang et al., 2005). Not restricted to VEGF, it stimulates abundance of matrix metalloproteinase (MMP)-production which cause extracellular matrix degradation and increased cellular migration and invasion. During metastases, usually majority of cells undergo anoikis, a kind of apoptosis which is induced by the detachment of anchorage-dependent cells from the adjacent extracellular matrix. However, CD147 was identified as a contributor to anoikis-resistance which leads to tumor cells metastases (Ke et al., 2012). Recently, CD147 has been indicated to be one of the critical cell-surface proteins in promoting chemoresistant and survivability of cancer stem cells (CSC) (Kang et al., 2013). In this regard, down regulation of its expression using RNA interference is an attractive way to suppress CD147-dependent cell proliferation, invasion and metastases activity of cancer cells to eventually induce anoikis.

Before entering into human clinical trials, the safety of the incipient therapeutic gene has to be substantiated with animal studies. As consideration to safety in gene therapy, it is customary to assess the biodistribution of the DNA plasmid in order to cater information on potential toxicities. The development of new, safe and effective cancer therapy for 21st century are at the midst of explosion. Scientists have achieved
an advance experimental in the simultaneous use of two or more agents for treating cancer. However, new technologies in combination therapy need innovative approaches, new models, standards and assays.

The aim of this study is to understand the effect and interaction between the tumor and its blood vessel during combinatorial pro-apoptotic and anti-tumorigenic therapy. Recently developed genomic and proteomic technologies, gene silencing against CD147 will be applied as anti-tumorigenic therapy. Due to short half-life of the siRNA, here, gene silencing will be applied in a vector form for long term expression. Anti-tumorigenic agent would be expected to inhibit tumor cell proliferation and invasion, while enhancing sensitization to pro-apoptotic agent induced apoptosis. In this study, tumors will be treated with combined pro-apoptotic and anti-tumorigenic therapy. This combinative approach may improve effectiveness of the tumor treatment.

1.2 Hypothesis of This Study

It is hypothesized that by utilizing inducible GRP78 promoter to drive cancer-selective VP3 expression would improve the expression in solid tumor while combinative approach of pro-apoptotic and anti-tumorigenic gene therapy would provide better and more effective means in treating and inducing apoptosis in tumor-bearing model.

1.3 Objectives of This Study

This study investigates the effects and efficiency of plasmid DNA-mediated VP3 and shCD147 genes therapy in murine model for colon cancer including:

1) To develop recombinant plasmids harboring VP3 (pVIVO1-GFP/VP3) and shCD147 (psiRNA-CD147).

2) To compare between GRP78 and CMV driven VP3 expression in vitro.

3) To select the best shCD147 construct with highest silencing effect in vitro.

4) To evaluate in vivo biodistribution and acute toxicity effect of pVIVO1-GFP/VP3 using effective dose and up to 4X effective dose.

5) To investigate the inhibition of tumor growth and apoptosis analysis of VP3, shCD147/2 or combination treatment in tumor-bearing murine model.

6) To examine the effect of VP3, shCD147/2 or combination treatments against cell proliferation and invasion in post-treated tumor mass.
REFERENCES


