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OPTIMIZATION FOR DETECTING THE EFFICIENCY OF VIRAL-VECTORED GENE DELIVERY VIA INTRATUMOURAL ROUTE IN MURINE MODEL

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By

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The journey of cancer and its therapy involve a committed process of discoveries either at preclinical set-up or clinical trials. The attractive features of viral-vectored therapy have shown significant progress at the level of animal models. Efficacy of the preclinical outcome eventually determines the successful rate of clinical endpoints. This study was conducted in order to evaluate the response of the replicative-incompetent-retroviral-based VP3 (rRV-VP3) treatment towards TROP-2 and CRIPTO-1 tumour markers in tumour modelling of Balb/c mice for seven subsequent days. TROP-2 and CRIPTO-1 were among the tumour markers involve in human tumour development while thorough investigation in murine model was still underway.

The recovered rRV-VP3 particles produced 1.67 x 10^4 CFU/ml titer from Colony-Formation-Assay. The rRV-VP3 titer was then correlated with a standard curve generated from Real-Time PCR assay, giving the ratio of rRV-VP3 particles-to-infectious particles at 1:6.0 x 10^{10} in order to initiate virus transduction. Gene expression of TROP-2 and CRIPTO-1 were detected in both rRV-VP3 treated tumour-bearing murine model and the control group. Expression of VP3 protein can be detected only in rRV-VP3-treated group. The main causes of over expression of tumour markers could be due to the introduction of the xeno-therapeutic gene and the short half-life of rRV-VP3 particles. Since gene expression study through conventional RT-PCR assay was merely a tool to detect the effectiveness of VP3 delivery *in vivo*, additional sensitive confirmation of protein behaviour was done. Therefore, optimization for protein-protein interaction and expression was investigated using Biacore Surface-Plasmon-Resonance (SPR) assay as it could mimic the *in vivo* system while minimizing animal usage for *in vivo* work.

Optimization of the ligands in the flow cells of the CM5 Sensor Chip was successful using 10 mM of sodium acetate of pH 5.0. Ligands were critical in detecting the presence of tumour markers and VP3 protein effectively. Eventually, the combination of 50mM NaOH and 10 mM glycine of pH 2.0 was the best regeneration buffer to disrupt the antigen-antibody complex. *In vivo* observation of rRV-VP3-treated group

for seven consecutive days revealed dramatic VP3 protein expression especially in Day 2 and Day 5, hence implies the efficient transduction of gene in the tumour. However, TROP-2 expression was almost similar to VP3 expression ($p>0.05$). One plausible explanation of the similarity is the shortage of VP3 protein to associate with anaphase-promoting complex in G2/M phase of cell cycle and subsequently unable to sufficiently inhibit the tumour cells proliferation, thus allowing the continuous expression of TROP-2 during the mitosis stage. Contrarily, a minimal expression of VP3 in tumour cells down regulated the expression of CRIPTO-1 protein ($p<0.05$) and this could relate to VP3 involvement in interfering the CRIPTO-1/ mitogen-activated-protein-kinase (MAPK) signaling pathway. Unlike the rRV-VP3-treated group, the control untreated groups showed insignificant changes in the pattern of protein expressions ($p>0.05$).

In summary, the usage of antibodies as the immobilized ligands for SPR was proven to be optimal for detecting tumour marker responses against VP3 treatment in *in vivo* analysis ($p<0.05$). In comparison to SDS-PAGE, the optimized SPR analysis managed to track the traces of protein expressions throughout the seven days of observation, enabling the interpretation of treatment analysis via real-time protein behavioural pattern.

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

PENGOPTIMUMAN UNTUK MENGESAN KECEKAPAN PENGHANTARAN VIRUS-VEKTOR GEN MELALUI INTRATUMOURAL DALAM MODEL MURIN

Oleh

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Mei 2015

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Perjalanan kanser dan terapi melibatkan proses penemuan komited samaada di peringkat pra-klinikal atau ujian klinikal. Ciri-ciri yang menarik terapi virus-vektor telah menunjukkan kemajuan yang ketara di tahap model haiwan. Keberkesanan hasil pra-klinikal akhirnya menentukan kadar kejayaan titik hujung klinikal. Kajian ini dijalankan untuk menilai tindakbalas daripada rawatan VP3 berasaskan replikasi-ketidakcekapan-retroviral (rRV-VP3) terhadap penanda tumour TROP-2 dan CRIPTO-1 dalam model tumour mencit BALB/c selama tujuh hari secara berterusan. TROP-2 dan CRIPTO-1 adalah antara penanda tumour yang terlibat dalam pembangunan tumour manusia sementara siasatan menyeluruh dalam model murin masih dijalankan.

Zarah rRV-VP3 yang dipulihkan menghasilkan 1.67 x 10^4 CFU/ml titer dari Ujian-Pembentukan-Koloni. Titer rRV-VP3 kemudiannya dikaitkan dengan lengkung piawai dijana daripada cerakin berasaskan Real-Time PCR, memberikan nisbah rRV-VP3 zarah ke zarah berjangkit pada 1: 6.0 x 10^{10} untuk memulakan virus transduksi. Ekspresi gen daripada TROP-2 dan CRIPTO-1 telah dikesan dalam model tumour mencit untuk kedua-dua kumpulan rRV-VP3 dirawat dan kumpulan kawalan. Ekspresi protein VP3 boleh dikesan hanya dalam kumpulan rRV-VP3 dirawat. Punca utama ekspresi tumour penanda berlebihan adalah mungkin disebabkan pengenalan gen xeno-terapeutik dan separuh hayat pendek zarah rRV-VP3. Oleh kerana kajian gen melalui cerakinan konvensional berasaskan RT-PCR adalah semata-mata alat untuk mengesan keberkesanan penghantaran VP3 dalam haiwan, pengesahan sensitif tambahan mod protein telah dilakukan. Oleh itu, pengoptimuman untuk interaksi dan ekspresi protein-protein disiasat menggunakan cerakinan Biacore Surface-Plasmon-Resonance (SPR) kerana ia seakan-akan menyamai sistem *in vivo* serta dapat meminimumkan penggunaan *in vivo* dalam kajian.

Pengoptimuman ligan dalam sel-sel aliran Sensor Chip CM5 berjaya menggunakan 10 mM natrium asetat pH 5.0. Ligan penting dalam mengesan kehadiran penanda tumour dan protein VP3 secara berkesan. Didapati, gabungan 50 mm NaOH dan 10 mM

glycine pada pH 2.0 adalah penampan regenerasi yang terbaik untuk mengganggu kompleks antigen-antibodi. Kumpulan haiwan yang dirawat dengan rRV-VP3 selama tujuh hari berturut-turut mendedahkan ekspresi protein VP3 yang dramatik terutamanya di hari ke-2 dan hari ke-5, membayangkan kecekapan transduksi gen dalam tumour. Walaubagaimanapun, ekspresi TROP-2 hampir sama dengan ekspresi VP3 ($p>0.05$). Satu penjelasan yang munasabah untuk persamaan ini adalah kekurangan protein VP3 bersekutu dengan anafase-mempromosikan-kompleks pada fasa G2/M kitaran sel dan seterusnya tidak cukup menghalang percambahan sel-sel tumour. Maka dengan itu membolehkan ekspresi TROP-2 yang berterusan ke peringkat mitosis. Sebaliknya, ekspresi VP3 yang minimum dalam sel-sel tumour dapat mengawalselia penurunan ekspresi protein CRIPTO-1 ($p<0.05$) dan hal ini boleh dikaitkan dengan penglibatan VP3 dalam mengganggu isyarat laluan CRIPTO-1/mitogen-diaktifkan-protein-kinase (MAPK). Tidak seperti kumpulan yang dirawat rRV-VP3, kumpulan kawalan yang tidak dirawat menunjukkan perubahan ketara dalam corak ekspresi protein ($p>0.05$).

Kesimpulannya, penggunaan antibodi sebagai ligan untuk SPR telah terbukti optima untuk mengesan respon penanda tumour terhadap rawatan VP3 di dalam analisis *in vivo* ($p<0.05$). Berbanding dengan SDS-PAGE, analisis SPR yang telah dioptimakan berjaya mengesan jejak ekspresi protein untuk pemerhatian sepanjang tujuh hari, membolehkan tafsiran analisis rawatan melalui masa-nyata corak mod protein.

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I certify that a Thesis Examination Committee has met on 21 May 2015 to conduct the final examination of NOR HIDAYAH BINTI MUSTAFA on her thesis entitled "OPTIMIZATION FOR DETECTING THE EFFICIENCY OF VIRAL-VECTORED GENE DELIVERY VIA INTRATUMOURAL ROUTE IN MURINE MODEL" 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 fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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bp	base pair
BLAST	Basic Local Alignment Search Tool
CAV	Chicken Anaemia Virus
Ca^{2+}	calcium
CEA	Carcinoembryonic antigen
CFU	Colony-forming-unit
CO_2	carbon dioxide
CRC	Colorectal cancer
Da	Dalton
DMEM	Dulbecco Eagle Media
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
DTT	dithiothreitol
e.g.	*Exempli gratia* (for example)
ERK	extracellular signal regulated kinase
HCl	hydrochloric acid
i.e.	*id est* (that is)
ITGA4	integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)
kb	kilo base
KCl	potassium chloride
kDa	kilo Dalton
M	molar
mA	milliampere
MAPK	mitogen-activated protein kinase
$MgCl_2$	magnesium chloride
mM	milli molar
MSDS	material safety datasheet
NaCl	sodium chloride
NCBI	National Center for Biotechnology Information
neo	neomycin
$(NH_4)_2SO_4$	ammonium sulfate
OD	optical density
PCR	polymerase chain reaction
pMSCV	plasmid murine stem cell virus
RNA	ribonucleic acid
RPM	revolution per minute
RPMI 1640	Roswell Park Memorial Institute 1640
RT PCR	reverse transcription polymerase chain reaction
RU	response unit
SAM	self-assembled monolayer
SPR	Surface plasmon resonance
TAA	Tumour-associated antigens
UV	ultraviolet
V	volt
VP3	Virus Protein 3
3-D	three dimensional

CHAPTER 1

INTRODUCTION

Historically, tumour modelling in murine model has been a popular trend in preclinical testing. In fact, it remains to be a powerful tool in the development of cancer and immunotherapy research and evaluation (Schuh, 2004). Bridging the gap between preclinical efficacy and clinical trials endpoints is notably a challenging and critical factor for cancer and tumour treatment with approximately 95% discrepancy rate was reported between them (Wartha *et al.*, 2014). Nevertheless, the limitations could be overcome through several ways such as the selection of animal models based on tissue and drug targets and appropriate study design together with its data evaluation.

Years by years, statistics showed that the cancer and tumour occurrence spread widely over genders, races, age and so forth without prejudice in every segment of the populations (Siegel *et al.*, 2014; Wendy & Radzi, 2008). Many types of the cancers have been diagnosed, for example, the breast, lung, head and neck, and colourectal cancer. In Malaysia, one of the most occurring cancers is the colon cancer (Wendy & Radzi, 2008). Borrowing the definition from U.S. National Institute of Health, colon cancer can be referred as the progressive formation of tissue in the colon and may extend to the intestine, rectum and bowel.

In reality, there are no specific tumour marker best at diagnosis, prognosis or at any level of screening or monitoring (Schrohl *et al.*, 2003). The selection of specific tumour marker depends on one's purpose since the idea of one-fits-all sometimes does not applicable to all level of society (Deisboeck, 2009). As for the case of colourectal cancer, Khare and Verma (2012) revealed that oncogenes lead to the colon tumourigenesis. There are a lot of oncogenes that has been discovered, in which, TROP-2 and CRIPTO-1 are among of them. Besides their characteristics as oncogenes, both can functioned as tumour marker-targeted therapy due to their common malignancies presence in tissue of colon carcinomas or known as oncogenes-addiction (Corso & Giordano, 2014).

Basically, the root towards most of the cancer problem was merely due to the uncontrolled progression of cells in the affected area. This phenomenon may lead to tumour malignancy that eventually alarms the body with the presence of the tumour-associated antigens (TAA) (Sidransky, 2002). In the event where tumourigenesis happened, TAA may produces tumour marker which can be found anywhere in the body such as blood, urine, and the tumour itself and by which they can be evaluated quantitatively or qualitatively (Schrohl *et al.*, 2003).

The beauty of tumour marker lies in its special attributes of representing the true-face of the disease and extremely useful in monitoring responses towards intervention therapy (Duffy, 2001). However, the relevancy of using tumour marker as the benchmark for the successfulness of the treatment is yet to be an on-going issue and need validation with proper quality assurance.

Clinically, the standard forms of treatments that are being used are such as surgery, chemotherapy, radiation therapy and targeted therapy. Unlike conventional treatments,

targeted therapy provides less harm and few side-effects since it works selectively towards tumour cells only (Corso & Giordano, 2014). Underneath it, recombinant viral-vectored therapy has gained high impact in this area, for example, recombinant avipox-CEA (Zhu *et al.*, 2000) and recombinant vaccinia-CEA virus (Kass*et al.*, 1999). The modified viruses which have been widely used are for instance coming from the adenoviridae, poxviridae, herpesviridae and retroviridae families (Cawood *et al.*, 2012).These viruses were in the state of either replication- or non-replication-competent.

According to Cawood *et al.* (2012), up to 40 clinical trials utilized recombinant viral vaccines in cancer treatment. Adenovirus of Ad2 and Ad5 serotypes were among the pioneers in the cancer vaccination studies due to its well-characterized genome and easily manipulation of exogenous DNA in the genome segment. As for poxviruses, the common types were such as vaccinia virus, fowlpox virus and canarypox virus, with their special features of having potent immunogenicity and broad range of host tropism. Besides adenovirus and poxvirus, replicating viruses which are also known as oncolytic viruses have been employed vastly and having said to promote better vaccine effect since it can boost the immune system within the tumour microenvironment (Robert-Guroff, 2007).

Moreover, involvement of engineered retrovirus in gene therapy clinical trials is not new due to its specialty in transferring therapeutic gene stably into the chromosomes while also having a broad range of host tropisms (Andreadis *et al.*, 1999; McTaggart & Al-Rubeai, 2002). Recently, a clinical trial conducted in University of California, San Diego, US has successfully delivered the modified retroviral harbouring anti-cancer flucytosine (Toca 511) into the patient's tumour brain (Miranda *et al.,* 2013).

Insertion and expression of exogenous DNA into the recombinant viral vector is a key to have successful viral-based vaccines once it is administered into the affected area. Apparently, TAAs is the popular choice besides immunomodulatory agents (Cawood *et al.*, 2012). One of the most crucial elements is to have a vaccine that can target and destroy tumour cells selectively. For instance, the discovery of Viral Protein 3 (VP3) gene of Chicken Anaemia Virus (CAV) or apoptin, has known for its nuclear localization signals (NLS) in tumour cells only, have taken many eyes to study it in depth (Maddika *et al.*, 2006; Ruzila *et al.*, 2010; Tavasolli *et al.*, 2005; Teodoro *et al.*, 2004).

Therefore, in order to detect the efficacy and efficiency of the viral-based vaccines, the approach should consider many critical factors such as the titre of the virus and its infectious state upon delivery. For *in vivo* applications, titers in between 10^7 to 10^{14} are recommended to produce a therapeutic effect (Andreadis & Palsson, 1996; Trasnfiguracion *et al.*, 2004). After that, validation of the transgene and proteome expressions of the vaccine are another factors need to be thoroughly monitored through any state-of-the-art technology, especially when it is related to tumour-targeted therapy (Altman & Royston, 2000; Chau *et al.*, 2008; Taylor *et al.*, 2008). Not forgetting is the cost that governs the issue (Greenland, 2008).

Considering the possibilities and drawbacks, proteome advances such as Surface Plasmon Resonance (SPR) assay which involve biomarker are seemingly in favours as they usually can reveal in depth information on protein profiling (Campagnolo *et al.*,

2004; Su *et al.*, 2008). Besides, SPR itself may serve as the platform for protein-protein behavioural study and may correlates with the mechanism and regulation of cell cycle.

In general, the study herein aims to evaluate the efficiency of recombinant retrovirus (rRV) harbouring VP3 gene (rRV-VP3) as anti-tumour treatment in tumour-bearing mice model. Therefore, the scope of the study is segmented into these several objectives:

1. To validate and reactivate the recovery of infectious rRV-VP3 particles for viral-vectored VP3-based treatment.
2. To deliver and analyze the expression of VP3 protein and tumour markers *in vivo*.
3. To optimize chip-based detection system for the determination of efficiency of anti-tumour treatment in preclinical set up.

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