CONSTRUCTION OF RECOMBINANT NEWCASTLE DISEASE VIRUS EXPRESSING GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR

By

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Chair : Associate Professor Chia Suet Lin, PhD
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Cancer continues to surpass human intervention for decades. The cancer mortality rate increases every year and there is neither a single treatment that is suitable for all tumours at different stages nor it could eliminate tumour completely. Clearly, alternative treatments to accommodate the demanding disease are anticipated. One of such treatments is oncovirotherapy – the use of virus as a therapeutic agent for cancer. Newcastle disease virus (NDV) is a promising anti-cancer agent because it selectively infects and replicates in cancer cells without harming the normal cells. To further enhance the oncolytic activity, NDV can be manipulated via reverse genetics to harbour and express immunomodulatory gene in NDV-infected cancer cells. The aim of this study was to construct two recombinant NDVs (rNDV) that express human and murine granulocyte-macrophage colony-stimulating factor (hGM-CSF and mGM-CSF). These genes were amplified from lipopolysaccharide (LPS)-induced human myeloid leukaemia cells and murine colorectal carcinoma cells, respectively, and were cloned into NDV antigenome plasmid, pOLTV5 (rAF-GFP). The plasmids were co-transfected with helper plasmids (pCIneoNP, pCIneoP, and pCIneoL) into BSR T7/R5 cells to produce the recombinant NDVs, designated as rAF-GFP/hGM-CSF and rAF-GFP/mGM-CSF. Genomes of the viruses were extracted and verified by DNA sequencing followed by a large-scale propagation of the virus using 9-day old embryonated egg. The expression of both GM-CSF genes was determined via ELISA. High hGM-CSF and mGM-CSF glycoproteins were expressed by both rAF-GFP/hGM-CSF and rAF-GFP/mGM-CSF virus during viral infection in human colorectal carcinoma cells. In migration assay, human myeloid leukaemia cells and mouse macrophage cells that were seeded on top of collagen matrix gel were shown to be attracted towards the human colorectal carcinoma infection supernatant containing hGM-CSF and mGM-CSF in 24 hours and 12 hours, respectively. In conclusion, rAF-GFP/hGM-CSF and rAF-GFP/mGM-CSF virus produced in this study successfully express hGM-CSF and mGM-CSF genes upon infection and they are biologically active as verified through migration assay. This warrant a further investigation for their potential to be used for cancer treatment.

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

PEMBINAAN VIRUS PENYAKIT NEWCASTLE REKOMBINAN YANG MENGEKSPRESIKAN FAKTOR PERANGSANG KOLONI MAKROFAJ GRANULOSIT

Oleh

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Kanser terus mengatasi intervensi manusia selama beberapa dekad. Kadar kematian disebabkan kanser meningkat setiap tahun dan tidak ada satu pun rawatan yang sesuai untuk semua tumor pada tahap yang berbeza dan tidak dapat menghilangkan tumor sepenuhnya. Jelaslah, rawatan alternatif untuk merawat penyakit yang rumit tersebut diharapkan. Salah satu rawatan tersebut adalah *oncovirotherapy* - penggunaan virus sebagai agen terapeutik untuk kanser. Virus penyakit Newcastle (NDV) adalah agen anti-kanser yang menjanjikan kerana ia secara selektif menjangkiti dan mereplikasi sel-sel kanser tanpa membahayakan sel-sel normal. Untuk meningkatkan lagi aktiviti onkolitik, NDV dapat dimanipulasi melalui genetik terbalik untuk menyimpan dan mengekspresikan gen imunomodulator dalam sel kanser yang dijangkiti NDV. Tujuan kajian ini adalah untuk membina dua NDV rekombinan (rNDV) yang mengekspresikan faktor perangsang koloni makrofag granulosit manusia dan tikus (hGM-CSF dan mGM-CSF). Gen-gen ini diperkuat dari sel leukemia mieloid manusia yang diaruhi oleh *lipopolysaccharide* (LPS) dan sel karsinoma kolorektal tikus, masing-masing, dan diklon ke dalam plasmid antigenom NDV, pOLTV5(rAF-GFP). Plasmid tersebut telah ditransfeksi bersama dengan plasmid penolong (pCIneoNP, pCIneoP, dan pCIneoL) ke dalam sel BSR T7/R5 untuk menghasilkan NDV rekombinan, yang dinamkan sebagai rAF-GFP/hGM-CSF dan rAF-GFP/mGM-CSF. Genom virus diekstraksi dan diverifikasi dengan urutan DNA diikuti dengan pembiakan virus secara besar-besaran menggunakan telur embrio berusia 9 hari. Ekspresi kedua-dua gen GM-CSF ditentukan melalui ELISA. Glikoprotein hGM-CSF dan mGM-CSF yang tinggi diekspresi oleh kedua-dua virus rAF-GFP/hGM-CSF dan rAF-GFP/mGM-CSF semasa jangkitan virus pada sel karsinoma kolorektal manusia. Dalam ujian migrasi, sel leukemia mieloid manusia dan sel makrofag tikus yang diletakkan di atas gel matriks kologen telah menunjukkan tertarik ke arah supernatan jangkitan karsinoma kolorektal manusia yang mengandungi hGM-CSF dan mGM-CSF, masing-masing dalam 24 jam dan 12 jam. Kesimpulannya, virus rAF-GFP/hGM-CSF dan rAF-GFP/mGM-CSF yang dihasilkan dalam kajian ini berjaya mengekspresikan gen hGM-CSF dan mGM-CSF apabila dijangkiti dan mereka aktif secara biologi seperti yang disahkan melalui ujian migrasi.

Ini memerlukan penyelidikan lebih lanjut mengenai potensi mereka untuk digunakan untuk rawatan kanser.

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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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LIST OF ABBREVIATIONS

%	Percent
×g	times gravity
°C	degree celsius
µg	Microgram
µL	Microliter
µm	micrometre
∞	Infinity
ANTRX1	anthrax toxin receptor 1
bp	base pair
CAR	coxsackievirus and adenovirus receptor
CD	cluster of differentiation
CD14	cluster of differentiation 14
cDNA	complementary deoxyribonucleic acid
CO_2	carbon dioxide
CSF2	colony stimulating factor 2
DAF	decay-accelerating factor
ddH_2O	double-distilled water
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
F	Fusion
F1	fragment 1

F2	fragment 2
F3	fragment 3
FBS	foetal bovine serum
g	gram
GE	gene end
GM-CSF	granulocyte-macrophage colony-stimulating factor
GM-CSFR	granulocyte-macrophage colony-stimulating factor receptor
GS	gene start
h	hour
hGM-CSF	human granulocyte-macrophage colony-stimulating factor
HN	haemagglutinin-neuraminidase
hpi	hour-post induction
IG	intergenic
JAK-2	janus kinase 2
JAM-A	junctional adhesion molecule A
L	litre/large
LOD	limit of detection
LPS	lipopolysaccharide
M	matrix
mg	miligram
mGM-CSF	murine granulocyte-macrophage colony-stimulating factor
min	minute
mL	millilitre
MOI	multiplicity of infection
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NDV	newcastle disease virus
ng	Nanogram
NP	nucleocapsid
OD	optical density
P	phosphoprotein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pfu/mL	plaque forming units/millilitre
PMA	phorbol 12-myristate 13-acetate
RNA	ribonucleic acid
rNDV	recombinant newcastle disease virus
RNP	ribonucleoprotein
s	Second
SOC	super optimal broth with catabolite repression
ssRNA	single-stranded ribonucleic acid
STAT5	signal transducer and activator of transcription 5
TfR	transferrin receptor
TLR4	toll-like receptor 4
v/v	volume/volume
w/v	weight/volume
α	Alpha
β	Beta
γ	Gamma

CHAPTER 1

INTRODUCTION

Cancer has caused approximately 10 million deaths globally and the number of mortalities in 2030 and 2040 are estimated to be 12.9 million and 16.3 million, respectively (World Health Organization, 2021). Despite the availability of cancer treatments, the estimated number of cancer mortalities is increasing. Cancer is formed by the accumulation of genetic abnormalities of the normal cells that may be induced by several factors such as unhealthy lifestyle, viruses, exposure to ultraviolet radiation and many more (National Cancer Institute, 2021 & World Health Organization, 2021). Such genetic abnormalities affect the homeostasis of the normal cells resulting in unregulated replication and eventually tumours formation (National Cancer Institute, 2021).

Cancer can be treated through surgery, radiotherapy, chemotherapy or a combination of them depending on the tumour type and patients' health condition. Although they are the gold standard treatment in many hospitals, these treatments are not potent enough to eliminate tumours completely (Kelly & Russell, 2007; Schirrmacher, 2019). Consequently, innovative intervention using biological agents for cancer treatment such as immunotherapy and virotherapy is continuously investigated in many preclinical and clinical studies (Schirrmacher, 2019). In virotherapy, virus that possesses oncolytic activity is manipulated and used as the anti-cancer agent (Russell & Peng, 2018). A virus that is suitable to be an oncolytic virus must possess certain traits. It must be non-pathogenic to humans and is able to lyse cancer cells without affecting the normal cells. The oncolytic virus can gain the oncolytic ability naturally or through genetic modification (Maroun et al., 2017; Russell & Peng, 2018). Out of the many oncolytic viruses available, Newcastle disease virus (NDV) has gained the researchers' interest to be used as an anti-cancer agent.

NDV is an avian pathogen that causes Newcastle disease in the avian species (Kaleta & Baldauf, 1988). It is an RNA virus with a genome that is non-segmented, single-stranded, and negative-sensed (3'→ 5') (Yusoff & Tan, 2001; Song et al., 2019). Its RNA genome consists of six genes encoding for six structural proteins and two non-structural proteins (Wilde et al., 1986; Yusoff & Tan, 2001). NDV is classified into three groups based on its pathogenicity; non-virulent (lentogenic strain), moderately virulent (mesogenic strain) and highly virulent (velogenic strain) (Ganar et al., 2014). Although it is an infectious avian virus, it is non-pathogenic to human causing only mild flu-like symptoms (Kaleta & Baldauf, 1988). The virus is a promising anti-cancer because it can specifically lyse cancer cells without going through any genetic modification (Zamarin & Palese, 2012). In addition, NDV oncolytic activity could also be enhanced via reverse genetics for genetic manipulation (Bukreyev & Collins, 2008). Integration of immunostimulatory gene in the NDV genome has been proven to improve the elimination of cancer cells (Janke et al., 2007; Cheng et al., 2016).

In 2015, talimogene laherparepvec (T-VEC) is the first viroimmunotherapy that was approved by the U.S. Food and Drug Administration (FDA) to be used on patients with non-resectable metastatic melanoma (Fountzilas et al., 2017). T-VEC is genetically modified herpes simplex virus-1 (HSV-1) that express human granulocyte-macrophage colony-stimulating factor (GM-CSF) which is an immunostimulatory gene and it was proven to be clinically benefit in a randomized phase III with an acceptable safety profile in patients with advanced melanoma (Fountzilas et al., 2017; Bommareddy et al., 2018).

The approval of T-VEC by FDA as a treatment for melanoma patients has shown that viroimmunotherapy is a dark horse in cancer treatment and this sparked researchers' interest to develop more biological anti-cancer agents including Cheng et al. (2016). They generated recombinant NDV that express human GM-CSF using NDV-73T mesogenic strain even though the strain is classified as selective agents. This is due to the correlation of NDV strains and their oncolytic ability which, the more virulent it is, the better it can lyse cancer cells (Kalyanasundram et al., 2018). The addition of the human GM-CSF gene with the mesogenic strain of NDV-73T enhanced immune cells infiltration without affecting its ability to lyse cancer cells (Cheng et al., 2016).

Currently, there is no report on the recombinant velogenic strain of NDV expressing GM-CSF even though it has the best oncolytic ability out of other NDV strains and the immunogenic effects after the integration of GM-CSF in the velogenic viral genome also remain unknown. This study aimed to generate recombinant NDV expressing human and murine granulocyte-macrophage colony-stimulating factors, rAF-GFP/hGM-CSF and rAF-GFP/mGM-CSF, respectively, from NDV rAF-GFP velogenic strain. It was hypothesised that upon infection of the viruses in the cancer cells, they would be able to release GM-CSF to attract macrophages. To achieve the objective, three sub-objectives were derived:

1. To clone human and murine GM-CSF gene into pOLTV5(rAF-GFP) plasmids
2. To recover the recombinant NDV harbouring the GM-CSF gene
3. To verify the expression and biological activity of GM-CSF during infection

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