UNIVERSITI PUTRA MALAYSIA

SAFETY AND EFFICACY OF RECOMBINANT NEWCASTLE DISEASE VIRUS EXPRESSING HUMAN INTERLEUKIN-12 AS A POTENTIAL VACCINE IN BREAST CANCER

ZAHIAH BINTI MOHAMED AMIN

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By

ZAHIAH BINTI MOHAMED AMIN

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

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

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March 2019

Chairman : Associate Professor Noorjahan Banu bt Mohamed Alitheen, PhD
Faculty : Biotechnology and Biomolecular Science

In this developing era, breast cancer still remains a life-threatening disease globally. However, oncolytic virotherapy has taken over interest as a promising non-conventional alternative to treating breast cancers. Newcastle disease virus (NDV), an avian paramyxovirus has been demonstrated with significant oncolytic activity against cancer based on numerous preclinical studies. Today, genetically modified viruses coding for immunomodulatory agents, such as cytokines or chemokines, have come into focus. Such engineered viruses are able to promote efficient immune responses against tumour cells. The overall of this project aims to study the effects of a recombinant NDV expressing human interleukin 12 (rAF-IL12) in the apoptotic and metastatic process in MCF7 and MDA-MB231 human breast cancer cell lines. The parental NDV AF2240 was used as a positive control in this study. Notably, rAF-IL12 was able to maintain its stability when passaged in specific pathogen free (SPF) eggs up to ten passages. Furthermore it is considered safe as it selectively induced cytotoxic effects in chicken and breast cancer cell lines while sparing non-cancerous breast cell line as demonstrated through the MTT assay. The stability of each passaged rAF-IL12 was verified *via* haemagglutination assay (HA), mean death time (MDT) and intracerebral pathogenicity index (ICPI), while the IL12 was quantified through Enzyme-Linked Immunosorbent Assay (ELISA). Comparable to AF2240, rAF-IL12 was also able to induce apoptosis significantly based on several apoptotic assays. Both rAF-IL12 and AF2240 managed to increase the percentage of G2/M and S phase in the cell cycle analysis while inducing the percentage of apoptosis. Although both AF2240 and rAF-IL12 demonstrated comparable *in vitro* apoptosis results, rAF-IL12 possessed significant ($p<0.05$) anti-metastatic activity in comparison to AF2240 based on metastasis related assays including the *in vitro* scratch assay, migration/ invasion assay, human umbilical vein endothelial cell (HUVEC) tube formation and rat aortic ring assay. Additionally, to further evaluate the anti-tumour and anti-metastatic mechanism of rAF-IL12, *in vivo* studies were conducted using 4T1-challenged BALB/c mice as a model of this study. The rAF-IL12

was proven to function as an improved tumour vaccine as it significantly (p<0.05) reduced the size of tumour in comparison to the parental AF2240 virus. Apoptotic results showed that the number of cancer cells in the tumour significantly (p<0.05) reduced after 28 days of intra-tumoural treatment with rAF-IL12 (2^7 HAU). In addition, rAF-IL12 was able to inhibit the migration of cancer cells to other vital organs as opposed to the untreated group. To further elucidate the apoptotic and anti-metastatic mechanism of rAF-IL12 at molecular level, NanoString nCounter was conducted. Even though both AF2240 and rAF-IL12 exhibited similar mechanism of action, rAF-IL12 was more potent than AF2240 in terms of apoptosis and anti- metastasis activity. In conclusion, both rAF-IL12 and AF2240 were able to inhibit the proliferation of breast cancer cells and induce apoptosis in the *in vitro* studies; however, rAF-IL12 was able to demonstrate significant (p<0.05) improved functionality in comparison to AF2240 alone based on the *in vivo* studies. This proved that interleukin 12 was able to increase the immune response against tumour cells by inducing cell death, anti-angiogenesis and anti-metastasis effects, further improving the function of AF2240 as a potential vaccine in breast cancer.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KESELAMATAN DAN KECEKAPAN REKOMBINAN VIRUS PENYAKIT SAMPAR MENGEKSPRESI INTERLEUKIN-12 MANUSIA SEBAGAI VAKSIN BERPOTENSI DI DALAM KANSER PAYU DARA

Oleh

ZAHIAH BINTI MOHAMED AMIN

Mac 2019

Pengerusi : Profesor Madya Noorjahan Banu bt Mohamed Alitheen, PhD
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Dalam era membangun ini, kanser payudara masih menjadi penyakit yang mengancam nyawa di seluruh dunia. Walaubagaimanapun, terapi viro onkolitik telah mengambil alih kepentingan sebagai alternatif yang tidak konvensional bagi merawat kanser payudara. Virus penyakit sampar (NDV) merupakan paramyxovirus burung telah menunjukkan aktiviti onkolitik yang ketara terhadap kanser berdasarkan pelbagai kajian pra-klinikal. Pada masa kini, virus pengubahsuaian genetik untuk ejen imunomodulator, seperti sitokin atau chemokin telah menjadi tumpuan. Ini adalah kerana virus kejuruteraan itu dapat menggalakkan tindak balas imun yang cekap terhadap sel-sel tumor. Projek ini bertujuan untuk mengkaji kesan NDV rekombinan yang mengekspresi interleukin 12 manusia (rAF-IL12) melalui proses apoptotik dan metastatik dalam sel-sel kanser payudara MCF7 dan MDA-MB231. NDV AF2240 digunakan sebagai pemalar positif dalam kajian ini. Dilihat rAF-IL12 mampu mengekalkan kestabilannya apabila dilancarkan dalam telur bebas patogen spesifik (SPF) hingga sepuluh petikan dan keselamatan secara selektif melalui kesan sitotoksik di dalam sel-sel kanser ayam dan payudara tanpa memberi kesan sitotoksik pada sel payudara bukan kanser seperti yang ditunjukkan melalui asai MTT. Kestabilan setiap rAF-IL12 disahkan melalui asai hemaglutinan (HA), purata waktu kematian (MDT) and indeks patogenisiti antara serebral (ICPI), manakala interleukin 12 dikuantiti melalui asai imunoserapan berkaitan enzim (ELISA). Sebanding dengan AF2240, raf-IL12 juga dapat menyebabkan apoptosis dengan ketara berdasarkan beberapa ujian apoptosis. Kedua-dua raf-IL12 dan AF2240 berjaya meningkatkan peratusan fasa G2/ M dan S dalam analisis kitaran sel sambil meningkatkan peratusan apoptosis. Walaupun kedua-dua AF2240 dan rAF-IL12 menunjukkan hasil apoptosis in vitro yang sebanding, rAF-IL12 mempunyai aktiviti anti-metastatik yang ketara (p<0.05) berbanding AF2240 berdasarkan ujian metastasis berkaitan termasuk asai gores in vitro, asai migrasi/ invasi, pembentukan tiub human umbilical vein endothelial cell (HUVEC) dan asai cincin aorta tikus. Selain itu, untuk menilai lagi mekanisma anti-tumor dan anti-metastatik rAF-IL12, kajian *in vivo*

dijalankan menggunakan tikus BALB/ c yang dicabar 4T1 sebagai model kajian ini. Terbukti rAF-IL12 berfungsi sebagai vaksin tumor yang lebih baik kerana ia secara signifikan (p<0.05) dapat mengurangkan saiz tumor secara ketara berbanding dengan virus AF2240. Keputusan apoptotik menunjukkan bahawa bilangan sel kanser dalam tumor berkurangan secara signifikan (p<0.05) 28 hari selepas rawatan intra-tumoral dengan raf-IL12 (2^7 HAU). Di samping itu, rAF-IL12 mampu menghalang penghijrahan sel-sel kanser ke organ penting berbanding kumpulan yang tidak dirawat. Bagi menerangkan mekanisme apoptotik dan anti-metastatik raf-IL12 pada tahap molekul, NanoString nCounter telah dijalankan. Walaupun kedua-dua AF2240 dan rAF-IL12 mempamerkan mekanisma tindakan yang sama, raf-IL12 lebih berkesan secara signifikan (p<0.05) berbanding AF2240 dari segi apoptosis dan aktiviti anti-metastasis. Kesimpulannya, kedua-dua rAF-IL12 dan AF2240 dapat menghalang percambahan sel-sel kanser payudara dan mendorong apoptosis dalam kajian *in vitro*, bagaimanapun, rAF-IL12 dapat menunjukkan fungsi peningkatan yang lebih ketara (p<0.05) berbanding dengan AF2240 sahaja berdasarkan kajian *in vivo*. Ini membuktikan bahawa interleukin 12 dapat meningkatkan tindak balas imun terhadap sel tumor dengan menggalakkan kematian sel, anti-angiogenesis dan kesan anti-metastasis, sambil meningkatkan lagi fungsi AF2240 sebagai vaksin berpotensi terhadap kanser payudara.

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LIST OF ABBREVIATIONS

AO	Acridine Orange
ANG-2	Angiotensin 2
ATCC	Animal Tissue Culture Collection
ATM	Ataxia telangiectasia mutated
BAX	Bcl-2 Associated X-Protein
BCL2	B-cell lymphoma 2
BRCA1	Breast Cancer 1
BrdU	Bromodeoxyuridine
CO_2	Carbon dioxide
CXCL-1	Chemokine Ligand 1
EGFP	Enhanced green fluorescent protein
F	Fusion glycoprotein
DNA	Deoxyribonucleic acid
ELISA	Enzyme-Linked Immunosorbent Assay
DMEM	Dulbecco's Modified Eagle Medium
FITC	Fluorescent isothiocyanate
HA	Haemagglutination
HEGF	Human Endothelial Growth Factor
HN	Haemagglutinin-neuraminidase glycoprotein
HUVEC	Human Umbilical Vein Endothelial Cells
IC_{50}	Half maximal inhibitory concentration
IACUC	Institutional Animal Care and Use Committee
ICPI	Intracerebral Pathogenecity Index

IFN-γ	Interferon-gamma
IL-12	Interleukin-12
IL-2	Interleukin-2
JC-1	5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide
L	Large polymerase protein
M	Matrix protein
MDT	Mean Death Time
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N	Nucleocapsid protein
NDV	Newcastle Disease Virus
OIE	World Organisation for Animal Health
P	Phosphoprotein
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PI	Propidium Iodide
RNA	Ribonucleic acid
RNAse	Ribonuclease
RT-QPCR	Real Time quantitative PCR
SSC	Saline-sodium citrate
SPSS	Statistical Package for the Social Sciences
TMB	3,3',5,5'-Tetramethylbenzidine
TRAIL	TNF-related apoptosis inducing ligand
TUNEL labeling	Terminal deoxynucleotidyl transferase dUTP nick end
VEGF A	Vascular endothelial growth factor A

CHAPTER 1

INTRODUCTION

Cancer is defined as uncontrollable cell division and the invasion ability of these cells to other tissues causing tumour mass formation, metastasis and vascularization (Vogelstein and Kinzler, 2004). In addition, angiogenesis (formation of new blood vessels) is also an important process for growth, development and to cause tumours to become malignant (Jyothi, 2012). After lung cancer, breast cancer ranks the second most common cancer estimating 1.7 million cases since 2012, worldwide and is the fifth cause of death (522,000 deaths) (Ferlay *et al*., 2015). One of the main approaches to treat breast cancer is chemotherapy. Chemotherapy utilizes anti-breast cancer drugs either given intravenously or orally (Maughan *et al*., 2010). The drugs travel through the bloodstream reaching breast cancer cells (Maughan *et al*., 2010). However, chemotherapy induces cytotoxic effects to the cancer cells as well as normal cells (Jyothi, 2012). In addition, resistance towards the drug(s), disturbed biodistribution and clearance of the drug may arise (Alfarouk *et al*., 2015; Jyothi, 2012). Therefore, the development of new therapeutic strategies is required to overcome the latter complications.

Oncolytic virotherapy is an adjunctive strategy to minimize tumour burden through selective virus replication in rapidly proliferating cells (Aurelian, 2013). It is currenty studied to be used as a future novel strategy in cancer treatments that can be later combined with conventional therapies for improved efficacy. Various viruses from different families are currently being studied as oncolytic agents at pre-clinical and clinical levels (Haddad, 2017; Singh *et al*., 2012; Sugiyama *et al*., 2012). Newcastle disease virus (NDV), also known as avian paramyxovirus serotype 1 (APMV-1), is a single negative-stranded RNA virus of nearly 15 kb, which belongs to the *Avulavirus* genus member in the family of *Paramyxoviridae*. NDV is a promising oncotherapeutic agent for human cancers as it is normally non-pathogenic to humans (Ghrici *et al*., 2013). AF2240 is a viscerotropic velogenic strain of NDV which was isolated during an outbreak in the Malaysia in the 1960s resulting in high mortality rate and is now being used as the challenge virus in vaccine trials in Malaysia (Molouki *et al*., 2011).

NDV is known to efficiently infect and kill cancer cells and is consequently being investigated as a novel cancer therapy (oncolytic virotherapy) (Schirrmacher *et al*., 2014). The first oncolytic study on NDV was reported back in 1965 by Cassel and Garret (1965) whereby the virus was studied as an antineoplastic agent on Ehrlich ascites carcinoma of the mouse, the human adenocarcinoma and HeLa cells. The virus kills the human cancer cells by apoptosis *via* either the intrinsic or extrinsic pathway resulting in the cell death (Ekert and Vaux, 1997). NDV possesses several advantages over other oncolytic viruses such as poxvirus, measles, HSV-1 or reovirus (Zamarin and Palese, 2012). It is considered to be safe for use in cancer treatments since it is an avian and non-pathogenic to humans and does not affect the immune system. The problem of pre-existing immunity can be overcome as 96% of the human population is seronegative towards NDV (Charan *et al*., 1981; Miller *et al*., 1971).

Vaccines are known to be highly effective. The production of viral vaccines involves either modifying living attenuated virus or using viruses which are chemically inactivated. The drawback of utilising such conventional methods in vaccine development relates to safety, efficiency and cost of the vaccine (Huang *et al*., 2003). Therefore, the development of genetically engineered viruses has come into focus as such viruses improve in safety, efficiency and cost.

The first study of genetically engineered virus was conducted to induce immune response against hepatitis B in chimpanzees using recombinant engineered to exhibit hepatitis B surface antigen in animal cells (Moss *et al*. 1984). Ever since then, several studies have been made in the fields of various viral vectors which were evaluated for immunotherapeutic and vaccine applications for a wide range of diseases (Choi and Chang, 2013). Methods of producing infectious paramyxoviruses from c-DNA clones (reverse genetics) have been developed during the last 20 years (Schirrmacher *et al*., 2014). Some of the genetically engineered NDV vaccines reported include Hitchner B1strain armed with IL2 in treating malignant melanoma (Zamarin et al., 2009), LaSota strain armed with IL-2, TNF-related apoptosis inducing ligand (TRAIL), IL-2-TRAIL, or enhanced green fluorescent protein (EGFP) as anti-neoplastic (Bai *et al*., 2014) and LaSota strain armed with IFN-λ1 against gastric adenocarcinoma cells (Bu *et al*., 2016). However, no studies have been reported on NDV AF2240 armed with IL12 against breast cancer yet.

In animal models and in clinical testing, cytokines have been found to be effective immunomodulators, suggesting that anti-tumour effect is closely related to its expression levels (Pan *et al*., 2016). Interleukin-12 (IL12) is a heterodimeric cytokine produced specifically by phagocytic cells and antigen-presenting cells. It exhibits a crucial role in activating anti-tumour immunity as it increases differentiation of T-cells, initiates natural killer cells as well as functions to inhibit angiogenesis and metastatic process enhancing anti-tumour activity in pre-clinical models (Derin *et al*., 2018). Additionally, IL12 was able to efficiently eradicate experimental liver cancer through intra-tumoural injections while reducing systemic toxicity (Sangro *et al*., 2004). Promising anti-tumour properties of IL12 have been proven on cancers such as liver, colorectal and pancreatic (Sangro *et al*., 2004), renal cancer (Gollob *et al*., 2000) and gastrointestinal primary malignancy (Lenzi *et al*., 2002). However, further in-depth mechanisms including anti-metastatic effects of IL12 are yet to be revealed, especially in breast cancer. The safety profile of NDV AF2240 expressing IL12 should also be tested although the anti-cancer activities of both AF2240 and IL12 are promising. The problem statement of this study is that IL12 has potent anti-tumour activities with strong clinical relevance to cancer therapy. However, the operating mechanism of NDV armed with IL12 is not fully understood. Other than that, the anti-tumour effect of NDV armed with IL12 in breast cancer has not been reported yet. Therefore, the hypothesis of this study is; the newly developed recombinant NDV expressing human IL12 genes is safe and stable with the ability to selectively replicate in cancer cells and induce anti-tumour effect. . Hence, the novel recombinant NDV armed with human IL12 as a potential cancer vaccine in breast cancer studies will be the first reported.

The objectives of this study are:

1. To assess the pathogenicity and stability of rAF-IL12 in chickens through multiple passaging *in vivo*.
2. To investigate the *in vitro* cytotoxic effects and the mechanism of rAF-IL12 in the induction of cell death and anti-metastatic abilities in MCF-7 and MDA-MB231 breast cancer cell lines.
3. To determine the *in vivo* anti-tumour activity of rAF-IL12 in 4T1-breast cancer challenged mice.

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LIST OF PUBLICATIONS / AWARDS

Zahiah, M. A., Tan, S. W., Noorjahan, B. M. A. & Yeap, S. K. Safety, stability and efficacy of recombinant Newcastle disease virus expressing human interleukin 12 (rAF-il12) as an anti-breast cancer vaccine. The 3rd AMDI IBSC 2018 on Emerging Infectious Diseases, January 19-20, 2018.

Awarded "Best Oral Presenter" at The 3rd AMDI IBSC 2018 on Emerging Infectious Diseases, January 19-20, 2018.

Zahiah, M.A, Muhammad, A.C.A, Sheau, W.T, Swee, K.Y, Noorjahan, B.A. et al. & Khatijah, Y. (2018). Safety and cytotoxicity effects of a recombinant NDV strain AF2240 expressing human interleukin 12 (Submitted to Scientific Reports, Nature). Submission Date: 4th July 2018; Submitted with Revision: 1st October 2018.

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Zahiah, M.A, Muhammad, A.C.A, Sheau, W.T, Swee, K.Y, Noorjahan, B.A. et al. & Khatijah, Y. In vivo anti-tumour effects of a recombinant NDV strain AF2240 expressing human interleukin 12 in 4T1 breast cancer cell-challenged mice. (Manuscript in Preparation).

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