UNIVERSITI PUTRA MALAYSIA

THE EFFECTS OF NEWCASTLE DISEASE VIRUS (NDV) ON BREAST CANCER CELL LINES

HARYATI SHILA MOHAMAD WALI

FPV 2003 8

THE EFFECTS OF NEWCASTLE DISEASE VIRUS (NDV) ON BREAST CANCER CELL LINES

By

HARYATI SHILA MOHAMAD WALI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of Science

August 2003

Specially dedicated to

My beloved parents,

TUAN HJ. MOHAMAD WALI B. HASAN and

PUAN HJH. SHAMSIAH BT. HJ. MOHD. SHAH

My loving husband,

EN. SAHALAN B. SUBAAT

My three sisters,

HARYANI LIANA

HARYANTI AZURA

HAZWANI DIYANA

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Master of Science

THE EFFECTS OF NEWCASTLE DISEASE VIRUS (NDV) ON BREAST CANCER CELL LINES

By

HARYATI SHILA MOHAMAD WALI

August 2003

Chairman: Professor Aini bt. Ideris, PhD

Faculty : Veterinary Medicine

Three Newcastle disease virus (NDV) isolates namely; F, Ijuk and 01/C were tested for their anticancer properties against two breast cancer cell lines, MCF-7 and MDA-MB-231. Each virus strain was propagated in 10-day old embryonated eggs. Purification was carried out by density gradient centrifugation using sucrose. The titer of each virus strain was determined by hemagglutination (HA) test. Screening of NDV strains for anticancer properties on breast cancer cells was performed by a colorimetric cytotoxic assay using tetrazolium salt (MTT). F strain displayed cytotoxic activity on both breast cancer cells with an IC_{50} value of 8 HAU for MDA-MB-231 cells and 2048 HAU for MCF-7 cells. Meanwhile Ijuk showed cytotoxic activity against MDA-MB-231 cells only with an IC_{50} value of 8.6 HAU. Strain 01/C did not exhibit any cytotoxic activity towards both breast cancer cells. Inactivation of the virus at 100^0C for 30 minutes destroyed its ability to kill the

breast cancer cells. Positive control experiment involved treatment of the cells with tamoxifen, an estrogenic antagonist agent. Negative control experiment was carried out by infecting the virus onto normal mouse fibroblasts (3T3 cell). No cytotoxic activity was observed on 3T3 cells following infection at low virus titer. However, infection at higher virus titer resulted in 50% inhibition of cell growth. Infection of the virus displayed clear evidence of apoptosis which was detected as a ladder-like pattern on agarose gel electrophoresis. This was further confirmed by TEM which provided ultrastructural changes of the infected cells. The role of sialic acid receptor was also studied based on neuraminidase (NA) and sialyllactose (SLL) treatment. Treatment of the cells with NA did not destroy the ability of the virus to cause apoptosis. Meanwhile a reduction in the ability of the virus to cause apoptosis was observed in the treatment of SLL. However there was no significant difference between the SLL-treated virus (27.05%) and untreated virus (30.87%). Based on the results obtained, this study showed that NDV strains, F and Ijuk have the potential to be developed as an anticancer agent. Mechanisms by which the virus infects and kills the cells need further studies. The role of sialic acid receptors in NDV-induced oncolytic effects requires further studies.

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

KESAN VIRUS PENYAKIT NEWCASTLE (NDV) KE ATAS SEL KANSER PAYUDARA

Oleh

HARYATI SHILA MOHAMAD WALI

Ogos 2003

Pengerusi: Profesor Aini bt. Ideris, PhD

Fakulti : Perubatan Veterinar

Tiga isolat virus penyakit Newcastle (NDV) iaitu; F, Ijuk dan 01/C telah diuji bagi kesan anti kanser terhadap dua jenis sel kanser payudara, MCF-7 dan MDA-MB-231. Setiap strain virus dibiakkan dalam telur berembrio berusia 10 hari. Penulenan virus dijalankan melalui kaedah pengemparan gradien berasaskan ketumpatan menggunakan sukrosa. Titratan virus tersebut ditentukan melalui ujian hemaglutinasi (HA). Proses penyaringan strain NDV bagi ciri-ciri antikanser ke atas sel kanser payudara melibatkan esei sitotoksik berasaskan warna menggunakan garam tetrazolium (MTT). Strain F mempamerkan aktiviti sitotoksik ke atas kedua-dua jenis sel kanser payudara dengan nilai IC_{50} bersamaan 8 HAU bagi sel-sel MDA-MB-231 dan 2048 HAU bagi sel-sel MCF-7. Manakala Ijuk menunjukkan aktiviti sitotoksik hanya pada sel-sel MDA-MB-231 sahaja dengan nilai IC_{50} sebanyak 8.6 HAU. Strain 01/C tidak menunjukkan sebarang aktiviti sitotoksik

terhadap kedua-dua jenis sel kanser payudara. Penyahaktifan virus tersebut pada 100^0C selama 30 minit memusnahkan keupayaannya untuk membunuh sel-sel kanser payudara. Ujikaji kawalan positif melibatkan rawatan sel tersebut dengan tamoxifen, sejenis agen antagonis estrogen. Ujikaji kawalan negatif dijalankan melalui jangkitan virus pada sel fibroblast tikus normal (sel 3T3). Tiada aktiviti sitotoksik diperhatikan pada sel 3T3 tersebut pada titratan virus yang rendah. Walau bagaimanapun, jangkitan pada titratan virus yang tinggi menyebabkan perencatan pertumbuhan sel sebanyak 50%. Jangkitan oleh virus menunjukkan bukti jelas berlakunya apoptosis yang dikenalpasti melalui corak tangga pada elektroforesis gel agar. Keadaan ini disahkan melalui TEM yang menunjukkan perubahan ultrastruktur pada sel-sel yang dijangkiti. Kajian ke atas peranan penerima sialic asid dijalankan menerusi rawatan neuraminides (NA) dan sialilaktosa (SLL). Rawatan NA ke atas sel-sel tersebut tidak memusnahkan keupayaan virus tersebut untuk menyebabkan apoptosis. Manakala penurunan keupayaan virus dalam menyebabkan apoptosis diperhatikan pada rawatan menggunakan SLL. Walau bagaimanapun tiada perbezaan ketara diperhatikan antara virus yang telah dirawat dengan SLL (27.05%) dan virus tanpa rawatan (30.87%). Berdasarkan keputusan yang didapati dalam kajian ini, strain NDV, F dan Ijuk mempunyai potensi untuk dibangunkan sebagai agen antikanser. Mekanisme bagaimana virus tersebut menjangkiti dan membunuh sel masih perlu kajian lanjutan. Kajian berkenaan peranan penerima asid sialik di dalam kesan onkolitik yang diakibatkan oleh NDV memerlukan kajian lanjut.

ACKNOWLEDGEMENTS

In the name of Allah, the Most Gracious, the Most Merciful.

I would like to extend my deepest and heartiest gratitude to my supervisor, Prof. Dr. Aini Ideris, Dean, School of Graduate Studies for her guidance and suggestions throughout the course of this study despite her hectic schedule. Special thanks are due to Assoc. Prof. Dr. Abdul Rahman Omar, my co-supervisor and coordinator of Biologics Laboratory for his continuous guidance and assistance in facilitating the course of this project. The same appreciation goes to my co-supervisors; Assoc. Prof. Dr. Fauziah Othman and Prof. Dr. Abdul Manaf Ali for their ideas and valuable suggestions.

My utmost appreciation is conferred to my friends and colleagues; Siti Hasmah Mohtar, Balkis Hj. A. Talip, Fariza Zainul, Kong Lih Ling, Tan Sheau Wei, Madihah Zawawi, Narayani Meyyappan, Wan Keng Fei and Zulkefley Othman, for their contributions in one way or another. Their friendship and support are highly appreciated. Not to forget Puan Rodiah Husin, staff of Biologics Laboratory for her assistance and cooperation throughout this study. I would also like to convey my deepest gratitude to the staffs of Microscopy and Microanalysis Unit, Laboratory of Enzyme and Microbial Technology; especially to Mr. Ho Oi Kuan, Azilah Abd. Jalil, Nooraini Mohd. Ain and Rafiuz Zaman Haroun.

I would also like to acknowledge and thank the National Cancer Council (MAKNA) for the financial support towards this research.

Last but not least, credit should be given to my beloved family especially my parents and my husband for their patience and faith in me. My gratefulness is also dedicated to my three siblings for their support and encouragements.

I certify that an Examination Committee met on 30th August 2003 to conduct the final examination of Haryati Shila Mohamad Wali on her Master of Science thesis entitled "The Effects of Newcastle Disease Virus (NDV) on Breast Cancer Cell Lines" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

ASMAH RAHMAT, Ph.D.
Associate Professor,
Faculty of Medicine and Health Science,
Universiti Putra Malaysia.
(Chairman)

AINI IDERIS, Ph.D.
Professor,
Faculty of Veterinary Medicine,
Universiti Putra Malaysia.
(Member)

ABDUL RAHMAN OMAR, Ph.D.
Associate Professor,
Faculty of Veterinary Medicine,
Universiti Putra Malaysia.
(Member)

ABDUL MANAF ALI, Ph.D.
Professor,
Faculty of Food Science and Biotechnology,
Universiti Putra Malaysia.
(Member)

FAUZIAH OTHMAN, Ph.D.
Associate Professor,
Faculty of Medicine and Health Sciences,
Universiti Putra Malaysia.
(Member)

GULAM RUSUL RAHMAT ALI, Ph.D.
Professor/Deputy Dean,
School of Graduate Studies,
Universiti Putra Malaysia.

Date: 08 DEC 2003

This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee are as follows:

AINI IDERIS, Ph.D.
Professor,
Faculty of Veterinary Medicine,
Universiti Putra Malaysia.
(Member)

ABDUL RAHMAN OMAR, Ph.D.
Associate Professor,
Faculty of Veterinary Medicine,
Universiti Putra Malaysia.
(Member)

ABDUL MANAF ALI, Ph.D.
Professor,
Faculty of Food Science and Biotechnology,
Universiti Putra Malaysia.
(Member)

FAUZIAH OTHMAN, Ph.D.
Associate Professor,
Faculty of Medicine and Health Sciences,
Universiti Putra Malaysia.
(Member)

AINI IDERIS, Ph.D.
Professor/Dean,
School of Graduate Studies,
Universiti Putra Malaysia.

Date: 16 DEC 2003

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

HARYATI SHILA MOHAMAD WALI

Date: 15/12/2003

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS

µl	Microlitre
^0C	Degree Celcius
APC	Antigen presenting cell
ATCC	American Type Culture Collection
ATV	Antibiotic-trypsin-versine
bp	Base pair
CAM	Complementary and alternative medicine
cm	Centimeter
cm^2	Centimeter square
cm^3	Centimeter cube
CO_2	Carbon dioxide
CTL	Cytotoxic T lymphocytes
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DTH	Delayed type hypersensitivity
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Estrogen receptor
F	Fusion
Fig.	Figure
g	Gram
g/cm^3	Gram per centimeter cube
HA	Hemagglutination
HAU	Hemagglutination unit
HN	Hemagglutinin-neuraminidase
HSV	Herpes simplex virus
IC_{50}	Inhibition concentration
IFN	Interferon
IFN-α	Interferon-alpha
IFN-β	Interferon-beta
IL	Interleukin
MAKNA	Majlis Kanser Nasional
MDT	Mean death time
mg	Milligram
MHC	Major histocompatibility complex
ml	Millilitre
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NA	Neuraminidase
NDV	Newcastle disease virus
NK	Natural killer

nm	Nanometer
NO	Nitric oxide
NTE	NaCl-Tris-HCl-EDTA buffer
OD	Optical density
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PEC	Peritoneal effector cell
PFU	Plaque forming unit
PI	Propidium iodide
RBC	Red blood cells
RNA	Ribonucleic acid
rpm	Revolution per minute
ssRNA	Singe stranded RNA
TEM	Transmission electron microscopy
TNF	Tumour necrosis factor
TNF-α	Tumour necrosis factor-alpha
UPM	Universiti Putra Malaysia
USM	Universiti Sains Malaysia
UV	Ultra violet
w/v	Weight/volume

CHAPTER I

GENERAL INTRODUCTION

Cancer is a much feared word when it comes to our health. Cancer is a general name given to a group of about 210 diseases and it can affect any organ of the body. Cancerous cells behave abnormally where they keep on dividing and forming more cells without control or order (Brock, 1993). These cells will form a mass of extra tissue called growth or tumor. In general, a tumor can be either benign or malignant. Both types differ in their ability to spread to other parts of the body. Benign tumors are not cancerous and can be removed where usually they do not come back. Furthermore benign tumors do not spread to invade other parts of the body. On the other hand, malignant tumors are cancerous and can spread or metastasize and damage nearby tissues and organs. Cancer cells can also break away from the initial mass and enter the bloodstream or lymphatic system thus forming new tumors in other parts of the body. Cancer cells differ in very minute ways from a normal cell that the immune system is able to tolerate it rather than attacking it.

Breast cancer is the common malignancy affecting women worldwide. It happens when cells in the breast begin to grow out of control and invade nearby tissues or spread throughout the body. Breast cancer can metastasize and spread to the lymph glands and other parts of the body. The most common type of breast cancer is ductal carcinoma, which begins in the cells of the ducts. Among the risk factors

responsible for breast cancer are age, a family history of breast cancer, previous history of breast cancer, never having given birth and menstruating at an early age. Standard treatments commonly used for this type of cancer are surgery, radiation therapy, chemotherapy and hormone therapy (Aikin, 1996).

Since the past century, novel approaches for the treatment of cancer have been developed. Besides conventional method such as surgery, radiotherapy and chemotherapy, immunotherapy with virus have received adequate attention as cancer treatment (Horvath *et al.*, 1999). This type of treatment is also known as complementary and alternative medicine (CAM). Therapies are being developed focusing on how the body fights cancer on its own. Although it is still experimental, cancer vaccines are gaining interest as a method of cancer treatment. However, this type of treatment is therapeutic which stresses on treating the cancer rather than preventing it. The main role of cancer vaccines is preventing cancer from recurring after the elimination of primary tumor via surgery, radiation or chemotherapy. Cancer vaccines initiate the immune system to attack existing cancerous cells. Since fighting established cancers are quite a task, cancer vaccines are usually used in combination with additional substances such as cytokines or adjuvants to help stimulate the immune system. Besides trying to provoke an immune response, cancer vaccines must also be able to stimulate the immune system to overcome tolerance against the cancerous cells. A cancer vaccine can be made either of whole

tumor cells or antigens. The antigens could comprise of proteins, genetic material (RNA or DNA), antibodies or viruses.

Over 30 viruses have been tested in various cancers including Newcastle disease virus (NDV), herpes simplex virus (HSV), mumps, measles and influenza. Although genetically engineered viruses are being tested for this matter, some naturally occurring viruses were shown to possess oncolytic activity. Observations in early 1920s indicated that certain viruses replicated in and lysed murine and other experimental tumors. Human cervical carcinoma regressed following inoculation of the patient with attenuated rabies vaccine (De Pace, 1912). Remissions of Burkitt's and Hodgkin's lymphomas following natural infection with measles virus were also reported (Bluming and Ziegler, 1971). The contribution of the virus to tumor regression is possibly mediated by the virus stimulating an anti-tumor immune response rather than infecting and destroying the tumor directly (Ring, 2002). Some naturally occurring viruses of veterinary importance have been adapted by serial passage in tumor cells to increase their oncolytic efficacy (Hammon *et al.*, 1963; Yohn *et al.*, 1968). NDV strains adapted to Ehrlich ascites carcinoma cells (Cassel *et al.*, 1983) or human melanoma cells (Ahlert and Schirrmacher, 1990) possess this trait.

Newcastle disease virus (NDV) or avian paramyxovirus 1 is a renowned pathogen of fowl. It contributes to substantial loss to the poultry industry economically. The virus is transmitted via ingestion or inhalation depending on its pathotypes. Despite recent advances in the control of disease by vaccination and mass slaughtering, outbreaks still occur occasionally as was the case in Australia (Westbury, 2001) and Malaysia (Yusoff, 2001). Despite being a threat to the poultry industry, NDV has been exploited as a potential treatment of cancer. It has been shown to display a distinguished ability as an oncolytic agent (Sinkovics *et al.*, 2000a). Immunisation with autologous (or allogeneic) NDV 73T strain post-surgically prevents relapses of malignant melanoma in human (Cassel and Murray, 1992). Other study shows that NDV has been successfully used to prevent relapses of surgically removed kidney carcinoma (Kirchner *et. al.*, 1995). Among other cancer type that shows relapses following NDV treatment are Daudi Burkitt's lymphoma cells, neuroblastoma, fibrosarcoma, melanoma, colon and kidney carcinoma. Naturally occurring viruses showing potential as an oncolytic agent must be amenable to genetic manipulation. It should be well characterised in terms of replication in different cell types and pathogenesis in human population. Studies indicate that while NDV replicates and kills cancer cells selectively, it does not affect normal cells (Reichard *et al.*, 1992). Furthermore, NDV has the advantage of relative safety and will only cause mild conjunctivitis and laryngitis in human, in some cases. Low-grade fever was also observed in clinical trials using low dose of NDV strain 73T (Cassel, 1965).

In Malaysia, cancer is reported as the fifth major cause of deaths. A report by Majlis Kanser Nasional, Malaysia (MAKNA) stated that an estimation of about 40,000 cases of cancer is diagnosed every year. The major cancers occurring in Malaysia are the cancer of lung, mouth, stomach, liver, breast and cervix. Breast cancer is the major cause of cancer deaths among Malaysian women. It is the most common cancer and the number one cause of cancer deaths amongst women in Malaysia. Screening for breast cancer involves mammograms, clinical breast examinations, and breast self-examinations. Conventional method of cancer treatment such as surgery, radiotherapy and chemotherapy also applies to breast cancer. This study was conducted to look into other possible methods in treating breast cancer.

Newcastle disease was reported in Malaya as early as 1934 (reviewed by Yusoff and Ideris, 2003; reviewed by Aini, 1990). Recent outbreaks in Malaysia occurred in the year 2000 and 2001 which resulted in mass eradication of infected flocks. Several different strains of NDV have been isolated from these outbreaks. However, the oncolytic ability of local NDV strains is not known. Hence, a collaborative project between UPM, USM and MAKNA has been established in identifying local NDV with oncolytic properties on breast cancer cells. Furthermore, the oncolytic effects of NDV were tested against two types of breast cancer cell lines; estrogen receptor positive (ER+) and estrogen receptor negative (ER-).

The objectives of this study are therefore:

i) To propagate and purify different NDV isolates.

ii) To screen NDV strains with cytotoxic effects against breast cancer cell lines.

iii) To determine the cytolytic effects and the nature of cell death caused by NDV strains.