UNIVERSITI PUTRA MALAYSIA

ONCOLYTIC EFFECT OF NEWCASTLE DISEASE VIRUS ON THE MCF-7 AND MDA-MB-231 BREAST CANCER CELL LINES

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By

NARAYANI MEYYAPPAN

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

August 2003

This thesis is especially dedicated to:

My parents, who are infinitely precious to me

My brother-in law, sisters and niece, who have filled my life with joy and happiness,

And

To Assoc. Prof. Dr. Fauziah Othman

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

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Chairman: **Associate Professor Fauziah Othman, Ph.D.**

Faculty: **Medical and Health Sciences**

The study was conducted to find a new anti-cancer agent for the treatment of breast cancer. The oncolytic effect of Newcastle disease virus (NDV) on MCF-7 and MDA-MB-231 breast cancer cell lines was investigated using various methods. The V4-UPM and AF2240 strains of NDV were propagated in the allantoic fluid of 11-days-old embryonated eggs. The allantoic fluid was harvested, purified and stored at -20°C. The haemagglutination (HA) test was conducted on the purified viruses to determine the HA titre of the V4-UPM and AF2240 strains, which were 16 384 and 128 HA units consecutively. The microculture tetrazolium (MTT) assay was carried out to detect the cytotoxic effect of the NDV strains on breast cancer cell lines using monolayer and co-culture techniques to determine the IC_{50} value. The IC_{50} values for the MCF-7 treated with V4-UPM strain were 4096 and 128 HA units for the monolayer and co-culture, respectively, while, the MDA-MB-231 had an IC_{50} value of 96 HA units for both techniques. When treated with AF2240 strain, MCF-7 showed IC_{50} value of 96 and 64 HA units for the monolayer and co-culture methods, respectively. Whereas, the IC_{50} value for MDA-MB-231 cells was 4 HA units for both techniques. Further studies were done to observe the ultrastructural changes in the treated cells and viewed under an energy filter transmission electron microscope

filter transmission electron microscope (EFTEM). The morphological features were also observed under a confocal microscope whereby the cells were stained with acridine orange (AO) and propidium iodide (PI). The treated cells showed apoptotic features such as cell shrinkage, nuclear fragmentation, chromatin condensation, cytoplasmic vacuolisation, cell blebbing and formation of apoptotic bodies. However, control cancer cells did not show any prominent changes and intact nucleus and clear cytoplasm were noticed. For the identification of the presence of the NDV, the cells were labelled with a polyclonal antibody and anti-chicken FITC. The NDV was found in the cytoplasm of the MCF-7 and MDA-MB-231 cells and occasionally in the nucleus of the MDA-MB-231 cells at day 3 post-inoculation. The TdT-mediated dUTP nick-end labelling (TUNEL) assay was conducted to specifically label and quantify the percentage of apoptotic cells via lesion scoring under a fluorescence microscope. The statistical analysis using independent t-test revealed that the apoptotic activity of V4-UPM and AF2240 strains on the MCF-7 and MDA-MB-231 cells are significantly different ($p \leq 0.05$) and the susceptibility of these cell lines towards the V4-UPM and AF2240 strains are also significantly different ($p \leq 0.05$). From the data obtained, the AF2240 strain was found to be significantly more effective, while the MDA-MB-231 cells demonstrated to be significantly more responsive towards the NDV strains. These findings confirmed that the mode of cell death induced by the NDV strains is apoptosis. This study also suggests that the V4-UPM and AF2240 strains of NDV can be a potential anti-cancer agent for the treatment of breast cancer.

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

KESAN ONKOLITIK VIRUS NEWCASTLE DISEASE TERHADAP MCF-7 DAN MDA-MB-231 SEL-SEL KANSER PAYUDARA

Oleh

NARAYANI MEYYAPPAN

Ogos 2003

Pengerusi: **Profesor Madya Fauziah Othman, Ph.D.**

Fakulti: **Perubatan dan Sains Kesihatan**

Kajian ini dijalankan untuk mencari agen anti-kanser baru bagi merawat kanser payudara. Kesan onkolitik virus Newcastle disease (NDV) terhadap MCF-7 dan MDA-MB-231 sel-sel kanser payudara telah disiasat mengggunakan pelbagai kaedah. Virus V4-UPM dan AF2240 NDV telah dipropagasi dalam cecair alantoid telur embrio berusia 11 hari. Cecair alantoid tersebut telah di kumpul, dipurifikasi dan disimpan dalam -20°C. Ujian heamagglutinasi (HA) telah dilakukan terhadap virus yang dipurifikasi untuk menentukan titer HA bagi virus V4-UPM dan AF2240, iaitu 16 384 HA unit untuk V4-UPM dan 128 HA unit untuk AF2240. Ujian microculture tetrazolium (MTT) telah dijalankan untuk mengenalpasti kesan sitotoksik virus NDV terhadap sel-sel kanser payudara menggunakan 2 kaedah, iaitu kaedah 'monolayer' dan 'co-culture' bagi menentukan nilai IC_{50}. Nilai-nilai IC_{50} sel MCF-7 yang dirawat dengan virus V4-UPM ialah 4096 HA unit untuk kaedah 'monolayer' dan 128 HA unit untuk kaedah 'co-culture'. Sementara, sel MDA-MB-231 mempunyai IC_{50} bernilai 96 HA unit untuk kedua-dua kaedah. Apabila dirawat dengan virus AF2240, sel MCF-7 menunjukkan IC_{50} bernilai 96 HA unit untuk kaedah 'monolayer' dan 64 HA unit untuk kaedah 'co-culture'. Nilai IC_{50} untuk sel MDA-MB-231 pula ialah 4 HA unit untuk kedua-dua kaedah. Kajian selanjutnya

telah dijalankan untuk meneliti perubahan ultrastruktur sel-sel yang dirawat dengan menggunakan mikroskop elektron pancaran penuras tenaga (EFTEM). Ciri-ciri morfologi juga dikaji di bawah mikroskop konfokal dimana sel-sel diwarnakan dengan acridine orange (AO) dan propidium iodide (PI). Sel-sel yang dirawat menunjukkan ciri-ciri apoptotik seperti pengecutan sel, fragmentasi nukleus, pengkondensasian kromatin, pembentukan vakul-vakul sitoplasma, 'blebbing' sel dan pembentukan jasad-jasad apoptotik. Walau bagaimanapun, sel-sel kanser kawalan tidak mempunyai sebarang perubahan yang ketara dan menunjukkan nukleus dan sitoplasma yang sempurna. Untuk mengenalpasti kehadiran NDV, sel-sel dilabel dengan antibody 'anti-chicken' poliklonal dan 'anti-chicken FITC'. NDV telah dikesan di dalam sitoplasma sel-sel MCF-7 dan MDA-MB-231 dan kadang-kadang didapati di dalam nukleus sel MDA-MB-231 pada hari ketiga inokulasi. Ujian 'TdT-mediated dUTP nick-end labelling' (TUNEL) telah dilakukan untuk melabel secara spesifik dan mengkuantifikasi peratus sel-sel apoptotik melalui kaedah skor lesi dengan menggunakan mikroskopi floresens. Analisis statistik menggunakan 'independent t-test' menunjukkan bahawa aktiviti apoptotik virus V4-UPM dan AF2240 terhadap sel-sel MCF-7 dan MDA-MB-231 adalah berbeza secara signifikan ($p \leq 0.05$) dan sensitiviti sel-sel ini terhadap virus V4-UPM dan AF2240 adalah juga berbeza secara signifikan ($p \leq 0.05$). Dari maklumat yang diperolehi, kesan virus AF2240 didapati lebih secara signifikan, sementara, sel MDA-MB-231 menunjukkan bahawa ia lebih reaktif terhadap virus NDV. Penemuan ini mengesahkan bahawa mod kematian sel yang dirangsang oleh virus NDV ialah apoptosis. Kajian ini juga mencadangkan bahawa virus V4-UPM dan AF2240 NDV boleh menjadi agen anti-kanser yang berpotensi untuk rawatan kanser payudara.

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LIST OF ABBREVIATIONS

AO	Acridine orange
ATV	Antibiotic-trypsin-versin
bp	Basepair
CO_2	Carbon dioxide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamenetetraacetate
ELISA	Enzyme-linked immunosorbent assay
ER	Estrogen receptor
FITC	Fluorescein isothiocynate
HA	Haemagglutination
HCl	Hydrochloric acid
HN	Haemagglutination neurimidase
IC_{50}	Inhibition concentration at 50 percent
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
NaCl	Sodium Chloride
NDV	Newcastle disease virus
NTE	NaCl, Tris-HCL and EDTA
OD	Optical density
PBS	Phosphate buffered saline
pH	Hydrogen ion concentration
PI	Propidium iodide
RBC	Red blood cell
RNA	Ribonucleic acid
rpm	Rotation per minute
SSC	Saline-sodium citrate
TdT	Terminal deoxynucleotidyl transferase
TEM	Transmission electron microscope
TNF	Tumour necrosis factor
TUNEL	Terminal deoxynucleotydil transferase-mediated dUTP nick end-labeling
UPM	Universiti Putra Malaysia
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

Cancer is a group of diseases that cause cells in the body to change and grow out of control. Most types of cancer cells form a lump or mass called a tumour, and are named after the part of the body where the tumour first starts. Breast cancer begins in breast tissue, which is made up of glands for milk production, called lobules, and the ducts that connect lobules to the nipples. The remainder of the breast is made up of fatty, connective, and lymphatic tissue. Most types of tumours that form in the breast are benign. Benign breast tumours are abnormal growths, but they do not grow and spread like cancer, and are not life-threatening. Some breast tumours are cancerous, but are called *in situ*, because they have not spread beyond the area where they began. *In situ* breast cancers are confined within the ducts or lobules of the breast. The majority of these tumours will not progress to become an invasive tumour, and at this early stage, nearly all of these cancers can be cured. However other cancerous breast tumours are invasive, or infiltrating. These cancers start in the ducts or lobules of the breast but have broken through the duct or gland walls to invade the surrounding fatty tissue of the breast. The seriousness of invasive breast cancer is strongly influenced by the stage of the disease, or how far the cancer has spread when it is first diagnosed.

Breast cancer is the most common cancer among woman, and is the main cause of cancer death among women in Malaysia. The rate of the cancer incidence differs between different countries. The highest rate is in America and Europe while the

lowest rate is in Asia and Africa. The estimated breast cancer incidence in Malaysia is about 35 for every 100 000 population in the year 2000 (Yip, 2002). The incidence of breast cancer has been rising for the past two decades, while mortality has remained relatively stable since the 1950s. In 2001, approximately 192 200 new cases of invasive breast cancer were diagnosed among women in America (Thomas *et al.*, 2002).

Important risk factors for female breast cancer include early age at onset of menarche, late age at onset of menopause, first full-term pregnancy after age 30, a history of pre-menopausal breast cancer or of benign proliferative breast disease. Obesity, nulliparity, and urban residence also have been shown to be associated with increased risk of breast cancer (Thomas *et al.*, 2002).

Scientists and research groups are working for the improvement for the treatment of breast cancer. The currently available treatments for breast cancer patients are chemotherapy, hormone therapy and radiotherapy, but they are not always effective and often have unpleasant side effects. The efficacy of the treatment depends on the type and the stage of the cancer. Some research is directed to find better ways to use the available drugs, while other research focused on development of new drugs for the treatment of breast cancer that includes the development of anti-cancer from natural products, cancer vaccines and gene therapy. With the development of advanced molecular biology techniques, viruses of animal origin have been tested for virus therapy of human cancers. The Newcastle disease virus (NDV) has been shown to exhibit outstanding anti-cancer effect on various cancer cells.

NDV is a paramyxovirus that causes Newcastle disease in a wide variety of birds and chickens. This fatal disease is characterised by inflammation of respiratory tract and or either brain or the gastrointestinal tract. NDV can also infect humans, but it is generally not very virulent, causing only mild-flu-like symptoms or conjunctivitis or laryngitis. The perception that NDV can replicate up to 10 000 times better in human cancer cells than in most normal human cells has prompted much interest in this virus as a potential anti-cancer agent. Numerous in-vitro and in-vivo studies have been carried out on the oncolytic activity of NDV, which includes clinical studies in the United States, Germany and Hungary. NDV strains that were used in most of these studies are the 73-T, Ulster, MTH-68, Italien, Hickman and PV701 (Cassel and Garret, 1965; Lorence *et al.,* 1988; Schild *et al.,* 1988; Bohle *et al.,* 1990; Reichard *et al.,* 1992; Csatary *et al.,* 1993; Lorence *et al.,* 1994a; Lorence *et al.,* 1994b; Plaksin *et al.,* 1994; Sinkovics and Hovarth, 1995; Csatary *et al.,* 1999; Anonymous, 2001; Fabian *et al.,* 2001; Phuangsab *et al.,* 2001; Schirrmacher *et al.,* 2001; Washburn and Schirrmacher, 2002).

In this study, the oncolytic effect of a local strain of NDV, which is the AF2240 and two modified vaccine strains, namely V4-UPM and S strains were tested on the breast cancer cell lines in-vitro. These strains of NDV have been characterised by Universiti Putra Malaysia (UPM) researchers. However, no studies have been carried out to investigate the anti-cancer effect of the virus for cancer treatment in Malaysia. According to current literature review, the NDV-based anti-cancer therapies have been reported to be of benefit (Reichard *et al.,* 1992; Csatary *et al.,* 1993; Lorence *et al.,* 1994a; Lorence *et al.,* 1994b; Plaksin *et al.,* 1994; Sinkovics and Hovarth, 1995).

The NDV demonstrated to be a potential anti-cancer agent and it could be the choice of treatment for cancer patients in Malaysia in the future.

A new perspective on anti-cancer therapeutic efficacy and the possibility of new targets for anti-cancer agent is offered by the principle that cytotoxic drugs operate primarily by causing apoptosis in tumour cells and participate in tumour regression. Apoptosis (from the Greek apo: off and ptosis: a falling off or dropping off) is defined as 'a single deletion of scattered cells by fragmentation into membrane-bound particles which are phagocytosed by other cells, believed to be due to programmed cell death (Boise *et al.,* 1995). With the realization of the importance of this process, a variety of techniques have been used to study apoptosis. The process of apoptosis can be used to evaluate drug toxicity and therapeutic efficacy in the studies involving NDV, as a new approach for cancer treatments.

The general objective of this study is to:

Identify a new anti-cancer agent for the treatment of breast cancer.

The specific objectives are to:

1) determine the IC_{50} value of the breast cancer cell lines inoculated with NDV strains using the microculture tetrazolium (MTT) assay to evaluate the cytotoxic effect,

2) observe the ultrastructural changes of the cells inoculated with NDV strains, under energy filter transmission electron microscope (EFTEM),

3) observe the morphological changes of the cells stained with a combination of acridine orange and propidium iodide stains under confocal microscope,

4) detect the presence and location of the virus by immunolabelling with a polyclonal antibody and goat-anti-chicken FITC,

5) detect DNA strand breaks in apoptotic cells using TUNEL labelling assay (DeadEnd Fluorometric TUNEL System), and

6) quantify the percentage of apoptotic cells by conducting lesion scoring under fluorescence microscope and followed by statistical analysis.

CHAPTER 2

LITERATURE REVIEW

2.1 Breast Cancer

Breast cancer is the most frequently diagnosed cancer in women. The incidence of breast cancer has been increasing constantly over the past several decades. The lifetime risk of developing breast cancer is 12.2%, or 1 in 8 women. The lifetime risk of dying of breast cancer is 3.6%, or 1 in 28 women (Dorr, 1998). The incidence of breast cancer is rare below the age of 30 but increases beyond the age of 50, as it continues to rise but more slowly compared to premenopausal women (Taylor *et al.,* 2000).

2.2 Etiology and Risk Factors

About a third of women with breast cancer relate to a family history on one or more first-degree relatives with breast cancer and of these 4 to 9% have hereditary breast cancer. Hereditary breast cancer is, however, believed to be responsible for up to 25% of breast malignancies that occur prior to age 30 (Dorr, 1998).

Factors leading to an increased risk of breast cancer include mutations in the breast cancer susceptibility gene BRCA-1 and BRCA-2. Mutation in these genes causes majority of hereditary breast cancers. However, hereditary breast cancer account for only 5% to 10% of all breast cancers (Taylor *et al.,* 2000).