UNIVERSITI PUTRA MALAYSIA

# CELLULAR APOPTOSIS OF 4T1 BREAST CANCER CELLS INDUCED BY V4-UPM NEWCASTLE DISEASE VIRUS

MAHANI MAHADI

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# CELLULAR APOPTOSIS OF 4T1 BREAST CANCER CELLS INDUCED BY V4-UPM NEWCASTLE DISEASE VIRUS

**By**

**MAHANI MAHADI**

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

**January 2007**

***Dedicated to my mother,***

***Patimah Jantan, my greatest source of inspiration***

***Also to my brothers and sisters:***

***Especially to Along, Azmi Mahadi,***

***Mashitah Mahadi***
***Zaid Mahadi***
***Maimon Mahadi***
***Abdul Aziz Mahadi***
***Zubir Mahadi***
***Azizan Mahadi***
***Mokhtar Mahadi***
***Azizul Mahadi***

***To my husband:***

***Mohd Firdaus Hamat***

***Thank you for the everlasting support and advice.***

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

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**Chairman: Professor Aini Ideris, PhD**

**Institute: Bioscience**

This study was carried out to investigate the effects of Newcastle disease virus (NDV) strain V4-UPM in eliminating breast cancer cells through the apoptosis machinery process and the potential use of the virus as an agent for breast cancer therapy. Oncolytic effects of V4-UPM NDV on 4T1, a mouse mammary cancer cell line was investigated via *in-vitro* and *in-vivo* assays, and three of the apoptosis characteristic were evaluated through various methods. Propagation of V4-UPM NDV was conducted in the allantoic fluid of 10 day old embryonated chicken eggs after 5 to 7 days incubation. The fluid was harvested, purified, and the haemagglutination (HA) test was carried out to determine the HA titre of the virus. The HA titre obtained from purified V4-UPM NDV was 131 072 or $2^{17}$. Cytotoxic effects of V4-UPM NDV on 4T1 cell line were first carried out using microculture tetrazolium (MTT) assay to determine the amount required to kill 50% of cancer cells. It was observed that 32 768 or $2^{15}$ HA unit was required to kill

50% of the 4T1 cells. Further studies were done by observing the morphological changes in treated cells under scanning electron microscope (SEM). The cells treated with V4-UPM NDV showed apoptotic characteristics such as shrinkage and reduction in cell size, cell indention, membrane blebbing and dispersion of cells, compared with oval to round, smooth surface of untreated 4T1 cells. By using confocal microscope, localization of tumor suppressor gene p53 and mitochondria activity in treated cells were evaluated to identify the involvement during the process of apoptosis. Positive localization of p53 in the nucleus of untreated cells was observed after labeling with anti-p53 monoclonal antibody and the localization of p53 outside the nucleus was clearly seen after treatment. V4-UPM NDV is suggested to enhance the function of p53 to cause 4T1 cells to commit suicide. The mitochondrial activity was investigated by using mitotracker red staining and low involvement of mitochondria activity in cancer cells was observed in untreated cells. Greenish fluorescence was observed in treated cells showing higher involvement of mitochondrial activity during apoptosis.

Further investigations were carried out based on the *in-vitro* studies as a preclinical trial on an animal breast cancer model (*in-vivo*) to evaluate the effects of V4-UPM NDV on cancer tissue. Female inbred Balb/c mice were used as an animal model and induction of cancer was done through inoculation of 4T1 cells into subcutaneous mammary fat pad. After 10 to 14 days, the tumor growth was observed in all induced mice. The statistical

analysis of tumor development showed a significant difference ($p \leq 0.05$) of tumor volume between control cancer cells and cancer cells treated with V4-UPM NDV. However, no significant changes were observed in body weight and tumor mass. Cell proliferation was significantly reduced as shown by the measurement of apoptotic:mitotic cell via lesion score counted under light microscope. Confirmation of apoptotic cells by specific labeling of DNA fragment with TdT mediated dUTP nick end labeling (TUNEL) assay showed a higher apoptotic percentage counted in cancer cells treated with V4-UPM NDV as compared with cancer control cells. Ultrastructural features of treated tissue were viewed under energy filtered transmission electron microscope (EFTEM) to confirm that cell death due to V4-UPM NDV is via apoptotic pathway. Cells were observed to be tightly connected with other cells, with clear boundaries and with the normal structure of organelles in cancer control cells. The distinct ultrastructural changes prominently seen in 4T1 cells treated with V4-UPM NDV were the apoptotic characteristics, such as, cell shrinkage and resulting spaces in between cells, membrane blebbing, shrunken nucleus and also the presence of numerous numbers of mitochondria and endoplasmic reticulum (ER). From these findings, it was confirmed that the mode of cell death induced by V4-UPM NDV, to eliminate the cancer cells is by apoptosis. This suggested that V4-UPM NDV is a potential agent for breast cancer treatment.

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

**SELLULAR APOPTOSIS PADA 4T1 SEL KANSER PAYUDARA YANG DIRANSANG OLEH V4-UPM VIRUS PENYAKIT NEWCASTLE**

**Oleh**

**MAHANI MAHADI**

**APRIL 2007**

**Pengerusi: Professor Aini Ideris, PhD**

**Institut : Biosains**

Kajian ini dilakukan untuk menyelidik kesan virus penyakit Newcastle (NDV) strain V4-UPM dalam menghapuskan sel kanser payu dara melalui proses apoptosis dan juga potensi virus ini bertindak sebagai agen untuk terapi kanser payudara. Kesan onkolitik NDV V4-UPM pada 4T1, sel kanser payudara mencit diselidik secara *in-vitro* dan *in-vivo*, dan tiga ciri apoptosis disiasat melalui pelbagai kaedah. V4-UPM NDV di propagasi dalam cecair alantoik telur ayam berembrio berusia 10 hari yang telah dieramkan selama 5 hingga 7 hari. Cecair alantoik tersebut dikumpulkan, dipurifikasi dan ujian hemagglutinasi (HA) dilakukan untuk menentukan HA titer virus ini. HA titer yang diperolehi daripada virus V4-UPM NDV yang telah ditulenkan ialah 131 072 atau $2^{17}$ . Kesan sitotoksik V4-UPM NDV pada sel 4T1 telah dilakukan kali pertama dengan menggunakan ujian 'microculture tetrazolium' (MTT) untuk menentukan jumlah yang diperlukan bagi membunuh 50% sel kanser. Ianya didapati sebanyak 32 768 atau $2^{15}$ HA unit diperlukan untuk

membunuh 50% sel 4T1. Kajian seterusnya dilakukan dengan melihat perubahan morfologi dalam sel yang dirawat dengan menggunakan mikroskop pengimbas elektron (SEM). Sel yang dirawat dengan V4-UPM NDV menunjukkan ciri-ciri apoptosis seperti sel mengecut and saiznya mengecil, sel melekuk ke dalam, membran menggelembung dan sel pecah dibandingkan dengan sel 4T1 yang tidak dirawat, yang menunjukkan sel yang membulat serta permukaan membran yang licin. Dengan penggunaan mikroskop konfokal, lokasi gen perencat tumor, p53 dan aktiviti mitokondria di kaji untuk mengenalpasti penglibatan semasa apoptosis berlaku. Lokasi p53 terletak di dalam nukleus pada sel 4T1 yang tidak dirawat setelah dilabel menggunakan antibodi monoklonal anti p53, dan perubahan lokasi p53 diluar nukleus dilihat dengan jelas selepas dirawat. V4-UPM NDV dijangka meningkatkan fungsi p53 untuk menyebabkan sel 4T1 mati. Aktiviti mitokondria dikaji dengan menggunakan perwarnaan ‘mitotracker red’ dan aktiviti mitokondria yang rendah dalam sel kanser dilihat pada sel yang tidak dirawat. Warna hijau floures diperolehi dalam sel yang dirawat menunjukkan penglibatan aktiviti mitokondria yang tinggi semasa apoptosis berlaku.

Kajian seterusnya dilakukan berdasarkan hasil kajian *in-vitro* sebagai percubaan pra klinikal menggunakan model haiwan kanser payudara (*in-vivo*) untuk melihat kesan V4- UPM NDV pada tisu kanser. Balb/c mencit betina yang sebaka digunakan sebagai model haiwan dan kanser induksi dilakukan dengan menginokulasi sel 4T1 ke dalam lemak di bawah lapisan

mamari. Selepas 10 hingga 14 hari didapati semua mencit yang di induksi menunjukan pertumbuhan tumor. Statistikal analisis pertumbuhan tumor menunjukan perbezaan yang signifikan ($p \leq 0.05$) isipadu tumor di antara sel kanser kawalan dan sel kanser yang dirawat dengan V4-UPM NDV. Walau bagaimanpun, tiada perubahan yang signifikan diperolehi ke atas berat badan dan jisim tumor. Proliferasi sel menurun dengan signifikan seperti yang ditunjukkan selepas mengukur sel apoptotik:mitotik melalui kaedah skor lesi yang di kira di bawah mikroskop cahaya. Pengenalpastian sel apoptotik menggunakan label spesifik pecahan DNA iaitu ujian 'TdT mediated dUTP nick end labeling' (TUNEL) menunjukkan peratusan apoptotik yang tinggi pada kanser sel yang dirawat dengan V4-UPM NDV dibandingkan dengan kanser sel kawalan. Bentuk ultrastruktur tisu yang dirawat di kaji menggunakan mikroskop elektron pancaran penuras tenaga (EFTEM) untuk menentukan bahawa kematian sel disebabkan oleh V4-UPM NDV adalah melalui mekanisma apoptosis. Sel didapati bersusun dengan rapat diantara satu sama lain dengan sempadan yang jelas serta struktur normal organel pada kanser sel kawalan. Perubahan ultrastruktur yang jelas dan ketara dilihat pada sel 4T1 yang dirawat dengan V4-UPM NDV menunjukan ciri-ciri apoptosis, seperti sel mengecil dan menyebabkan kehadiran ruang diantara sel, membran menggelembung, nukleus mengecut dan juga mitokondria dan endoplasmik retikulum yang banyak. Daripada penemuan ini, didapati cara sel mati yang diransang oleh V4-UPM NDV untuk menghapuskan sel kanser adalah melalui apoptosis. Ini menunjukan

V4-UPM NDV adalah agen yang berpotensi sebagai rawatan kanser payudara.

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I certify that an Examination Committee has met on 26th January 2007 to conduct the final examination of Mahani Bt Mahadi on her Master of Science thesis entitled "Cellular Apoptosis of 4T1 breast Cancer Cells Induced By V4-UPM Newcastle Disease Virus" 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:

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This thesis 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 are as follows:

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Date: 10 MAY 2007

## DECLARATION

I hereby declare that the thesis is based on my original work except for quotation 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.

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**MAHANI MAHADI**

Date: 27 MARCH 2007



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| | |
|---|---|
| AB | Apoptotic Bodies |
| AIF | Apoptosis Inducing Factor |
| ANOVA | Analysis of Variance |
| AI | Apoptotic Index |
| ATP | Adenosine Triphosphate |
| ATCC | American Type Culture Collection |
| BSE | Breast Self Examination |
| DMBA | Dimethylbenz(a)anthracene |
| DMSO | Dimethyl Sulfoxide |
| DNA | Deoxynucleic Acid |
| dUTP | Deoxynucleotide Triphosphate |
| EDTA | Ethylenediaminetetraacetic Acid |
| EFTEM | Energy Filtered Transmission Electron Microscope |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| ER | Endoplasmic Reticulum |
| F | Fusion Protein |
| FITC | Fluorescein isothycyanat |
| GLOBOCAN | Global of Cancer |
| H&E | Hematoxylin and Eosin |
| HA test | Heamagglutination Test |
| HN | Haemagglutinin-Neuraminidase |
| IP | Intraperitoneal |



| | |
|---|---|
| IT | Intratumor |
| $IC_{50}$ | Inhibition Concentration at 50 Percent |
| IHC | Immunohistochemistry |
| L | Large Polymerase Protein |
| MTT | Microculture Tetrazolium |
| ME | Numerous Mitochondria and Endoplasmic Reticulum |
| MB | Membrane Blebbing |
| MI | Mitotic Index |
| NTE | NaCl tris-HCl EDTA |
| NaCl | Sodium Chloride |
| NDV | Newcastle Disease Virus |
| NP | Nucleocapsid Protein |
| $O_sO_4$ | Osmium Oxide |
| P | Phosphoprotein |
| PBS | Phosphate Buffer Saline |
| PI | Propodium Iodide |
| RBC | Red Blood Cell |
| RT | Room temperature |
| RNA | Ribonucleic Acid |
| SC | Subcutaneous |
| SSC | Saline Sodium Citrate |
| SEM | Scanning Electron Microscope |
| SN | Shrunken Nucleus |

| | |
|---|---|
| TAM | Tamoxifen |
| TEM | Transmission Electron Microscope |
| TUNEL | Terminal Deoxynucleotide Transferase-Mediated dUTP Nick End Labeling |
| TdT | Terminal Deoxynucleotidyl Transferase |
| USA | United States of America |
| UPM | Universiti Putra Malaysia |

# CHAPTER 1

## INTRODUCTION

'Cancer' is a well-known word among people in the world and it often strikes fear in people. Indeed, cancer is considered to be one of the major causes of death around the world including Malaysia. Globocan (2000) reported that an estimated 5.4 million people all over the world are afflicted with cancer and 51% of those affected are in developing countries.

The incidence of various kinds of cancer in Malaysia has been estimated to be around 30,000 annually and constitutes 10.3% of medically certified death, which is the fourth leading cause of death (Lim *et al*., 1993; Gerard 2002). The most predominant cancer affecting males in Malaysia are cancers of the lung, nasopharynx, mouth, stomach and liver, while the most prevalent cancers among females are cancers of the breast, cervix, lung and stomach (Gerard, 2002).

Breast cancer is the first leading cause of death among women. It is the most frequently diagnosed in the western world with approximately 180,000 new cases identified annually in the United States of America (USA) alone and currently the second leading cause of cancer-related mortality in women in USA (McPherson *et al*., 2000). The estimated breast cancer incidence in