UNIVERSITI PUTRA MALAYSIA

VIRUS NEWCASTLE (BAKA/STRAIN AF-2240) EKSPRESI GEN HN KE DALAM SEL KANSER COLON APOPTOTIK MANUSIA (HT-29)

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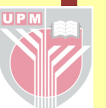

By

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

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To my dearest father and mother

Abstract of thesis presented to the Senate of Universiti Putra Malaysia
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MARCH 2009

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Newcastle disease virus (NDV) is the causative agent of the Newcastle disease (ND). The virus has been used as vaccines in veterinary medicine to protect poultry against pathogenic NDV strains which causes respiratory disease but NDV in humans has interesting anti-neoplastic and immune stimulating properties. NDV can be oncolytic and activate host immune cells to produce cytokines and to become cytotoxic against tumour cells via unknown mechanism. The recombinant haemagglutinin-neuraminidase (HN) protein was obtained from Malaysian viserotropic-velogenic NDV strain AF2240 through reverse transcription-polymerase chain reaction (RT-PCR) and cloned into the mammalian expression vector pEGFP-N2. The constructed recombinant plasmid which was named pEGFP-N2/HN was used to transform into *Escherichia coli* Top 10 for amplification. The extracted

plasmid from *E. coli* Top 10 was then used for transfection and apoptosis studies. The constructed plasmid, pEGFP-N2/HN, was transfected into HT-29 (human colon cancer) cell line and HeLa (human cervical cancer) cell line for protein expression and apoptosis studies, the 3T3 (normal mouse fibroblast) cell line was selected as control cell lines. At 72 h post-transfection, protein expression of constructed pEGFP-N2/HN was analysed using SDS-PAGE, Western Blot and fluorescence microscope. It was shown that the recombinant HN gene was expressed in HT-29 cell as well as HeLa cells and 3T3 cells. The cytotoxic effects of NDV strain AF2240 was determined by MTT assay. Results from MTT assay showed that 50% inhibitory concentration (IC_{50}) value in HT-29 cells obtained by 384 HA titer unit after 24 h while this amount decrease to 80 HA titer unit in HeLa cells during the same period. However after 48 h treatment IC_{50} in HT-29 cells obtained by 380 HA titer unit and in HeLa cells this amount decreased to 64 HA titer unit. At 72 h treatment IC_{50} of HT-29 cells was obtained by 300 HA titer unit, in HeLa cells this amount was only 4 HA titer unit. In 3T3 cells no inhibition effect was observed after infection with NDV strain AF2240. The apoptosis effects of NDV strain AF2240 infection and pEGFP-N2/HN expression on HT-29, HeLa and 3T3 cell were analysed by Flow cytometry in which Propidium Iodide was used for cell staining, the untreated cells were considered as control. Infection studies were carried out with NDV strain AF2240 at its IC_{50} HA titer unit for 24, 48 and 72 h. Transfection studies were performed with 4 µg of constructed plasmid, pEGFP-N2/HN, for 72 h. Flow cytometry results from pEGFP-N2/HN transfection studies showed the involvement of HN gene expression of NDV strain AF2240 in inducing apoptosis in tumour cells but not normal cells. Flow cytometry result from transfection studies

showed that the apoptosis induction of pEGFP-N2/HN transfection in HeLa cells was higher than apoptosis induction in HT-29 cells. Early apoptosis effect of NDV strain AF2240 infection and pEGFP-N2/HN transfection on HT-29, HeLa and 3T3 cell were analysed by Flow cytometry in which Annexin V staining was used, in all experiments untreated cells were considered as control. The Flow cytometry results showed that NDV strain AF2240 infection was able to trigger early apoptosis in tumour cell line after 12 h while transfection of tumour cells with pEGFP-N2/HN induced early apoptosis after 24 h, no early apoptosis induction has been observed in normal cells. In conclusions the recombinant HN gene of NDV strain AF2240 expressed in human colon cancer cells (HT-29) and human cervical cancer cells (HeLa) as well as mouse normal fibroblast cells (3T3). The expressed recombinant HN protein of NDV strain AF2240 induced apoptosis in human tumor cells (HT-29 and HeLa) without any cytotoxic effects on normal fibroblast cells.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia

sebagai memenuhi keperluan untuk ijazah Master punya Sains

VIRUS NEWCASTLE (BAKA/STRAIN AF-2240) EKSPRESI GEN HN KE DALAM SEL KANSER COLON APOPTOTIK MANUSIA (HT-29)

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Penyakit Newcastle adalah disebabkan oleh virus jenis NDV (Newcastle Disease Virus). NDV telah lama digunakan sebagai vaksin dalam perubatan vetrinari untuk melindungi ternakan daripada strain pathogeniknya yang merupakan penyebab penyakit respiratori. Berbeza dalam manusia, virus tersebut menunjukan ciri-ciri anti-neoplastik dan juga sebagai peransang sistem imun. Oleh itu, NDV mampu bertindak sebagai onkolitik lalu mengaktifkan sistem imun perumah untuk menghasilkan sitokinin yang kemudiannya bertukar menjadi sitotoksik terhadap sel-sel tumor melalui suatu mekanisme yang tidak dikenali.

Protein recombinan haemagglutinin-neuraminidase (HN) yang didapati daripada strain viserotropik-velogenik NDV Malaysia jenis AF2240 melalui reaksi rantai transkripsi polymerase berbalik (RT-PCR) diklonkan ke dalam vector ekspresi

mamalia pEGFP-N2. Plasmid recombinan (pEGFP-N2/HN) yang terhasil diamplifikasikan dengan bantuan *Escherichia coli* Top 10. Bagi tujuan transfeksi serta kajian apoptosis, pEGFP-N2/HN dipindahkan ke sel-sel HT-29 (kanser kolon manusia) dan sel-sel HeLa (kanser servikal manusia) di mana sel-sel 3T3 (fibroblast tikus normal) dijadikan sebagai kawalan.

Selepas 72 jam, protein ekspresi daripada pEGFP-N2/HN dianalisis dengan SDS-PAGE, Western Blot dan juga mikroskop fluorescene. Keputusan bagi ketiga-tiga jenis sel mengesahkan kehadiran gen recombinan HN padanya. Selain itu, sel-sel HT-29, HeLa and 3T3 juga dirawat dengan larutan NDV (strain AF2240) 512 HA unit titer bagi tempoh masa yang berlainan iaitu 24, 48 and 72 jam untuk mengetahui kadar sitotoksiknya juga dikenali sebagai ujian MTT assay. Bagi tempoh masa 24jam, IC_{50} (50% inhibitory concentration) bagi sel-sel HT-29 dan HeLa didapati 384 dan 80 HA unit titer masing-masing. Manakala bagi 48 jam pula bersamaan dengan 380 dan 64 HA unit yang kemudiannya menurun kepada 300 dan 4 HA unit titer masing-masing apabila mencecah 72 jam. Pemerhatian menunjukan tiada kesan pada sel-sel 3T3 bagi ketiga-tiga tempoh masa berkenaan.

Kesan apoptosis dianalisis menggunakan teknik flowsitometri dengan Propidium Iodide sebagai pewarna sel dimana sel-sel yang tidak diwarna dianggap sebagai kawalan. Kajian berkaitan infeksi dijalankan dengan sel-sel yang telah dirawat dengan NDV (strain AF2240) pada nilai IC_{50} HA unit titer tertentu bagi tempoh masa 24, 48 dan 72 jam. Manakala transfeksi pula dikaji dengan 4µg plasmid recombinan pEGFP-N2/HN selama 72 jam. Keputusan flowsitometri daripada kajian transfeksi

menunjukan penglibatan gen ekspresi HN daripada NDV dalam mencetuskan proses apoptosis pada sel-sel tumor dan bukannya pada sel-sel normal. Kadar induksi apoptosis terbukti lebih tinggi dalam sel-sel HeLa berbanding sel-sel HT-29. Pada peringkat awal apoptosis, pewarna jenis Annexin V telah digunakan dalam flowsitometri terhadap ketiga-tiga jenis sel tersebut manakala sel-sel yang tidak diwarna sebagai kawalan. Flowsitometri mengesahkan infeksi dengan NDV strain AF2240 mencetuskan apoptosis awal selepas 12 jam berbanding transfeksi dengan pEGFP-N2/HN yang mencetuskan apoptosis awal hanya selepas 24 jam. Tiada sebarang kesan apoptosis awal pada sel-sel yang normal diperhatikan.

Sebagai kesimpulannya, gen recombinan HN daripada NDV baka AF2240 telah di ekspresikan ke dalam sel-sel kanser kolon manusia (HT-29), sel-sel kanser servikal manusia (HeLa) dan juga pada sel-sel fibroblast tikus normal. Ternyata gen tersebut dapat mencetuskan apoptosis pada sel-sel tumor manusia (HT-29 and HeLa) manakala tiada sebarang kesan sitotoksik terhadap sel-sel fibroblast normal.

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I certify that a Thesis Examination Committee has met on 3rd of March 2009 to conduct the final examination of Samira Khodai on her thesis entitled "**Newcastle Disease Virus Strain AF2240 HN Gene Expression in Apoptotic Human Colon Cancer Cell Lines (HT-29)**" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U. (A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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DECLARATION

I declare that the thesis is my original work except for quotaions and citations wich have been duly acknowledged. I also declare that it has not been previously, and is not concurrenctly, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

SAMIRA KHODAI

Date: 9 July 2009

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LIST OF ABBREVIATIONS

AI	active immunotherapy
AIDS	acquired immune deficiency syndrome
AMV	avian myeloblastosis virus
APMV-1	avian paramyxovirus type-1
APS	ammonium persulfate
Arg	arginine
ASI	active specific immunization
ATP	adenosine-5′-triphosphate
ATV	autologous tumour cell vaccine
BCIP	bromochloroindolyl phosphate
Bcl-2	B-cell lymphoma-2
BCG	bacille calmette-guérin
c-myc	myelocytomatosis-c
BSA	bovine serum albumin
CAM	cell adhesion molecules
CD 19	cluster of differentiation-19
cDNA	complementary DNA
CEF	chicken embryo fibroblast
DEPEC	diethylpyrocarbonate
d	distilled water
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EFG	epidermal growth factor
ELISA	enzyme linked immunosorbent assay
ER	endoplasmid reticulum
F	fusion protein
g	gravity
Gal	galactose
GM-CSF	granulocyte macrophage-colony stimulating factor

H	hour
HA	haemagglutinin activity or haemagglutination
HAU	haemagglutination unit
HI	haemagglutination inhibition
HN	haemagglutinin-neuraminidase (protein)
IB	inclusion bodies
ICPI	intracerebral pathogenicity index
ICTV	international committee on the taxonomy of virus
Ig	immunoglobulin
IL	interleukin
INF	interferon
INF-α	interferon-alpha
IVPI	intravenous pathogenicity index
kb	kilobase
kDa	kiloDalton
L	large (protein)
LB	lubria-bertani medium
M	matrix (protein)
mAb	monoclonal antibody
MCS	multiple cloning site
min	minute
mRNA	messenger ribonucleic acid
NA	neuraminidase activity
NBT	nitro blue tetrazolium
ND	Newcastle disease
NDV	Newcastle disease virus
NK	Natural killer cell
NP	nucleocapsid (protein)
ORF	open reading frame
P	phosphoprotein
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffer saline
PBMC	Peripheral blood mononuclear cells

RBC	Red blood cell
RE	restriction enzyme
RNA	ribonucleic acid
RNase	ribonuclease
rpm	resolution per minute
RT	room temperature
RT-PCR	reverse transcription-polymerase chain reaction
SD	Shine-dalgarno
SDS	sodium dodecyle sulfate
Tag	*termus aquaticus*
TEMED	N,N,N′N′-tetramethyethylenediamine
TNF	tumour necrosis factor
TNF-α	tumour necrosis factor-α
TPBS	tween 20-phosphate buffer saline
TRAIL	TNF related apoptosis inducing ligand
tRNA	transfer ribonucleic acid
v/v	volume per volume
w/v	weight per volume

CHAPTER 1

INTRODUCTION

Newcastle disease virus (NDV) contains a single-stranded, negative-sense, non-segmented RNA genome and belongs to the genus *Avulavirus* in the family *Paramyxoviridae* (Mayo 2002 .a). The genomic RNA is 15,186 nucleotides in length (Krishnamurthy and Samal, 1998) and contains six genes that encode at least seven proteins (Steward *et al*., 1993). The envelope of NDV contains two glycoproteins, the hemagglutinin-neuraminidase (HN) and fusion (F) proteins: the HN protein mediates attachment of the virus to the cell, and the F protein mediates fusion of the viral envelope with cellular membranes (Scheid and Choppin 1974). NDV is a paramyxovirus that causes Newcastle disease in a wide variety of birds (Seal *et al.,* 2000). In humans NDV is generally not very virulent and causes only mild flu-like symptoms or conjunctivitis and/or laryngitis (Moss *et al.,* 1996). It has been labeled as a complementary and alternative medicine treatment because it is widely believed to be nontoxic for normal cells (Moss., *et al.,* 1996). The virus is able to raise host immune system against tumour and an innate capacity to stimulate the production of host cytokines that have potential anticancer activity (Beard and Hanson*,* 1984). NDV has been used in a clinical setting as an experimental oncolytic agent for more than 30 years (Csatary, 1971). Naturally occurring NDV has been reported to be an effective oncolytic agent in a variety of animal tumor models (Sinkovics and Horvath, 2000). It has been used in vaccination with tumor cell oncolysates in people with head and neck squamous cell carcinomas (Karcher *et al*., 2004) tumors of digestive tract (Liang *et al*., 2003) glioblastoma multiform (Schneider *et al*., 2001; Steiner *et al*., 2004) malignant melanoma (Cassel *et al*., 1988; Batliwalla *et al*., 1998;

Wallack *et al*., 1998) colorectal carcinoma (Ockert *et al*., 1996; Schlag *et al*., 1992) and other advanced cancers.

The mechanisms governing cytotoxicity effects of NDV remains to be fully characterised. Many NDV strains are known to evoke apoptosis in cancer cells through cell-to cell contact or stimulation of immune system (Washburn *et al.,* 2003). The differences observed in tumour cell cytotoxicity might be a reflection of the differences in the major surface glycoproteins, it has been shown that NDV strains with differences only in the HN proteins will have different levels of cytotoxicity against tumour cells (Zeng *et al*., 2002). The HN protein of NDV mediates apoptosis in NDV-infected cells. Since NDV enter all types of cells using sialic acid receptors, the observed differences in cytotoxicity against tumour cells by these viruses are likely to be due to other HN protein functional differences that do not alter receptor specificity (Zeng and Schirrmacher, 2002). It has been shown that the HN protein of the virus causes apoptosis in chicken embryo fibroblast cells (Ravindra *et al.,* 2008). Tumour cells expressing the HN protein demonstrated decreased DNA content, phosphatidylserine exposure and increased cytoplasmic vacuolation. Up-regulation of caspase-1, -9, -8, -3, loss of mitochondrial transmembrane potential and an increase in oxidative stress were also observed in cells expressing the HN protein. Based on above data it can be concluded that HN protein of NDV potentially causes apoptosis in tumour cells.