EFFECTS OF AUTOPHAGY INHIBITION ON NEWCASTLE DISEASE VIRUS-INDUCED ONCOLYSIS IN BREAST CANCER CELLS

By

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

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Researchers have been developing oncolytic viruses (OVs) as an alternative treatment to treat advanced cancer and to combat against the cancer cell resistance towards chemotherapy and radiotherapy. Newcastle disease virus (NDV) is an avian virus which selectively replicates in mammalian cancer cells due to the lack of antiviral immune response in these cells, thus making NDV a good candidate for oncolytic virotherapy. Recently, scientists have explored a novel strategy to fight cancer that is by inhibiting autophagy. Autophagy is a highly conserved cellular degradation mechanism which recycles unused cytoplasmic constituent into new nutrients. Importantly, studies have demonstrated that inhibition of autophagy enhanced NDV-induced oncolysis in several human cancer cells including gastric carcinoma, lung, and glioma cancer cells. Even though studies have been done to show NDV oncolytic effect in breast cancer cells, the effect of autophagy inhibition on NDV-induced oncolysis in breast cancer cells remains unknown. The main aim of this study was to examine the effect of autophagy inhibition on NDV-induced oncolysis in human breast cancer cells MCF7. Two approaches were utilised to inhibit autophagy which were pharmacological inhibitors and short-interfering RNA (siRNA)-mediated protein knockdown. Briefly, MCF7 cells were infected with the recombinant NDV strain AF2240 with GFP (rAF-GFP) with or without autophagy inhibition by the pharmacological autophagy inhibitors, SAR405 and chloroquine (CQ); or by siRNA-mediated knockdown of the autophagy protein Beclin-1 (BECN1). Autophagic activity was observed and quantified using fluorescence microscopy and fluorometer, respectively. MTT assay was used to measure cell death and viral replication was quantified using fluorometer. The results showed that NDV induced autophagy in MCF7 cells at 2 hours post-infection (hpi). Importantly, both autophagy inhibitors, SAR405 and CQ, had no significant effect on NDV-induced oncolysis in MCF7 breast cancer cells, as measured at 24, 48 and 72 hpi. Furthermore, in contrast to our hypothesis, siRNA knockdown of BECN1 significantly reduced the cell death of NDV-infected MCF7 cells at 24 hpi by ~10%, but not at 48 and 72 hpi. Further experiment suggests that this could

be due to the reduction of viral replication by more than 50% following treatment with BECN1-targeting siRNA at 24 hpi. In conclusion, NDV induces autophagy in breast cancer cells. Importantly, inhibition of autophagy does not enhance the oncolytic efficacy of NDV in breast cancer cells, instead it reduces the cell death, possibly by suppressing viral replication. Further work can be done to determine if induction of autophagy can enhance the oncolytic efficacy of NDV in breast cancer cells.

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

KESAN RENCATAN AUTOFAGI TERHADAP ONKOLISIS YANG DIARUH OLEH VIRUS PENYAKIT SAMPAR AYAM DALAM SEL BARAH PAYUDARA

Oleh

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Penyelidik telah mula membangunkan virus onkolitik sebagai rawatan alternatif untuk merawat kanser peringkat akhir dan juga rawatan untuk sel barah yang rintang terhadap kemoterapi dan radioterapi. Virus penyakit sampar ayam (NDV) adalah sejenis virus yang menjangkiti burung dan telah menjadi calon untuk viroterapi onkolitik kerana telah menunjukkan kebolehan untuk mereplikasi di dalam sel barah mamalia secara selektif kerana kekurangan tindak balas imun antivirus. Saintis telah meneroka strategi baru untuk melawan kanser dengan cara perencatan autofagi. Autofagi ialah mekanisme degradasi selular yang mengitar semula juzuk sitoplasma yang tidak digunakan kepada nutrien baharu. Kajian telah menunjukkan perencatan autofagi meningkatkan kesan onkolisis yang disebabkan oleh NDV di dalam beberapa sel kanser manusia termasuklah sel-sel barah karsinoma gastrik, peparu dan juga glioma. Walaupun kajian telah menunjukkan NDV mempunyai kesan onkolisis ke atas sel barah payudara, kesan rencatan autofagi terhadap kesan onkolisis oleh NDV ke atas sel barah payudara masih belum diketahui. Matlamat utama kajian ini adalah untuk mengkaji kesan rencatan autofagi terhadap kesan onkolisis yang disebabkan oleh NDV di dalam sel barah payudara MCF7. Dua jenis pendekatan telah digunakan iaitu perencat farmakologi dan penyahfungsian siRNA protein autofagi Beclin-1. Secara ringkas, sel MCF7 telah dijangkiti dengan NDV rekombinan strain AF2240 dengan GFP (rAF-GFP) bersama atau tanpa perencat farkamologi autofagi, SAR405 dan chloroquine (CQ); atau dengan penyahfungsian siRNA protein autofagi Beclin-1 (BECN1). Aktiviti autofagi telah dicerap dan diukur menggunakan mikroskop pendarflour dan fluorometer. Ujian MTT telah digunakan untuk mengukur kematian sel dan replikasi virus diukur menggunakan fluorometer. Keputusan menunjukkan NDV telah mengaruh autofagi di dalam sel MCF7 pada 2 jam selepas jangkitan (hpi). Kedua-dua perencat autofagi, SAR405 dan CQ tidak mempunyai kesan onkolisis oleh NDV yang ketara ke atas sel barah payudara seperti yang diukur pada 24, 48 dan 72 hpi. Walau bagaimanapun, penyahfungsian siRNA protein autofagi BECN1 telah

mengurangkan kematian sel MCF7 secara ketara sebanyak ~10% pada 24 hpi, tetapi tidak pada 48 dan 72 hpi. Eksperimen lanjutan mencadangkan hal ini mungkin disebabkan oleh pengurangan replikasi virus melebihi 50% selepas penyahfungsian siRNA pada 24 hpi. Kesimpulannya, NDV mengaruh autofagi di dalam sel barah payudara. Rencatan autofagi tidak meningkatkan kesan onkolisis oleh NDV ke atas sel barah payudara, sebaliknya mengurangkan kematian sel-sel kemungkinan dengan menyekat replikasi virus. Kajian lanjut boleh dijalankan untuk menentukan sama ada aruhan autofagi boleh meningkatkan keberkesanan onkolosis oleh NDV ke atas sel barah payudara.

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

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LIST OF ABBREVIATIONS

3-MA	3-Methyladenine
A549	Human lung cancer cell line (ATCC)
A549/DDP	Cisplatin-resistant A549
A549/PTX	Paclitaxel-resistant A549
Ambra 1	Autophagy and Beclin 1 Regulator 1
AP	Alkaline phosphatase
Atg	Autophagy-related
Baf A1	Bafilomycin A1
Bcl-2	B-cell lymphoma 2 gene
BECN1	Beclin 1
Bif-1	Bax interacting factor 1
CO2	Carbon dioxide
CQ	Chloroquine
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DTX	Docetaxel
eIF2α	Eukaryotic translational initiation factor 2α
ER	Endoplasmic reticulum
HA	Hemagglutination assay
HCQ	Hydroxychloroquine
hpi	Hours-post infection
IFN	Interferon
IRE1α	Inositol-requiring transmembrane kinase endoribonuclease-1α
JNK	c-Jun N-terminal kinase

LC3	Light chain 3
MCF7	Human breast cancer cell line (ATCC)
MDA-MD-231	Human breast cancer cell line (ATCC)
MOI	Multiplicity of infection
MRC5	Normal human lung fibroblasts cell line (ATCC)
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium reduction assay
NDV	Newcastle disease virus
NDV rAF-GFP	Recombinant Newcastle disease virus strain AF2240 with green fluorescent protein
OV	Oncolytic virus
PBS	Phosphate buffer saline
PE	Phosphatidylethanolamine
PERK	Protein kinase R-like ER kinase
PI3K	Phosphoinositide 3-kinase
PI3K3C	Class III PI3K complex
PVDF	Poly(vinylidene fluoride)
QC	Quinacrine
rAV	Average of the radius
RFU	Relative fluorescence unit
RPMI 1640	Roswell Park Memorial Institute 1640 Medium
Scr	Scrambled (non-targeting)
SDS-PAGE	Sodium dodecyl-sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
siRNA	Short-interfering RNA
SKVCR	VCR-resistant ovarian carcinoma cells

SQSTM1/p62	Sequestosome 1
SW620	Human colon adenocarcinoma cell line (ATCC)
UPM	Universiti Putra Malaysia
UVRAG	UV radiation resistance-associated gene
VCR	Vincristine
Vps15	Vacuolar protein sorting
XBP-1	X-box binding protein 1
xg	Relative centrifugal force

CHAPTER 1

INTRODUCTION

1.1 Background

Normal cells proliferate, mature, and die after a period by apoptosis or programmed cell death. Cancer cells, on the other hand, are aged cells that do not die but instead proliferate uncontrollably and lose their functionality. Cancer patients have undergone combination of either chemotherapy, radiotherapy or immunotherapy as surgery alone could not remove the tumour all the time. The emergence of chemo- and radio-resistant cancer cells has encouraged researchers to develop alternative cancer treatments (Russell et al., 2012). Hence, the interest in using and developing oncolytic viruses (OVs) as an alternative cancer treatment has grown in many researchers (Burke et al., 2015).

Newcastle disease virus (NDV) is a virus that can cause diseases to avian species around the world and the oncolytic property of NDV has first been reported by Prince & Ginsberg (1957) in Ehrlich ascites tumour cells. Like the other OVs, NDV has been demonstrated to infect and kill various cancer cells including leukaemia, lymphoma, melanoma, neuroblastoma, colon carcinoma, lung carcinoma, prostate carcinoma, breast carcinoma, gastric carcinoma, mesothelioma, and head and neck carcinoma at a much higher magnitude compared to its effect on normal cells (Zamarin & Palese, 2012). Even though NDV can effectively infect and kill cancer cells, the virus could also develop viral-resistant cancer cells (Chia et al., 2014). However, with the recent advancement of genetic engineering, few recombinant NDVs have been produced to enhance their oncolytic efficacy (Bai et al., 2014; Harper et al., 2021; Mohamed Amin et al., 2019; Najmuddin et al., 2020; Ramamurthy et al., 2021; Vijayakumar et al., 2020; Y. Wu et al., 2012).

Recently, researchers have discovered a novel strategy to fight cancer which is by inhibiting the autophagy pathway (Chude & Amaravadi, 2017). Autophagy is an intracellular degradation pathway which degrades and converts unused cytoplasmic constituents into new nutrients. It can be induced when the cell is starved, exposed to radiation, or infected to promote cell survival. Some studies have shown that in patients undergoing chemotherapy treatment, autophagy can be induced and acts as a pro-survival pathway to help the cancer cells such as colorectal, bladder, gastric, and breast cancer cells, to be chemo resistant (Li et al., 2016; Lin et al., 2017; Yang et al., 2015; Yu et al., 2015).

Several studies have reported that NDV infection can induce autophagy in glioma cells, lung cancer cells, and stomach adenocarcinoma cells (Bu et al., 2015; Hu et al., 2015; Jiang et al., 2014; Meng et al., 2012). Importantly, inhibition of autophagy increased the cancer cell death that was initially caused by NDV in

several lung cancer cell lines (Hu et al., 2015; Jiang et al., 2014; Meng et al., 2014). However, a study found that autophagy inhibition caused a reduction in NDV-induced oncolysis in stomach adenocarcinoma cells (Bu et al., 2015). Autophagy inhibition reduces NDV-induced immunogenic cell death in human melanoma cells (Shao et al., 2019).

Although several strains of NDV including the locally isolated strain AF2240 have been shown to have oncolytic effects on breast cancer cells (Ahmad et al., 2015; Al-Ziaydi et al., 2020; Ghrici et al., 2013; Kalantari et al., 2020; Mohamed Amin et al., 2019; Othman et al., 2010), no study has been done to determine the effect of autophagy inhibition on NDV-induced oncolysis in breast cancer cells. In this current study, it was hypothesised that inhibition of autophagy could enhance NDV-induced oncolysis in breast cancer cells.

1.2 Objectives

The objectives of this study were:

1) to determine the ability of NDV to induce autophagy in breast cancer cells;
2) to examine the effect of autophagy inhibition on NDV-induced oncolysis in breast cancer cells; and
3) to determine the effect of autophagy inhibition on NDV replication in breast cancer cells.

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