ENHANCING HUMAN BREAST CANCER CELLS DESTRUCTION USING COMBINATION OF ADENOVIRUS EXPRESSING P53 AND HYPERTHERMIA TREATMENT

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Specially for my beloved parents, Elengoe and Thavamani

My lovely sister, Suguna and Vaani

&

My wonderful brother, Tevanraj.

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ABSTRACT

In Malaysia, breast cancer is the most common cancer where 1 in 19 Malaysian women will be diagnosed with breast cancer by the age of 85. Moreover, lack of specific symptoms in the early stage of disease leading to delay in diagnosis. Unfortunately, current treatments by chemotherapeutic agents, surgery and radiation are not fully effective for the treatment of breast cancer. Thus, there is an urgency in developing new approaches for the treatment of breast cancer patients. In this study, a novel therapeutic regimen, combining the effects of recombinant adenovirus and hyperthermia was investigated. Firstly, Adenovirus serotype 5 was constructed by cloning of p53 gene into a defective recombinant adenovirus vector, Ad5-p53-DsRed Monomer N1. The Ad5-p53-DsRed Monomer N1 (MOI of 100) was then used to infect breast cancer cells (MDA-MB 231 and MCF-7) with or without combination of hyperthermia treatment (42ºC for 2 hours). The cell killing and viral concentration were then determined by MTT assay and viral plaque formation assay respectively. After that, the heat shock protein (Hsp70) and p53 protein expression in transfected cells were quantitated using ELISA assay. Activated-Caspase 3/7, 8 and 9 were also evaluated to study the apoptotic pathway of cancer cells. Furthermore, the novel protein interaction between nucleotide binding domain (NBD) Hsp70 and human Ad5 E1A 32 kDa motif (PNLVP); and NBD and p53 motif (SCMGGMNR) were investigated through bioinformatics tools such as Gromacs and Autodock softwares. It was found that MDA-MB 231 and MCF-7 cells infected with virus Ad5-p53-DsRed Monomer N1 alone resulted in 46.77±2.74% and 42.26±1.78% cell killing respectively while hyperthermia in combination with virus were 84.82±1.64% and 80.13±3.30% respectively. The Hsp70 expression of both cancer cells was also increased to 170.57% (MDA-MB 231) and 169.83% (MCF-7). Moreover, p53 expression in MDA-MB 231 and MCF-7 cells by virus combined with heat treatment (85.72 ng/L and 79.05 ng/L respectively) could lead to enhanced oncolytic property compared to virus treatment alone (47.82 ng/L and 40.54 ng/L respectively). In addition, caspase activity was first time reported that apoptosis process started at very early stage of infection in breast cancer cells with hyperthermia compared to virus alone. This was due to the evident that the highest kinetic energy was found in caspase 3 whereas virus alone the highest in caspase 8. In conclusion, Hsp70 induction by hyperthermia treatment enhanced Ad5-p53-DsRed Monomer N1 replication and oncolysis in MDA-MB 231 and MCF-7 cells through apoptotic pathway. Besides that, NBD of Hsp70 had the best interaction with PNLVP motif at 42°C. Thus, combining Ad5-p53 with hyperthermia treatment could be a potential approach for breast cancer treatment.

ABSTRAK

Di Malaysia, kanser payudara adalah kanser yang paling umum dimana 1 dalam 19 wanita Malaysia akan didiagnosis dengan kanser payudara menjelang usia 85. Tambahan pula, kekurangan tanda-tanda spesifik di peringkat awal penyakit yang membawa kepada kelewatan dalam diagnosis. Malangnya, rawatan semasa dengan agen kemoterapi, pembedahan dan radiasi tidak berkesan sepenuhnya untuk merawat kanser payudara. Oleh itu, strategi baru diperlukan dengan segera untuk merawat pesakit kanser payudara. Dalam kajian ini, potensi untuk mengabungkan regimen terapeutik novel adenovirus rekombinan dan 'hyperthermia' telah dikaji. Pertamanya, Adenovirus jenis 5 telah dibangunkan dengan pengklonan gen p53 ke dalam vektor adenovirus rekombinan, Ad5-p53-DsRed Monomer N1. Kepekatan 100 PFU bagi Ad5-p53-DsRed Monomer N1 telah digunakan untuk menjangkiti sel-sel kanser payudara (MDA-MB 231 dan MCF-7) dengan atau tanpa digabungkan dengan rawatan hyperthermia (42ºC selama 2 jam). Kemudian, tahap kemusnahan sel dan kepekatan virus telah ditentukan dengan asai MTT dan asai pembentukan plak virus. Selepas itu, pengekspresan protein kejutan haba (Hsp70) dan p53 dalam sel telah dianalisis dengan menggunakan asai ELISA. 'Caspase' teraktif 3/7, 8 dan 9 juga telah dikaji untuk tapak jalan apoptosis sel kanser. Tambahan pula, interaksi protein novel di antara domain pengikat nukleotida (NBD) bagi Hsp70 dan motif Ad5 E1A 32 kDa (PNLVP); dan NBD dan motif p53 (SCMGGMNR) telah dikaji dengan kaedah bioinformatik seperti perisian Gromacs dan Autodock. Kajian ini menunjukkan bahawa MDA-MB 231 dan MCF-7 yang dijangkiti virus Ad5-p53-DsRed Monomer N1 sahaja menyebabkan 46.77 ± 2.74% dan 42.26 ± 1.78% sel musnah manakala 'hyperthermia' dengan virus adalah 84.82 ± 1.64% dan 80.13 ± 3.30% masing-masing. Pengekspresan protein Hsp70 bagi kedua-dua sel kanser juga meningkat kepada 170.57% (MDA-MB 231) dan 169.83% (MCF-7). Selain itu, pengekspresan protein p53 dalam MDA-MB 231 and MCF-7 bagi gabungan virus dan 'hyperthermia' adalah 85.72 ng/L dan 79.05 ng/L masing-masing manakala perlakuan virus sahaja adalah 47.82 ng/L dan 40.54 ng/L masing-masing. Aktiviti 'caspase' telah dilaporkan kali pertamanya bahawa proses apoptotik bermula pada peringkat yang sangat awal bagi gabungan virus dan 'hyperthermia' berbanding dengan virus sahaja. Ini dibuktikan melalui tenaga kinetik yang paling tinggi didapati dalam caspase 3 manakala virus sahaja yang tertinggi dalam caspase 8. Kesimpulannya, induksi Hsp70 oleh perlakuan 'hyperthermia' meningkatkan replikasi Ad5-p53-DsRed Monomer N1 dan 'oncolysis' dalam sel MDA-MB 231 dan MCF-7 melalui proses apoptotik. Selain itu, NBD bagi Hsp70 mempunyai interaksi yang terbaik dengan PNLVP motif pada 42°C. Oleh itu, penggabungan Ad5-p53 dengan 'hyperthermia' mungkin boleh menjadi pendekatan bagi rawatan kanser payudara.

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LIST OF ABBREVIATION

AAV	-	Adeno-associated viral
Ad5	-	Adenovirus serotype 5
Akt	-	Serine or threonine kinase
Ala	-	Alanine
Arg	-	Arginine
Asn	-	Asparagine
Asp	-	Aspartic acid
ATP	-	Adenosine triphosphate
BLAST	-	Basic Local Alignment Search Tool
BLASTP	-	Protein BLAST
CAR	-	Coxsackie adenovirus receptor
CCSB	-	Center for Cancer Systems Biology
CO_2	-	Carbon dioxide
CTLs	-	Cytotoxic T-lymphocytes
Cys	-	Cysteine
DC	-	Dendritic cells
dH_2O	-	Distilled water
DNA	-	Deoxyribonucleic acid
dNTPs	-	Deoxyribonucleotide triphosphates
E.coli	-	*Escherichia coli*
Eg.	-	Example
ELISA	-	Enzyme-linked immunosorbent assay
GRAVY	-	Grand average of hydropathicity
G-factor	-	Goodness factor
Gln	-	Glutamine
Glu	-	Glutamic acid

Gly	-	Glycine
GUI	-	Graphical User Interface
HDACs	-	Histone deactylases
HIF	-	Hypoxia-inducible factor
HILP	-	Hyperthermic isolated limb perfusion
HIPEC	-	Hyperthermic intraperitoneal chemotherapy
His	-	Histidine
HLS	-	Helical lid subdomain
Hsp	-	Heat shock protein
Hsp70	-	Heat shock 70 kDa protein
HSV	-	Herpes simplex virus
Ile	-	Isoleucine
IPHC	-	Intraperitoneal hyperthermic chemotherapy
ITR	-	Inverted terminal repeat
LB	-	Luria-Bertani
Leu	-	Leucine
Lys	-	Lysine
MD simulation	-	Molecular dynamics simulation
$MgCl_2$	-	Magnesium chloride
MDM2	-	Murine double minute gene 2
M.wt	-	Molecular weight
NaCl	-	Sodium chloride
NBD	-	Nucleotide binding domian
NCBI	-	National Center for Biotechnology Information
NLS	-	Nuclear localization signal
PBC	-	Periodic boundary condition
PBS	-	Phosphate buffer saline
PCR	-	Polymerase Chain Reaction
PDB	-	Protein Data Bank
PDF	-	Probability density function
Phe	-	Phenylalanine
pI	-	Isoelectric point
PKB	-	Protein kinase B
PME	-	Particle Mesh Ewald

Pro	-	Proline
ProSA	-	Protein Structure Analysis
PTEN	-	Phosphatase and tensin homolog deleted on chromosome ten
RF	-	Radiofrequency
RMSD	-	Root mean square deviation
RMSF	-	Root mean square fluctuation
SBD	-	Substrate binding domain
SBSD	-	Substrate-binding subdomain
Ser	-	Serine
SPC	-	Simple point charge
TAE	-	Tris-Acetate electrophoresis buffer
Thr	-	Threonine
Trp	-	Tryptophan
Tyr	-	Tyrosine
UV	-	Ultraviolet
Valine	-	Valine
WBH	-	Whole-body hyperthermia
WHO	-	World Health Organization
3-D	-	Three-dimensional

LIST OF SYMBOLS

cm	-	Centimetre
cm^2	-	Square centrimetre
g	-	Gram
h	-	Hour
K	-	Kelvin
kDa	-	Kilo Dalton
Kcal/mol	-	Kilocalorie per mole
L	-	Litre
M	-	Molarity
$M^{-1}cm^{-1}$	-	Molar absorptivity
mg	-	Miligram
mg/ml	-	Miligram/mililitre
mM	-	Mili molar
nm	-	Nano metre
ns	-	Nano second
ps	-	Pico second
rpm	-	Rounds per minute
s	-	Second
µl	-	Microlitre
µM	-	Micro molar
v	-	Volt
Å	-	Angstrom
α	-	Alpha
β	-	Beta
°C	-	Degree Celsius
ΔG_{bind}	-	Binding energy

>	-	Greater than
<	-	Less than

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CHAPTER 1

INTRODUCTION

1.1 Background of study

Currently, breast cancer is the fifth leading cause of cancer-related deaths for both men and women in the worldwide, accounting for 521,000 deaths in 2012 (World Health Organization, 2014). In Malaysia, breast cancer is the most common cancer where 1 in 19 Malaysian women will be diagnosed with breast cancer by the age of 85 (National Cancer Registry of Malaysia, 2014). Most cases occur during age 45-55. It is the most common cancer diagnosed in women (25.2% of all new cases in women) (World Health Organization, 2014). In addition, 10-15% of women treated for early breast cancer suffer a local recurrence (locally recurrent breast cancer, LRBC) within 10 years (Clemons *et al.*, 2001). Local failure causes significant physical and psychosocial morbidity (van der Zee *et al.*, 1999), and the majority of these patients die of their disease within 5 years of recurrence (Clemons *et al.*, 2001). This is due to the poor prognosis such as lack of specific symptoms in the early stage of disease leading to delays in diagnosis, the aggressive nature of disease, as evidenced by the high rate of local spread and/or distant metastasis at the time of diagnosis, diagnosis techniques that lack sufficient sensitivity and specificity to support screening for breast cancer. At present, the cancer treatment by chemotherapeutic agents, surgery and radiation has not been fully effective against the high incidence or low survival rate of breast cancer. Furthermore, these treatments cause negative side effects such as liver failure, cardiomyopathy and an increased risk of developing other types of cancer (Hawkins and Hermiston, 2001).

Thus, the development of a new therapeutic approach to breast cancer remains one of the most challenging area in cancer research.

Gene therapy is a new therapeutic approach for breast cancer. It specifically targets the tumour cells including metastatic cells in the body (Abaan and Criss, 2002). It has been shown to be effective with different types of diseases (Rubanyi, 2001). Therefore, it may be applicable for the treatment of breast cancer patients. Oncolytic adenoviruses are a class of promising anti-cancer agents, which are engineered to infect, replicate within, and lyses cancer cells (Yamamoto and Curiel, 2009). However, these agents alone failed to generate sustained clinical responses or to cause complete tumour regressions. This is because heterogeneity or indeed lack of expression of receptors (coxsackie adenovirus receptor, CAR) and co-receptors (integrin $\alpha_v\beta_3$ and $\alpha_v\beta_5$ classes) in tumours can be implicated in the poor efficiency of infectivity by adenovirus (Bauerschmitz *et al*., 2002; Kanerva and Hemmiki, 2004). In addition, many tumour cells fail to support adenovirus replication because of its replication deficiency. Thus, combination treatment is needed to improve the clinical outcome in breast cancer treatment.

Hyperthermia has been explored intensively to treat cancer patients. It is used to raise the temperature of a region of the body affected by cancer up to 41.5-43°C with minimal or no damaging healthy tissues (van der Zee, 2002). Several investigators suggested that hyperthermia might enhance viral replication, particularly in tumour cells (Thorne *et al*., 2005). Heat shock protein (Hsp) is the key player for the hyperthermia hypothesis. Glotzer *et al*. (2000) described that Hsp may play a vital role in the adenovirus life cycle because genome replication, synthesis of protein and virion assembly which are vital for viral replication, is dependent on the host cell. Hsp especially Hsp70 is the main responsible for import and colocalizes viral proteins in the nucleus with E1A gene products of adenovirus (Kao *et al*., 2005). Furthermore, Wickner *et al*. (1992) documented that bacterial DNAJ and DNAK, which are important for bacteriophage DNA replication, may depend on Hsp70 induction. Hsp40 and Hsp70 induction promotes production of viral proteins for avian adenovirus CELO (Glotzer *et al*., 2000).

Hyperthermia induces transgene expression, represents a promising strategy using the combination of hyperthermia with virotherapy (Huang *et al*., 2000; Lohr *et al*., 2000; Walther and Stein, 2009). Nevertheless, there are only few studies on this combination treatment against cancer. Based on Eisenberg *et al*. (2010) study, it has been demonstrated that the combination of hyperthermia and NV1066 (a recombinant herpes simplex virus-1) infection significantly increased the pancreatic cancer cell kill to approximately 80% without damaging normal cells. Therefore, adenovirus in combination with hyperthermia can be a potential treatment for breast cancer patients.

1.2 Problem statement of research

There have been numerous strategies attempted in the past to treat breast cancers with limited success. One of the latest approaches is adenovirus gene therapy. Although the oncolytic adenoviruses are promising anti-cancer agents, clinical studies demonstrated that viral therapy alone failed to produce sustained clinical responses or to destroy tumour completely. This is due to lack of expression of coxsackie adenovirus receptor and co-receptors in tumour cells which is crucial for adenovirus infection. Therefore, tumour cells hinder replication of adenovirus.

While the treatment effects of hyperthermia as a single agent are limited, its ability to potentiate the effects of standard chemo-radiotherapies has generated lasting interest. Yet, combination of hyperthermia with either chemotherapy, radiotherapy or both, led to improved clinical outcome in treatment of breast cancer; they have been shown potential side effects, such as impotence or incontinence that can greatly impair life quality (van der Zee, 2002). Thus, a novel approach of combining gene therapy and hyperthermia will be explored to be a new way to treat breast cancer cells.

1.3 Hypotheses of study

The hypotheses of this study are:

1. Can coupling of hyperthermia and Ad5-p53-DsRed Monomer N1 enhances killing of breast cancer cells (MCF-7 and MDA-MB 231)?
2. Can heat treatment induced Hsp70 and p53 expression in breast cancer cells?
3. Does the combination of hyperthermia and Ad5-p53-DsRed Monomer N1 involved in apoptosis pathway?
4. Is there any protein interaction between nucleotide binding domain (NBD) of Hsp70 and E1A 32 kDa motif (PNLVP)?
5. Is there any protein interaction between NBD of Hsp70 and p53 motif (SCMGGMNR)?

1.4 Objectives of study

The objectives of this study are:

1. To determine the cytotoxic effects of hyperthermia alone, Ad5-p53-DsRed Monomer N1 alone and combination of hyperthermia and Ad5-p53-DsRed Monomer N1 on breast cancer lines (MCF-7 and MDA-MB 231).
2. To determine the expression of Hsp70 in breast cancer cells after treated with Ad5-p53-DsRed Monomer N1 in combination with hyperthermia.
3. To determine p53 expression in breast cancer cells for combination treatment of Ad5-p53-DsRed Monomer N1 and hyperthermia.
4. To determine the possible pathway involved in apoptosis for MDA-MB 231 and MCF-7 cells after treated with the combination of Ad5-p53-DsRed Monomer N1 and hyperthermia.

5. To identify novel protein interaction between NBD of Hsp70 and E1A 32 kDa of human adenovirus serotype 5 motif (PNLVP).
6. To identify novel protein interaction between NBD of Hsp70 and p53 motif (SCMGGMNR).

1.5 Scope of research

This study involves construction of recombinant adenovirus, cytotoxicity, quantitation of viral replication, protein expression, protein modeling, molecular dynamic (MD) simulation of protein and protein-protein docking. Firstly, Ad5-p53 will be constructed by cloning p53 gene into defective recombinant adenovirus vector containing red fluorescent protein (DsRed Monomer N1). Then, Ad5-p53-DsRed Monomer N1 (multiplicity of infection of 100 PFU per cell, MOI of 100) will be infected with MCF-7 and MDA-MB 231 breast cancer cells. Cells will be treated at 42°C for 2 hours prior to viral treatment. The formation of viral plaques and cell survival (MTT assay) will be measured. After that, Hsp70 and p53 protein expression will be quantitated using ELISA assay. Activated-Caspase 3/7, 8 and 9 will also be performed to study the apoptotic pathway of cancer cells. Besides that, the novel protein interaction between NBD of Hsp70 and E1A 32 kDa of human Ad5 motif (PNLVP); and NBD and p53 motif (SCMGGMNR) will be investigated through bioinformatics tools such as Gromacs version 4.6.3 and Autodock version 4.2.

1.6 Significance of study

The beneficial outcome of this study is that the novel therapeutic regimen, combining the effects of recombinant adenovirus (Ad5-p53-DsRed Monomer N1) and hyperthermia (42ºC for 2 hours) can be explored as a potential breast cancer treatment. Furthermore, this combination treatment could be a useful application to develop adenovirus-based gene transfer to breast cancer cells. In spite of that, understanding the stability of Hsp70; the preferred sites of interaction between

Hsp70 and E1A 32 kDa of human Ad5; and the binding affinity and stability Hsp70-p53 motif complex structure through bioinformatics tools is the key to design rational drugs and vaccines in breast cancer treatment.

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