UNIVERSITI PUTRA MALAYSIA

CONSTRUCTION OF A SINGLE CHAIN VARIABLE FRAGMENT AGAINST MCF-7 BREAST CANCER CELLS

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IPM 2008 1

CONSTRUCTION OF A SINGLE CHAIN VARIABLE FRAGMENT AGAINST MCF-7 BREAST CANCER CELLS

By

ZUHAIDA ASRA AHMAD

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

March 2008

DEDICATED TO:

My husband, ANUAR, my son, MUHAMMAD AQIL FATHULLAH, my mother, MASNIRAH, my brothers and sister.

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

CONSTRUCTION OF A SINGLE CHAIN VARIABLE FRAGMENT AGAINST MCF-7 BREAST CANCER CELLS

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March 2008

Chairman: **Professor Abdul Manaf Ali, PhD**

Faculty: **Institute of Bioscience**

Recombinant antibody cloning and phage display technologies can be employed to produce and isolate single-chain antibodies (scFv) specifically against antigen of interest. The aims of this study are to construct single chain variable fragment (scFv) towards MCF-7 breast cancer cells and to characterize scFv antibodies that interacts with MCF-7. Initially, mRNA was extracted from previously well-characterized monoclonal antibody (C3A8) against MCF-7. The genes encoding heavy (V_H) and light (V_L) chains were amplified, linked in V_H-V_L orientation via PCR and cloned into a pCANTAB 5E phagemid vector. The protein was then expressed in a *sup*E strain of *E. coli* TG1. Phage particles displaying scFv were panned against MCF-7 and the selected clones were further used for infecting non-suppressor strain, *E. coli* HB2151. The scFv antibodies expressed were characterized by enzyme-linked

immunosorbent assay (ELISA) and immunoblotting. As demonstrated by ELISA result, the scFv antibodies could strongly bind to MCF-7 breast cancer cells. It retained the binding capacity of its parental C3A8 monoclonal antibody. Clone B7 was expressed mainly as soluble periplasmic protein. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the recombinant antibody revealed a protein with apparent molecular weight of approximately 32 kDa. Nucleotide sequence analysis of C3A8 scFv showed high homology (99%) with published single chain antibody against rice stripe virus protein P20 [synthetic construct]. In conclusion, the recombinant antibody technology is an effective approach in the development of scFv antibody for the next generation of immunotherapy reagents especially towards MCF-7 breast cancer cells.

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

PEMBINAAN SATU RANTAIAN SERPIHAN BOLEH UBAH TERHADAP SEL KANSER PAYU DARA (MCF-7)

Oleh

ZUHAIDA ASRA AHMAD

Mac 2008

Pengerusi: **Profesor Abdul Manaf Ali, PhD**

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Pengklonan antibodi rekombinan dan teknologi pameran faj digunakan bagi menghasilkan dan memencilkan satu rantaian serpihan boleh ubah (scFv) antibodi secara spesifik terhadap sebarang antigen yang diminati. Objektif kajian adalah untuk membina satu rantaian serpihan boleh ubah (scFv) antibodi terhadap sel kanser payu dara (MCF-7). Sebagai permulaan, mRNA (pengutus RNA) diekstrak daripada hybridoma (C3A8) yang sebelum ini telah berjaya dicirikan sifatnya dalam menghasilkan monoklonal antibodi (Mab) terhadap MCF-7. Gen-gen yang mengkodkan rantaian berat (V_H) dan ringan (V_L) diamplifikasikan, disambungkan pada orientasi V_H-V_L melalui tindakbalas PCR dan diklonkan ke dalam vector fajmid pCANTAB 5E. Protein kemudiannya diekspreskan di dalam strain penindas *E. coli* TG1. Partikel-partikel faj yag mempamerkan scFv dilimpahkan ke atas MCF-7 dan klon-klon terpilih kemudiannya digunakan bagi menjangkiti strain tanpa

penindas, *E. coli* HB 2151. Antibodi scFv yang diekspreskan selanjutnya dicirikan melalui ujian ELISA dan immunopemblotan. Sepertimana yang didemonstrasikan oleh keputusan ELISA, antibodi scFv berupaya untuk mengikat secara kuat kepada sel kanser payu dara, MCF-7. Ianya dapat mengekalkan keupayaan mengikat yang sama seperti Mab yang asal (C3A8). Kebanyakan klon B7 mengekspreskan scFv antibodinya dalam bentuk protein periplasmik terlarut. Analisis elektroforesis gel SDS-PAGE ke atas antibodi rekombinan jelas menunjukkan protein yang bersaiz 32 kDa berjaya diekspreskan. Analisis penjujukan DNA bagi scFv C3A8 pula memberikan tahap persamaan yang tinggi (99%) dengan satu rantaian serpihan boleh ubah (scFv) antibodi terhadap protein virus berjalur beras, P20 (pembinaan sintetik). Secara keseluruhannya, teknologi antibodi rekombinan adalah merupakan satu pendekatan berkesan di dalam pembinaan antibodi scFv di mana ia juga berpotensi bagi perkembangan reagen immunoterapi untuk generasi yang akan datang terutamanya terhadap sel kanser payu dara, MCF-7.

ACKNOWLEDGEMENTS

Glory to God in the highest, whose grace and strength has enabled me to complete this thesis in spite of many obstacles faced throughout the study. This study would not have been possible without the contribution and support of many people to whom I have the pleasure of expressing my appreciation and gratitude. Many thanks to my supervisor, Professor Dr. Abdul Manaf Ali for his guidance, understanding, patience and encouragement that lead to the completion of this thesis and most importantly his kindness to give me a chance to gain knowledge and experience through a series of workshops as well as seminars that I have attended.

Also thanks to my supervisory committee members, Dr Noorjahan Banu Mohd Alitheen, Dr Muhajir Hamid as well as Dr Shuhaimi Mustapha for monitoring the progress of research, helpful comments, proofreading and editing this thesis. Thanks are extended to Datin Paduka Khatijah Mohd Yusoff and for allowing me to drop in her lab for a few months during my lab transition, to Prof Dr Abdul Rahman Omar and also to Prof Dr Sazaly Abu Bakar from UM, for the technical advice throughout this study.

Special thanks to my colleagues from Molecular Biomedicine Laboratory, Kak Asmah, Kak Aliza, Kak Izan, Kak Maddie, Kak Dah, Kee, Aya, Ainul, Ana, Mas, Wan, Teo, Elham and others for their kindness, support and encouragement during the hard times. To the Ministry of Science Environment

and Technology (IRPA-5495304), thanks for awarding me a GRA Fellowship, providing me with the financial means to complete this project.

And finally, thanks to my husband, son, my mother, father and siblings who endured this long process with me, always offering support and love.

I certify that an Examination Committee has met on 6 March 2008 to conduct the final examination of Zuhaida Asra binti Ahmad on her Master of Science thesis entitled "Construction of a Single Chain Variable Fragment against MCF-7 Breast Cancer Cells" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the student be awarded the degree of Master of Science.

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DECLARATION

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

_____

ZUHAIDA ASRA AHMAD

Date: 14 March 2008

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
bp	base pair
kb	kilo base pair
cDNA	complementary deoxyribonucleic acid
DNA	deoxyribonucleic acid
FBS	fetal bovine serum
HI-FBS	Heat inactivated fetal bovine serum
Mab	monoclonal antibody
DNase	nuclease
scFv	single chain fragment variable
V	variable
V_H	heavy chain
V_L	light chain
Fv	fragment variable
Fab	antigen-binding fragment
mRNA	messenger RNA
PCR	Polymerase chain reaction
kDa	kilo Dalton
HAMA	human anti-mouse response
Ig	immunoglobulin
CDRs	complementarily-determining regions
FW	Framework regions
AP	alkaline phosphatase
μm	micrometer
μl	micro liter
U	units
mL	milliliter
L	liter
mg	milligram
ng	nanogram
μg	microgram
cm	centimeter
mM	millimolar
M	molar/molarity
nm	nanometer
UV	ultra violet
CO_2	carbon dioxide
v/v	volume per volume
DEPC	diethyl sulphoxide
BSA	bovine serum albumine
dATP	deoxyadenine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanine triphosphate

dTTP	deoxythymine triphosphate
DTT	dithiothreitol
$MgCl_2$	magnesium chloride
TE	tris-EDTA
Tris	tris[hydroxymethyl]aminomethane
EDTA	ethylene diamine tetracetate
H_2O_2	hydrogen peroxyde
$CaCl_2$	calcium chloride
OD	optical density
κ	kappa
SDS	soium dodecyl sulfate
x	times
scFv	single chain fragment variable
pfu	plaque forming unit
rpm	revolution per minute

CHAPTER I

INTRODUCTION

Beginning in 1975, monoclonal antibodies (Mabs) have been favored as they can be produced in unlimited quantities to practically bind to any antigen and are more easily standardized. However, there are limitations to their clinical applications, as they are almost exclusively murine in origin which could create human anti-mouse response (HAMA) when introduced to human (Klimka *et al*., 2000). The monoclonal antibody producing antibody technology is also very laborious and time consuming and small mammals like mice do not always provide the high-affinity antibody response to particular antigen needed for sensitive assay development (O'Connor *et al.*, 1997). These limitations of traditional techniques have led several research groups to investigate the use of phage display in producing single chain Fv (scFv) antibodies.

The generation of scFv has now become an established technique used to produce a completely functional antigen-binding fragment in bacterial systems. The variable fragment (Fv) portion of an antibody, consisting of variable heavy chain (V_H) and variable light chain (V_L) domains is the smallest fragment that consistently maintains the binding specificity and affinity of the whole antibody (Glockshuber *et al*., 1990). Previously, active Fv fragments have been produced by proteolytic digestion of the antibody molecule (Hochman *et al.*, 1973). However, the proteolysis often does not

result in a homogenous population of Fv fragments but rather produces a number of partially digested protein molecule, which may cause difficulties in both purification and characterization. Therefore, the advances in antibody engineering have now facilitated a more efficient and generally applicable method to produce Fv fragments. Nevertheless, the utility of the Fv fragments is limited by disassociation of the variable domains at low protein concentrations and in the absence of antigen (Glockshuber *et al*., 1990; Jager and Pluckthun, 1999).

The recombinant scFv construction represents a further technological advance in stabilized Fv production by means of joining both of V_L and V_H domains covalently into a single polypeptide chain (~30 kDa in size) using an engineered flexible polypeptide linker (Bird *et al*., 1988). The designed linker used should also be stable against proteolysis and should minimize protein aggregation, in addition to contributing to scFv solubility (Whitlow *et al*., 1993). For these reasons, an established flexible peptide linker, $(Gly_4Ser)_3$, was chose in this study for bridging the variable domains in which it consists of 15 amino acid and specifically designed to bridge the 3.5 nm gap between carboxy-terminus of the V_H chain and the amino terminus of the V_L chain (Huston *et al*., 1988 and Whitlow *et* al., 1991).

In the early 90's, McCafferty and coworkers (McCafferty *et al*., 1990) had developed one successful approach that relies on a phage-display system in which the fragments of antibodies (scFvs) are expressed as fusion proteins on

the minor coat protein (pIII) of the filamentous phage. In phage display system, an antigen is immobilized and a collection of binding proteins are displayed on the phage, together with the g3p as a fusion protein (Winter *et al*., 1994). All these systems share the principle of a relation between genotype (the Ig genes) and phenotype (antigen-binding). Therefore, the genetic information of the displayed protein is contained within the phage DNA in the same phage particle and thus, physically connected to the expressed protein. By using the polymerase chain reaction (PCR), both immunoglobulin variable (V_H and V_L) regions genes are first amplified from hybridomas or spleen cells followed by assembling into scFv fragment via a flexible linker, and expressed on the surface of the filamentous phages (phagebodies). In the selection procedure, so-called biopanning, the non-binding phage will be removed by washing while the remaining phages are used to infect *E. coli* for their amplification. The biopanning step can be repeated to reduce the number of clones to be assayed as well as to select clones with the highest affinity. It has also been proven by a number of researchers that extra rounds of panning could increase the selection efficiency of specific binding affinity clones (Maranhão and Brígido, 2000).

ScFv antibodies produced from phage display can be genetically fused to the marker proteins, such as fluorescent proteins (Didier *et al*., 2008) or alkaline phosphatase (Mousli *et al*., 2007). These bifunctional proteins having both antigen-binding capacity and marker activity can be obtained from transformed bacteria and used for one-step immunodetection of biological

agents. Alternatively, antibody fragments could also be applied in the construction of immunotoxins, therapeutic gene delivery as well as anti-cancer intrabodies for therapeutic purposes.

Because of these reasons, both recombinant antibody and phage display technologies have been exploited in the present study to produce an optimized reagent (scFv) starting from the mouse B-cell hybridoma cell line C3A8 (Ali *et al.,* 1996), which generates a monoclonal antibody against MCF-7 breast cancer cells.

Nowadays, breast cancer is the most common and frequent cause of cancer-derived death in women (Gross, 2001). There are conventional therapies such as surgery, chemotherapy, radiotherapy, anti-estrogen therapy which are not able to eliminate occult cancer cells and therefore to prevent metastatic diseases, relapses, and bears the risk of side effects on non-tumor tissues (Chlebowski *et al*., 1999). Therefore, sensitive detection of residual cancer cells in breast tissue would have important therapeutic and prognostic implications. The potential targets for immunotherapy could achieved by identification of tumor-specific or associated antigens on the surface of breast cancer cells (Carter, 2001). The first promising approaches which have been published in immunotherapy involved the application of monoclonal antibodies, immunotoxins, bispecific antibodies or vaccination with tumor specific antigens (Ghadersohi *et al*., 2002 and Presta, 2002). Until now, recombinant antibody fragments have been proven promising *in vitro* as well

as in phase I and II clinical trials in certain types of cancer. In 2006, the US Food and Drug Administration (FDA) has approved a quadrivalent recombinant vaccine for the prevention of diseases caused by human papillomavirus types 6, 11, 16, and 18 in females aged 9 to 26 years and also a new indication for topotecan HCl injection, allowing its use in combination with cisplatin for the treatment of recurrent or persistent stage 4B cervical cancer.

The MCF-7 breast cancer cells were chosen in this study since it is highly immunogenic and has distinct antigens that elicit antibodies specific for this type of cancer (Kida *et al.*, 1975; Yuan *et al.*, 1982 and Plessers *et al.,* 1990). Several Mabs obtained from the hybrids between murine myeloma cells with spleen cells from mice immunized with MCF-7 cell line demonstrated a high degree of specificity for neoplastic as well as normal epithelial cells (Yuan *et al.*, 1982). Therefore, these Mabs are very useful in diagnostic protocols against breast cancer. In addition, the changes on this murine antibody structure could then serve as immunotherapeutic reagents.

C3A8 hybridoma is a fusion between the lymphocytes of Balb/c mice sensitized with the MCF-7 breast carcinoma cell line with Sp2 myeloma cells. It was proven to be very stable in generating murine Mabs (IgM) which could specifically react against MCF-7 and T47-D cell lines while weak cross reactivities was found with ovarian cancer, Cao-3 and pancreatic cancer PANC-1. But no reactivity was observed when Mab C3A8 was tested with tissues of normal breast and other organs (Ali *et al.,* 1996). In consequence,