UNIVERSITI PUTRA MALAYSIA

EXPRESSION OF TUMOUR-ASSOCIATED ANTIGENS AND CHARACTERISTICS OF T CELL RESPONSES IN BREAST CARCINOMA

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LEONG POOI POOI

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

March 2005

Specially dedicated to,

My mother, husband, sister and brother

For their love, understanding, encouragement and patience

Good luck to you all.

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

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EXPRESSION OF TUMOUR-ASSOCIATED ANTIGENS AND CHARACTERISTIC OF T CELL RESPONSES IN BREAST CARCINOMA

By

LEONG POOI POOI

March 2005

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Breast cancer is the most common cancer among women in Malaysia. The standard conventional clinical management procedures use chemotherapy, radiotherapy and mastectomy. In the past decade, intense research towards the use of T-cell based immunotherapy as a treatment alternative has been made. The goals of our study are first to identify some of the tumour-associated antigens present in our tumour specimens from patients with infiltrating ductal carcinoma (IDC) of the breast, followed by antigenic peptide selection in order to develop an *in vitro* T-cell based cytotoxicity assay. At the same time, we also identified immunophenotypes of the tumour infiltrating lymphocytes (TILs) in the breast tumours. Isolated peripheral blood mononuclear cells (PBMCs) from patients with IDC were specifically stimulated with three combinations of cytokines and antibodies that were specific to the co-stimulatory molecule and HLA-A02 restricted antigen-specific peptides. Stimulated PBMCs were then used as effector cells in cytotoxicity assay using calcein-AM in which the MCF-7 breast adenocarcinoma cell line served as the target cells. Phenotypic investigation of tumour cell suspension was carried out by using specific lymphocyte cell differentiation markers. By using paraffin-embedded breast

tissues (n=49), immunohistochemistry studies showed significant expression of survivin (80.1%, $p<0.001$), cytoplasmic MUC-1 (38.3%, $p<0.05$) and membranous MUC-1 (63.8%, $p<0.001$) in the tumour area as compared to the apparently normal adjacent tissues. These results provided a guide for antigenic peptide selection for stimulating the T cells from the blood of the patients. Together in the presence of rIL-2 and rIL-7, 4 out of 9 peripheral blood mononuclear cells (PBMCs) from the patients responded to either survivin-derived peptide (S2) or Her2/neu specific peptide (H2) in a HLA-A02 restricted manner in order to produce sufficient amounts of effector cells for the subsequent cytotoxic assay. As effector/target (E/T) ratio increased, cytolytic activity of the effector cells became more efficient. For immunophenotypic analysis, CD8+ TILs at 23.4 ± 2.1% was found to be the major population in TILs and the presence of its effector counterpart, CD8+CD28+ TILs significantly correlated with low incidence of metastasis ($p<0.05$). At the same time, we noticed the predominance of CD4+CD25+ regulatory T cells (Treg) at 55.9 ± 3.9% in the Treg pool and its presence was significantly found in post-menopausal patients ($p<0.05$). In conclusion, survivin and MUC-1 (cytoplasmic and membranous) were over-expressed in breast cancer tissues. Further investigations are needed to determine the reasons as to why only a portion of PBMCs from the patients (4/9) responded to the specific peptide-based stimulation and showed effective cytolytic activity towards the target breast adenocarcinoma MCF-7 cell line. It is possible that other cytokine cocktails are needed to enhance the cytolytic property of the PBMCs. We also found that infiltration of effector TILs, CD8+CD28+, significantly reduced the metastatic event. Lastly, we noted that older women (≥ 50 years old) tend to possess higher amount of CD4+CD25+ Treg in TILs as compared to the younger patients (< 50 years old). The higher CD4+CD5+ Treg

in TILs may implicate poor disease outcome in older patients. We proposed that these Treg cells contribute to tumour escape mechanism.

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

TINDAK BALAS SEL T TERHADAP ANTIGEN YANG BERKAITAN DENGAN KANSER PAYU DARA

Oleh

LEONG POOI POOI

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Pengerusi : Profesor Seow Heng Fong, PhD

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Barah payudara merupakan kanser yang paling umum di kalangan wanita di Malaysia. Kaedah-kaedah perubatan klinikal yang biasa digunakan adalah kimiaterapi, radioterapi dan pembedahan. Dalam dekad yang lalu, banyak penyelidikan terhadap penggunaan imunoterapi sebagai kaedah perubatan alternatif telah dijalankan. Tujuan kajian ini adalah, pertama, untuk mengenalpasti beberapa antigen yang berkaitan dengan kanser (TAA) dalam specimen-specimen pesakit yang mengalami penyebaran sel–sel kanser ke salur duktur payudara (IDC), dan kedua, untuk memilih peptid antigen agar esei sitotoksik sel T dapat dilaksanakan. Pada masa yang sama, kami juga mengenalpasti imunofinotip dalam sel-sel limfosit yang tersebar dalam kanser payudara (TILs). Sel-sel mononuklear periperal darah (PBMC) yang diasingkan dari pesakit yang mengalami penyebaran sel –sel kanser ke salur duktur payudara telah dirangsangkan dengan menggunakan kombinasi sitokin dan antibodi yang specifik terhadap perangsangan berpandu dan peptid spesifik terhadap antigen terhad HLA-A02. PBMC yang terangsang digunakan sebagai sel efektor dalam esei sitotoksik calcein-AM di mana sel kultur

adenokarsinoma payudara MCF-7 digunakan sebagai sel target. Penyelidikan finotip terhadap sel-sel limforsit yang tersebar dalam kanser payudara dijalankan dengan menggunakan sel-sel kanser yang telah dicerai oleh enzim dan tanda-tanda perbezaan sel limfosit yang spesifik. Dalam tisu kanser payudara yang berparafin (n=49), kajian imunohistokimia mempaparkan ekpresi yang nyata terhadap survivin (80%, $P<0.001$), MUC-1 di sitoplasma (38.3%, $P<0.05$) and MUC-1 di membran (63.8%, $P<0.001$) di dalam kawasan sel-sel kanser berbanding dengan sel-sel normal yang bersebelahan. Keputusan ini memberi panduan dalam pemilihan antigen peptid untuk merangsang sel-sel T limfoit daripada darah pesakit tersebut. Dengan kehadiran rIL-2 dan rIL-7, empat daripada sembilan pesakit mempunyai sel-sel mononuklear periperal darah bertindakbalas terhadap peptide survivin atau peptid spesifik Her2/neu dalam keadaan HLA-A02 dihadkan agar dapat menghasilkan sel-sel efektor yang cukup untuk esei sitotoksik yang seterusnya. Apabila ratio efector/target (E/T) meningkat, sel efektor semkin cekap menjalankan aktiviti sitolisis. Dalam imunofenotip analisa, sebanyak 23.4 ± 2.1 % CD8+TILs merupakan kumpulan yang terbesar dalam TILs dan, dengan nyata sekali, kehadiran sel efektor CD8+CD28+TILs berkait rapat dengan insiden metastasis yang rendah ($P<0.05$). Pada masa yang sama, kami mendapati sel CD4+CD25+regulasi T (Treg) mendominasi kumpulan Treg dengan sebanayak 55.9 ± 3.9% dan kehadirannya hanya nyata dalam pesakit lebih tua ($\geq$ 50 tahun) ($p<0.05$). Sebagai kesimpulan, survivin dan MUC-1 (dalam sitoplasma dan pada membran) adalah terlebih ekspres dalam tisu kanser payudara. Penyelidikan yang lebih memdalam harus dilakukan untuk mengetahui sebab-sebab kenapa hanya sebahagian daripada pesakit (4/9) bertindakbalas terhadap rangsangan specifik peptid dan menonjolkan aktiviti sitolisis yang berkesan terhadap sel kultur adenokarsinoma payudara MCF-7.

Campuran beberapa sitokin mungkin akan meningkatkan aktiviti sitolisis PBMCs terhadap sel kanser. Kami juga mendapati sel efektor CD8+CD28+TILs berkesan mengurangkan insiden metastasis. Akhir sekali, kami mendapati wanita yang lebih tua cenderung memiliki jumlah sel CD4+CD25+Treg yang tinggi dalam Treg jika dibandingkan dengan pesakit lebih muda usianya. Jumlah sel CD4+CD25+Treg yang banyak mengimplikasi prognosi yang lemah dalam kalangan wanita susut haid. Kami mencadangkan bahawa Treg tersebut menyumbang kepada mekanisma di mana sel kanser terbebas daripada sistem imun.

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TABLE OF CONTENTS

LIST OF TABLE

LIST OF FIGURE

LIST OF ABBREVIATIONS

α	alpha
β	beta
γ	gamma
δ	delta
%	percentage
°C	Degree of Celsius
μg	microgram
ACD	acid citrate dextrose
AICD	activated-induced cell death
AMC	atypical medullary carcinoma
APC	allophycocyanin
APCs	antigen presenting cells
APES	aminoproeyltrimethoxysilane
BCG	bacilli Calmette-Guerrin
BCS	breast conservation surgery
bp	base pair
BRAC	breast cancer susceptibility protein
BSA	bovine serum albumin
Calcein-AM	calcein- acetoxymethyl
CD	cluster of differentiation
cm	centimeter
CO_2	carbon dioxide
COX-2	cyclooxygenase-2
CTLs	cytotoxic T lymphocytes
Cy-chrome	cyanine-chrome
DAB	diaminobenzidine tetrahydrochloride
DCIS	ductal carcinoma in situ
DCs	dendritic cells
DMSO	dimethylsulphoxide
DNA	deoxyribonuclease acid
DTH	delayed type hypersensitivity
EGFR	epithemal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
ER	oestrogen receptor
E/T	effector/target ratio
EthD	ethidium bromide homodimer
FADD	Fas-associated protein with death domain
FASL	Fas ligand
FBS	fetal bovine serum
Fc	forward scatter
FITC	fluorescein isothiocynate
FL	filter
FLICE	FADD homologous Interleukine-1 beta converting enzyme/ *Caenorhabdistis elegans* cell-death protein 3- like protease
FOXP	Foxhead/ winged-helix
GITR	glucocorticoid induced tumour necrosis receptor

GSK	glycogen synthase kinase
Her2/neu	human epidermal growth factor 2/ neu
HLA	human leukocyte antigen
HPV	human papillomavirus
HRT	hormone replacement therapy
HUKM	Hospital Universiti Kebangsaan Malaysia
IAP	inhibitory of apoptosis
ICAM-1	intracellular cell adhesion molecules-1
IDC	infiltrating ductal carcinoma
IFN-γ	interferon gamma
IL-	interleukin
iNKRs	inhibitory NK receptor
LAK	lymphocyte activated killer
LMP	latent membrane protein
LOH	loss of heterozygosity
MAGE	melanoma-associated antigen
MAPK	mitogen-activated protein kinase
MART-1	melanoma antigen recognized by T cell-1
MC	medullary carcinoma
MECL-1	multicatalytic endopeptidase complex like-1
MHC	major histocompatibility complex
ml	milliliter
mm	millimeter
MUC	mucin
NCCN	National Comprehensive Cancer Network
NKT	natural killer T
NSABP P1	National Surgical Adjuvant Breast and Bowel project –Phase 1
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR-SSP	polymerase chain reaction- sequence specific primer
PGE_2	prostaglandin E2
PE	phycoeythrin
PerCp	peridinin chlorophyll protein
PI	propidium iodide
PI3K-Akt	phosphaldylinositol 3- kinase/ Akt
PR	progesterone receptor
rIL-	recombinant interleukin
RNAi	interference ribosomal nucleic acid
RPMI 1640	Roswell Park Memorial Institute 1640
TAAs	tumour-associated antigens
TAE	Tris-acetate-EDTA
TAP	transporter associated with antigen processing
TCR	T cell receptor
TGF	transforming growth factor
Th	T helper
TILs	tumour infiltrating lymphocytes
TLR	toll-like receptor
TMC	typical medullary carcinoma
TNFR	tumour necrosis factor receptor
TRAIL	tumour necrosis factor receptor- related apoptosis inducing ligand

Treg	regulatory T lymphocyte
U	international unit
VEGF	vascular endothelial growth factor
VNTR	variable number of tandem repeats