UNIVERSITI PUTRA MALAYSIA

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

LEONG PO01 PO01

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

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

March 2005
EXPRESSION OF TUMOUR-ASSOCIATED ANTIGENS AND CHARACTERISTIC OF T CELL RESPONSES IN BREAST CARCINOMA

By

LEONG POOI POOI

March 2005

Chairman : Professor Seow Heng Fong, PhD
Faculty : Medicine and Health Sciences

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

March 2005

Pengerusi : Profesor Seow Heng Fong, PhD
Fakulti : Perubatan dan Sains Kesihatan

Barah payudara merupakan kanser yang paling umum di kalangan wanita di Malaysia. Kaedah-kaedah perubatan klinikal yang biasa digunakan adalah kimia terapi, 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 spesifik 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 diceraai oleh enzim dan tanda-tanda perbezaan sel limfosit yang spesifik. Dalam tisu kanser payudara yang berparafin (n=49), kajian imunohistokimia memaparkan ekspresi 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 (≥ 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.
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I certify that an Examination Committee met on 30th March 2005 to conduct the final examination of Leong Pooi Pooi on her Master of Science thesis entitled “Expression of Tumour-Associated Antigens and Characteristics of T-Cell Responses in Breast Carcinoma” in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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Date: 14 JUL 2005
DECLARATION

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

LEONG POOI POOI

Date: 9 August 2005
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<td>α</td>
<td>alpha</td>
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<tr>
<td>β</td>
<td>beta</td>
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<tr>
<td>γ</td>
<td>gamma</td>
</tr>
<tr>
<td>δ</td>
<td>delta</td>
</tr>
<tr>
<td>%</td>
<td>percentage</td>
</tr>
<tr>
<td>ºC</td>
<td>Degree of Celsius</td>
</tr>
<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>ACD</td>
<td>acid citrate dextrose</td>
</tr>
<tr>
<td>AICD</td>
<td>activated-induced cell death</td>
</tr>
<tr>
<td>AMC</td>
<td>atypical medullary carcinoma</td>
</tr>
<tr>
<td>APC</td>
<td>alloxycyanin</td>
</tr>
<tr>
<td>APCs</td>
<td>antigen presenting cells</td>
</tr>
<tr>
<td>APES</td>
<td>aminoproxytrimethoxysilane</td>
</tr>
<tr>
<td>BCG</td>
<td>bacilli Calmette-Guerrin</td>
</tr>
<tr>
<td>BCS</td>
<td>breast conservation surgery</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BRAC</td>
<td>breast cancer susceptibility protein</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>Calcein-AM</td>
<td>calcein- acetoxymethyl</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>cm</td>
<td>centimeter</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>COX-2</td>
<td>cyclooxygenase-2</td>
</tr>
<tr>
<td>CTLs</td>
<td>cytotoxic T lymphocytes</td>
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<tr>
<td>Cy-chrome</td>
<td>cyanine-chrome</td>
</tr>
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<td>DAB</td>
<td>diaminobenzidine tetrahydrochloride</td>
</tr>
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<td>DCIS</td>
<td>ductal carcinoma in situ</td>
</tr>
<tr>
<td>DCs</td>
<td>dendritic cells</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTH</td>
<td>delayed type hypersensitivity</td>
</tr>
<tr>
<td>EGFR</td>
<td>epithelial growth factor receptor</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>oestrogen receptor</td>
</tr>
<tr>
<td>E/T</td>
<td>effector/target ratio</td>
</tr>
<tr>
<td>EthD</td>
<td>ethidium bromide homodimer</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associated protein with death domain</td>
</tr>
<tr>
<td>FASL</td>
<td>Fas ligand</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>Fc</td>
<td>forward scatter</td>
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<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<td>FL</td>
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<td>FLICE</td>
<td>FADD homologous Interleukine-1 beta converting enzyme/ Caenorhabditis elegans cell-death protein 3- like protease</td>
</tr>
<tr>
<td>FOXP</td>
<td>Foxhead/ winged-helix</td>
</tr>
<tr>
<td>GITR</td>
<td>glucocorticoid induced tumour necrosis receptor</td>
</tr>
</tbody>
</table>
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
NK T  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
PGE2  prostaglandin E2
PE  phycoeythrin
PerCp  peridinin chlorophyll protein
PI  propidium iodide
PI3K-Akt  phosphathylinositol 3- kinase/ Akt
PR  progesterone receptor
rIL-  recombinant interleukin
RNAi  interference ribosomal nucleic acid
RPMI 1640  Roswell Park Memorial Institute 1640
TAA s  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