行政院國家科學委員會補助專題研究計畫 ■成果報告

利用基因微陣列技術來探究局部進行性乳癌的基因表現與對化學治療反應之相關性

Correlation between gene expression profile and chemotherapy response in locally advance breast cancer using cDNA microarray technique

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(二)中、英文摘要及關鍵詞(keywords)。

中文摘要

關鍵詞：局部進行性乳癌(locally advanced breast cancer), cDNA 微列陣(cDNA microarray), 腫瘤浸潤淋巴球(tumor infiltrating lymphocyte, TIL), T 細胞抗原受體 (T cell antigen receptor, TCR)

近年來由於飲食西化的影響，台灣乳癌的發生率急遽上升，死亡人數更超越子宮頸癌，成為婦癌的頭號殺手，乳癌之積極治療已是當前重要的課題。大部分的乳癌在診斷時被視為全身性疾病已經是醫學界的共識，因此，除了少數由篩檢中發現的早期乳癌之外，輔助性之化學治療在療程中扮演極其重要之角色，它可以有效減少復發的機會及延長存活的時間。根據 St. Gallen 國際乳癌共識會議的建議，超過 **70%的台灣乳癌患者符合接受化學治療的條件**。

化療的選擇，往往根據醫師本身的經驗，未能針對不同病人而有所調整，再加上復發及轉移多在接受治療後的數月或數年後才能發現，如何依據病人乳癌表現上的不同，個別選擇適當的藥物，是當務之急。

對於局部進行性的第三期及第四期乳癌，必需先接受化學治療使腫瘤縮小後再進行外科手術，這類病人提供了絕佳的模式可用來探討乳癌表現與化學治療反應之間的關係。然而，對於接近 30%化療反應不佳的患者，目前仍沒有臨床或分子檢驗能在治療前預測化療的反應。因此，本計劃將分三部分進行：

一. 26 位病人的 RNA 可進行 **cDNA 微列陣**的基因檢測，其中 14 位可同時檢測化療前後的基因表現，13 位接受 Epirubicn 治療的病人，可藉由基因的表現和化療反應之間的關係加以分析整理，可了解那些群組基因與化療反應有關或因化療有所改變，由於基因檢測的時程落後，目前結果仍在分析當中。

二. 我們成功由一位 25 歲的乳癌患者的腫瘤細胞培養成細胞株。生理及基因特性已透徹研究。可做為往後研究極佳的模型。也証實了建立細胞株的可行性。

三. **免疫系統在腫瘤治療上具有關鍵性的角色，在化療過程中，免疫系統的改變及其和腫瘤細胞之間的關係，確有釐清與探究的必要。本研究將化療前後腫瘤浸潤、正常組織、淋巴結及周邊血液的 T 淋巴球予以分離，更進一步依表面抗原 $CD4^+$、$CD8^+$及 DN 加以分類。**分析 T 細胞抗原受體的模組表現(TCR usage)。結果發現在化療後腫瘤浸潤及周邊血液的 T 淋巴球反應皆有明顯增強，但只集中在 $CD4^+$、$CD8^+$，DN 細胞的反應則減弱。正常組織幾乎沒有免疫反應，而 T 淋巴球單株或寡株的增生模式，更証明針對腫瘤的免疫系統有高度，發展以腫瘤浸潤淋巴細胞為基礎的癌症免疫療法的可行性大為提高。

英文摘要

Keywords: Locally advanced breast cancer (LABC), cDNA microarray, , Tumor infiltrating lymphocyte (TIL), T cell receptor(TCR)

Most breast cancer is thought to be **systemic**; thus **adjuvant chemotherapy** is necessary because it can substantially improve the disease-free and overall survival. According to the guideline for **adjuvant therapy of breast cancer** in **St. Gallen International Consensus Panel**, **more than 70% of the breast cancer patients in Taiwan are eligible for adjuvant chemotherapy**.

We know that for **locally advanced breast cancer (LABC, stage III/IV)**, preoperative chemotherapy improves local control and surgical outcome. It provides us with the best **in vivo** cases with which cancer's **responses to chemotherapy can be assessed.** Tracing the genetic differences of cancer cells before and after chemotherapy, we can locate the genes that may be involved in the tumor's chemosensitivity.

Our project including three parts: (1) change in gene expression of advanced breast cancer before and after chemotherapy; (2) establishment of breast cancer cell-line; (3) change of immune response after chemotherapy in breast cancer.

RNA extract from 26 of 36 locally advanced breast cancer(LABC) patients of patients are qualified for microarray analysis. 14 patients have paired pre- and post- chemotherapy sample for analysis of change in expression pattern induced by chemotherapy. Gene expression signature that related to chemotherapy response will be analysed in Epirubicin-subset of 13 patients. The analysis is on going because delay of the *core facility.* The comprehensive result is not available at the time this annual report is submitted.

One stable cell line from an 25 y/o breast cancer patient before chemotherapy has been well-characterized.

Summary of T-cell immune reaction during neoadjuvant chemotherapy, we find T-Lmphocyte reaction *increases* in tumor matrix and peripheral blood after chemotherapy. Increased response is found in *CD4+, CD8+ cells, but not DN cells. Monoclonal or at least oligoclonal expansion* of TILs to specific tumor antigen exists. Lymphocyte reaction *markedly decreases in normal breast.*

目錄

(三)報告內容：

前言

Keywords: Locally advanced breast cancer (LABC), cDNA microarray, , Tumor infiltrating lymphocyte (TIL), T cell receptor(TCR)

Most breast cancer is thought to be **systemic**; thus **adjuvant chemotherapy** is necessary because it can substantially improve the disease-free and overall survival. According to the guideline for **adjuvant therapy of breast cancer** in **St. Gallen International Consensus Panel, more than 70% of the breast cancer patients in Taiwan are eligible for adjuvant chemotherapy**.

Traditionally, **choice of chemotherapy is often empirical** based more on the histological appearance of the tumor than on an understanding of drug-resistant phenotypes. The former, which relies heavily on **physician's subjective experience**. On the other hand, the technique of **cDNA microarrays**, which provides expression profile of thousands of genes, makes it possible to give each cancer a distinct identification. Using this method, in **vitro** studies of **breast cancer cell line** have revealed that **different genetic expression patterns of tumor show different chemosensitivity;** besides**, chemotherapy can induce differences in expression pattern.** Although these researches bridge the gap between gene expression pattern and chemotherapy sensitivity, they, unfortunately, **fail to explain breast cancers' in vivo behavior.**

This project, therefore, attempts to investigate breast cancers' **in vivo** behavior through its genetic expressions. We know that for **locally advanced breast cancer (LABC, stage III/IV)**, preoperative chemotherapy improves local control and surgical outcome. It provides us with the best **in vivo** cases with which cancer's **responses to chemotherapy can be assessed.** Tracing the genetic differences of cancer cells before and after chemotherapy, we can locate the genes that may be involved in the tumor's chemosensitivity. Besides, it is known that **20~30% of advanced cancers have no response or respond poorly to chemotherapy.** By far, no clinical assessment or molecular test can predict response before treatment. This model can also serve as an excellent start to solve this mystery.

研究目的

In this study, with **cDNA microarray** technique, we will access gene expression profiles of tissue specimens of **locally advanced breast cancers(stage III/IV) before and after chemotherapy**. Serial sonography provides clinical assessment of tumor response. Frozen breast cancer tissue and its normal counter part in our tissue bank will also be evaluated by **cDNA microarry** as control. **Cell line** will be established simultaneously. All results achieved have to be confirmed by cell line experiments.

T Lymphocytes from tumor, its normal counterpart, metastatic lymph nodes and peripheral blood, will be isolated and further separated into $CD4^+$, $CD8^+$ and DN cells subsets. Their ***TCR usage*** before and after chemotherapy will be studied. The existence and abundance of T-cells with the ***invariant T cell receptor (TCR) α chain*** in relation to chemotherapy will also be investigated.

Specific aims and long-term objectives are as follows:

Specific aim(1). **Detection of differentially expressed genes in responder and non-responder.**

Specific aim(2). **Identification of novel targets(genetic markers) that affect chemosensitivity.**

Specific aim(3). **Detection of change in expression pattern induced by chemotherapy.**

Specific aim(4). **To elucidate TIL-tumor interactions based on their *TCR repertoire*;**

Specific aim(5). **An expectation to find a *distinct TCR repertoire specific to tumor antigen*.**

Long-term objectives:

1. **Development of cDNA chips for clinical diagnosis of breast cancer to choose proper chemotherapy agent and to early detect breast cancer.**
2. Our present study will produce the following substantial results concerning breast cancer: (1) **pathophysiological information**; (2)**diagnostic model establishment**; (3) **novel molecular targets.**
3. Development of **TIL-based immunotherapy** to **individualize** treatment in breast cancer patients.

文獻探討

Keywords: Locally advanced breast cancer (LABC), cDNA microarray, Tumor infiltrating lymphocyte (TIL), T cell receptor(TCR)

In Taiwan, breast is **the second popular site of female cancer** and is expected to be the first in the near future[1]. Because the screening program is not well-established, breast cancers were usually diagnosed in later stages. **Adjuvant chemotherapy** is necessary because it can substantially improve the disease-free and overall survival. According to the guideline for adjuvant therapy of breast cancer in **St. Gallen International Consensus Panel, more than 70% of the breast cancer patients in Taiwan are eligible for adjuvant chemotherapy**[2].

Traditionally, **choice of chemotherapy is often empirical** based more on the histological appearance of the tumor than on an understanding of drug-resistant phenotypes. The former relies heavily on **physician's subjective experience**. Because relapsed and metastatic cancers could only be detected in months or years after surgery, how to choose proper adjuvant chemotherapy agent is crucial in primary treatment. Yet, drug resistance is a major obstacle to successful therapy. Cancer cells exposed to anti-tumor drugs may directly induce the expression of genes that could confer resistance, thus allowing some cells to escape killing and form the relapsed resistant tumor. Alternatively, some cancer cells may express an array of genes, that could confer intrinsic resistance, and the exposure to cytotoxic drugs will screen out surviving cells that form the relapsed tumor.

Cancers are either primarily resistant to chemotherapy (intrinsic resistance), or respond to chemotherapy but later recur to form a multi-drug resistant tumor[3]. Several mechanisms of drug resistance in tumors are understood and include over-expression of the multi-drug resistance gene (MDR1)[4], the multi-drug resistance-associated protein[5], and increased DNA repair[6]. Various regulatory genes in the cell targeted for genetic alterations during tumor genesis may also influence cellular sensitivity to chemotherapeutic drug[7]. These genetic alterations involve a diverse group of gene products that include tumor suppressor genes, oncogenes, cell cycle regulators, transcription factors, growth factor receptors, DNA repair factors, and cell death regulators. Mechanisms of development of intrinsic drug resistance are not thoroughly understood and may involve the expression of multiple genes during tumor

progression. Therefore, a single mechanism pathway cannot explain the genesis of resistance in cancer. Rather, drug resistance seems to involve the altered expression of a diverse group of genetic factors influencing various biochemical pathways. The emergency of acquired resistance, on the other hand, may be associated with either drug induction or drug selection of tumor cells during chemotherapy, resulting in relapses that are refractory to treatment.

DNA microarray

The advent of **DNA microarray** technology and its capacity for simultaneous probing of the genome on high-density microarrays in yeast and man has enabled the analysis of the expression profiles of thousands of genes. In view of the complex array of genetic factors contributing to drug resistance, DNA microarray should be useful for examining the development of drug resistance in cancer[8-19]. These analyses ultimately may enable us to use the signature expression profiles of drug-resistant tumors to predict response to drugs and to design therapeutic regimens to circumvent drug resistance. **In vitro** study with **breast cancer cell line** and **cDNA array technique**, **different expression pattern showed different chemosensitivity** and **chemotherapy can induce difference in expression pattern**[20, 21]. Scherf and Ross combined the expression and drug response data with a view to determining whether correlations exist between gene expression and drug response. The fact that apparently significant clusters were obtained encourages the hope that the process has produced more than random noise as does the fact that a few of the correlations are consistent with known drug response. But, the proportion of these correlations that will prove productive leads unknown[20, 21]. By far, **no in vivo study** provided us direct evidence that correlated gene expression profile and chemotherapy.

Locally advanced breast cancer as in vivo model

For **locally advanced breast cancer (stage III/IV)**, pre-operative chemotherapy is applied in the primary treatment course[22]. Primary medical therapy in LABCs cancers improves local control and surgical outcome, offering a better cosmetic result as well as longer patient survival. This provides a perfect model to access chemotherapy response in vivo. Response to medical therapy varies widely. In

our unpublished data and previous reports, **70%~80% tumors shrink, a few disappear completely. The other 20~30% cases respond minimally or continue to progress**. This unpredictability makes the choice of the appropriate treatment difficult and so, to a large extent, selection of the initial regimen is arbitrary and may need to be changed or abandoned. Considering the limitations of conventional methods. i.e. clinical examination, mammography and B-mode ultrasound, in assessing tumor response to therapy, linking gene expression profile and drug response in advanced breast cancer may provide information that predict the response of breast cancer to therapy before treatment.

Cell lines

Cell lines derived from human tumors have been extensively used as models in the study of neoplastic diseases. Although such cell lines differ from both normal and cancerous tissues, however, the inaccessibility of human tumors and normal tissues make such cell lines still play an important role as an experimental model. For study the chemosensitivity before and after chemotherapy, we propose to establish breast cancer cell lines from early (stage I/II) and late (stage III/IV) breast cancer patients from our hospital as well as collect established breast cancer cell lines from ATCC to serve as comparison. In addition to the characterization (ex. estrogen receptor, progesterone receptor, p53, HER2/neu expression level; ploidy and cytogenetic analysis; histological and biochemical analysis...) of newly established breast cancer cell lines from patients, the gene expression profiles of different stages of breast cancer cell lines will also be analyzed using the same DNA microarray technique. Therefore, we can establish an extensive breast cancer cell bank and the related genetic expression profiles of breast cancer in Taiwan. Using this ***in vitro*** **cell model** and **DNA microarray technique**, the **correlation of known gene markers will be documented and the novel gene markers will also be characterized**. All of the breast cancer cell lines will be clustered on the basis of difference of gene expression and drug response before and after chemotherapy, to elucidate the possible correlation of gene-drug of the cell lines derived from 20-30% of chemotherapy resistant patients. The comparison results will provide useful pretreatment information for the decision of proper regimen.

Tumor infiltrating lymphocyte(TILs)

Lymphoid infiltration in tumor tissues has been demonstrated a **favorable sign for prognosis** of hosts in several malignant tumors[27-31]. Therefore, lymphocytes' infiltration is considered a result of **tumor targeted, specific interactions** rather than of an inflammatory response. Most of the infiltrating cells are **$CD3^+$ T cells** with a variable number of **$CD4^+$** and **$CD8^+$**. In most of the tumors, **no B cells** are found and natural killer cells constitute only a small minority of TIL[32-36].

In human breast cancer , there was a significant **reverse correlation between the intensity of the T-cell infiltration and the clinical stages**. In general, lymphocytes are found more frequently and more abundantly in cancer than in its normal counterparts. Furthermore, it is observed an increased **$CD4^+/CD8^+$ ratio** correlated with **tumor's size** and **lymph node metastases** [37]. Studies in experimental animals have shown that the adoptive transfer of **TIL is 50-100 times competent than LAK cell** in mediating tumor regression[38]. Thus, TIL is a potentially promising candidate for **adoptive immunotherapy**. TIL from primary breast carcinomas can be propagated in large numbers in vitro with rIL2 while still retaining autologous tumor specificity and MHC-restricted CTL activity [39].

We know that T cells play a critical role in human's immune defense as to recognize and eliminate malignant cells, and the highly specific **T cell antigen receptor (TCR)** is responsible for making distinction of them. Proteins of the TCR arise from gene segment rearrangements that provide the opportunity for virtually unlimited diversity[40-42]. Most of the work that has defined the T-cell response to tumors has come from analysis of cultured TIL. Tumor specific immune reaction has long been postulated, however, no study provided direct ***in vivo*** evidence so far. Analyses of **TCR usage** subclassify T-cells according to different V segment expression. By this method, even single T-cell can be characterized.

Initial results of TCR usage analyses come from studies of autoimmune diseases. In patients with autoimmune antiphospholipid syndrome, the TCR V_β segments of autoreactive $CD4^+$ T cells expanded oligoclonally after stimulation with β_2-glycoprotein I[43]. In nonobese diabetic mice (NOD), the analysis of the TCR repertoire of early islet infiltrates reveals enrichment for a small subset of TCR sequences. Reconstitution of these TCR in vitro demonstrates that these receptors confer reactivity to islet cells[44].

Different from traditional T lymphocytes that display a wide repertoire of antigen receptors, a new subset of T cells were found in humans, mice, and cattle. These cells bear an **invariant T cell receptor (TCR) α chain** containing hAV7S2 and AJ33 in humans and the homologous AV19-AJ33 in mice and cattle with a CDR3 of constant length. These T cells are $CD4^-$ $CD8^-$ double-negative (DN) T cells in the three species and also CD8αα in humans. In humans, their frequency was ~1/10 in DN, 1/50 in CD8α+, and 1/6,000 in $CD4^+$ lymphocytes. They preferentially use hBV2S1 and hBV13 segments and have an oligoclonal V_β repertoire and were probably selected for a nonclassical MHC class Ib molecule distinct from CD1. The conservation between mammalian species, the abundance, and the unique selection pattern, suggest an important role for cells using this novel canonical TCR α chain[45]. Recently, we identified a TCR α sequence that is abundant in our samples from healthy individuals but scarce, if any, in our samples from autoimmune patients. This sequence turned out to be identical to the above published second invariant sequence in humans.

Restricted repertoires seem to define discrete lymphocyte subpopulations at the frontier between innate and adaptive immunity. Cells with such a restricted repertoire may play an immunoregulatory role or another physiologic function. Their role in tumor progression is surely an interesting topic to investigate[46, 47].

Although a number of previous studies have addressed *in vitro* interaction between TIL and tumor, no report provided direct *in vivo* evidence of tumor specific immune response. Thus, in this part of the project we will study TCR usage of LABCs before and after chemotherapy ***in vivo***. Besides, the existence and abundance of T-cells with the invariant T cell receptor (TCR) α chain will also be investigated. We hope to find the meanings/correlations between TCR usage and chemotherapy response. The search for the potential role of T cells using the invariant T cell receptor (TCR) α chain in relation to chemotherapy response will help understanding the interaction between tumor, chemotherapy and T cell immunology. With our TCR usage information, to develop a **TIL-based immunotherapy** with which an individualized treatment can be achieved would be one of our final goals.

研究方法

Correlation between expression profile and chemotherapy response in locally advanced breast cancer patients using cDNA microarray

Using **cDNA microarray**, we plan to access gene expression profiles of tissue specimens of forty **LABCs before and after chemotherapy**. **Serial sonography will be used as a tool for monitoring tumor's responses**. Frozen breast cancer tissue(stage I/II) and its surrounding normal tissue in our tissue bank will also be evaluated by cDNA microarry as reference. The ***correlation between expression profile and drug response*** will pick up genes that affect chemosensitivity. The comparison of expression profile before and after chemotherapy will ***detect changes in the expression patterns induced by chemotherapy***. The difference between early(stage I/II) and late(stage III/IV) breast cancer can find ***genetic changes during tumor progression***.

Cell line validation

We propose to establish breast cancer ***cell lines*** from the **LABC before and after chemotherapy**. In addition to the characterization (ex. estrogen receptor, progesterone receptor, p53, HER2/neu expression level; ploidy and cytogenetic analysis; histological and biochemical analysis...) of newly established breast cancer cell lines from patients, in order to avoid experimental biases, **all results achieved from first year study have to be confirmed by cell line experiments**.

Changes of the Tumor infiltrating lymphocyte (TIL) during chemotherapy and its relation to chemotherapy response

We plan to elucidate **TIL-tumor interaction and chemotherapy influence** on this issue. T Lymphocytes from tumor, its normal counterpart, metastatic lymph nodes and peripheral blood of **LABC patients before and after chemotherapy** will be isolated and further sorted into ***$CD4^+$, $CD8^+$ and DN T cell subpopulations*** by **immunoaffinity** fractionation. In each subset of T-cells, we will establish a **cDNA library** from extracted RNA. Upon this library, the **TCR usage** of different V_α segments in each subset will be analyzed and compared. The V_α segments evaluated will include $V_\alpha 1$, $V_\alpha 14$,$V_\alpha 15$, HFR, $V_\delta 1$, $V_\delta 2$, $V_\delta 3$, $V_\delta 4$, $V_\delta 5$.

結果與討論(含結論與建議)

Our project includes three parts: (1) investigation on changes in breast cancer's gene expressions before and after chemotherapy; (2) establishment of pre- and post-treatment breast cancer cell-lines; (3) investigation on the immune responses of breast cancer to chemotherapy. These subprojects were introduced as they were performed separately year by year; however, in reality they were executed around the same period because of their interrelated nature. In the following we will briefly describe their progress and the results found accordingly.

(1) ***Changes in breast cancer's gene expressions before and after chemotherapy***

We have collected samples from 36 locally advanced breast cancer patients. RNA extract of 26 patients are qualified for microarray analysis. 14 of 26 patients have paired pre- and post- chemotherapy sample for analysis.

Among these twenty-six patients, thirteen patients received neoadjuvant chemotherapy of Epirubicin, seven of Taxotere, Five of Phyxol and one of Navorelbin. Because the comparison among patients with different chemotherapy agent is difficult, we chose the Epirubicin subset for analysis. This subset includes five responder and eight non-responders. This analysis will help us to probe gene expression signature that related to Epirubicin-chemosensitivity.

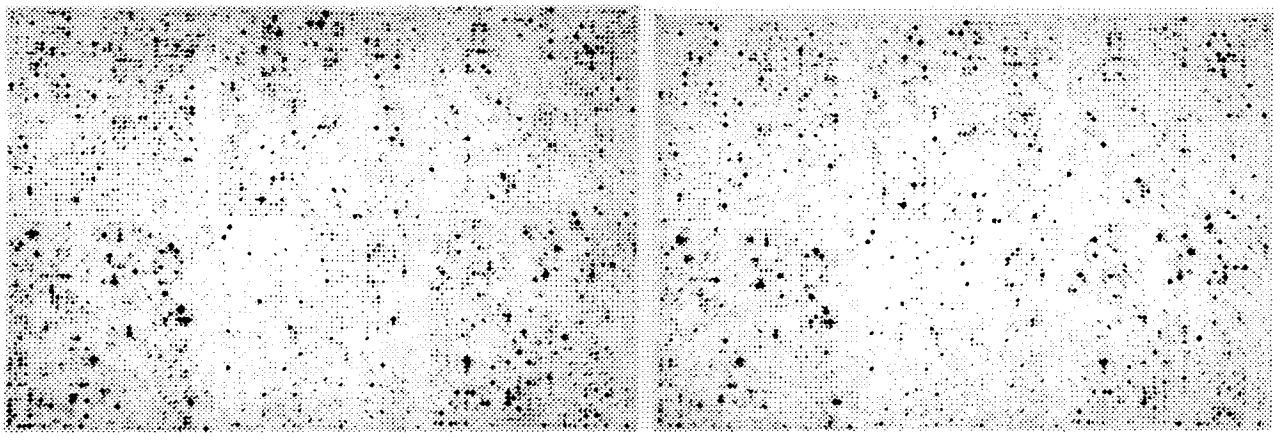
Chemotherapy agents of fourteen patients paired samples includes nine of Epirubicin, three of Taxotere and two of Phyxol. Change in expression pattern induced by chemotherapy in general and in differrent agents will be detected.

The microarray assessment did not finish until early November because the delay of the *core facility*. The analysis is on going. The comprehensive result is not available at the time this annual report is submitted. We are sorry about that. After the analysis is complete, we will revise the data as soon as possible.

Preliminary analysis of microarray data of one pair sample shows that gene expression pattern in breast cancer before and after chemotherapy is comparable (Fig. 1). The left is gene expression profile of pre-chemotherapy sample on a 9600 spots gene-chip. The right is after chemotherapy sample. The graph on the left side is the density plot of microarray data after normalization. As you see, the distribution

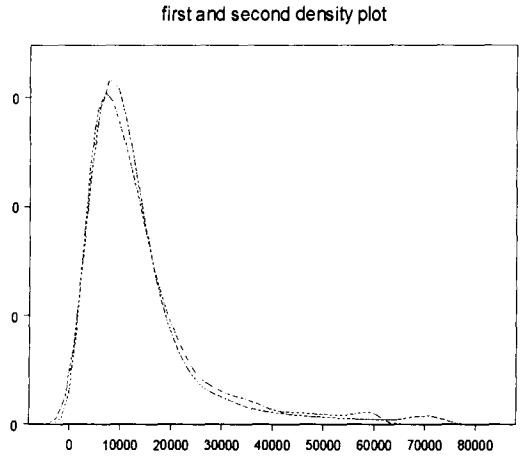
curves of these two samples are alike. The difference plot on the right side shows us the distribution of magnitude and frequency of differences in each gene. The expression of most genes is similar in two samples. Only a few genes markedly downregulate or upregulate after chemotherapy.

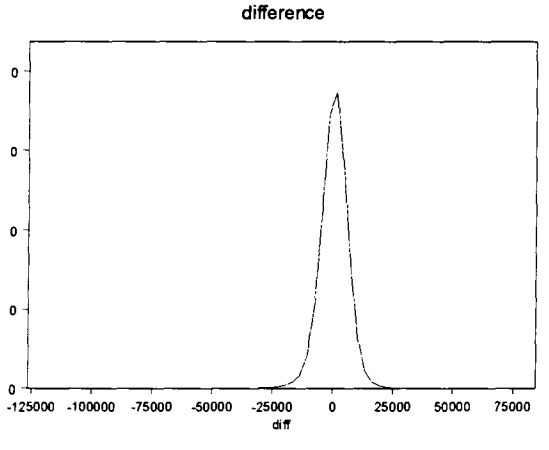
Fig. 1 Data of microarray assessment in one paired samples



before chemotherapy

after chemotherapy





The genes listed below are those noticeably downregulated or upregulated after chemotherapy, the chance is less than 0.1 percentile.

Down-regulate

Human B-cell receptor associated protein (hBAP)

mRNA, partial cds

EST

guanylate cyclase activator 1A (retina)

UDP-glucose dehydrogenase

Homo sapiens mRNA for KIAA0802 protein,

partial cds

KIAA0104 gene product

CD4 receptor {exons 1 and 2} [human,

T-lymphocyte, mRNA, 3429 nt]

Homo sapiens fetal unknown mRNA, complete

cds

Homo sapiens mRNA; cDNA DKFZp586H201

(from clone DKFZp586H201)

antigen identified by monoclonal antibodies 12E7,

F21 and O13

Homo sapiens DNA sequence from PAC 845O24

on chromosome 1p36.1-36.2. Contains a gene for

a Heterogenous Nuclear Ribonucl

KIAA0705 gene product

RBCL

Homo sapiens Arp2/3 protein complex subunit

p34-Arc (ARC34)

Homo sapiens mRNA for C2 domain containing

PI3-kinase

Human Ig J chain gene

nitric oxide synthase 2A (inducible, hepatocytes)

ESTs, Highly similar to COATOMER ZETA

SUBUNIT [Bos taurus]

angiotensin receptor-like 2

Homo sapiens clone 23555 mRNA sequence

B lymphoid tyrosine kinase

Janus kinase 1 (a protein tyrosine kinase)

Up-regulate

Homo sapiens chromosome 9, P1 clone 11659	cytochrome P450, subfamily IIA
heat shock transcription factor 1	(phenobarbital-inducible), polypeptide 7
Homo sapiens mRNA for kinesin-like DNA	Human 1-8D gene from interferon-inducible gene
binding protein, complete cds	family
Human clone 137308 mRNA, partial cds	ESTs, Highly similar to CAMP-DEPENDENT
SCO1, S. cerevisiae, homolog of 2	PROTEIN KINASE INHIB
GAPDH	MAP/ERK kinase kinase 1
myxovirus (influenza) resistance 1, homolog of	unc-51 (C. elegans)-like kinase 1
murine (interferon-inducible protein p78)	ASA1
vav 3 oncogene	GAPDH
	RBCL

(2) ***Establishment of pre- and post-chemotherapy breast cancer cell-lines***

We have developed a stable cell line from a 25-year-old patient before chemotherapy, and its characteristics has been well studied (for details, see Fig. 2). The stable cell lines collected from pre-chemotherapy cases of early-onset breast cancer has been well-characterized as follows: sixty-one passages, no tumorgenicity in nude mice was found; yet tests for estrogen and progesterone receptor showed positive. Karyotype analysis reveals us a normal chromosome number of forty six (Fig. 3), and the growth curve for it is shown in Figure 4. The test for BRCA1 & 2 mutations is in process; more data and discussions will be expected for future publications.

Figure 2. Cell line from a pre-chemotherapy 25y/o breast cancer patient(Phase contrast micrograph)

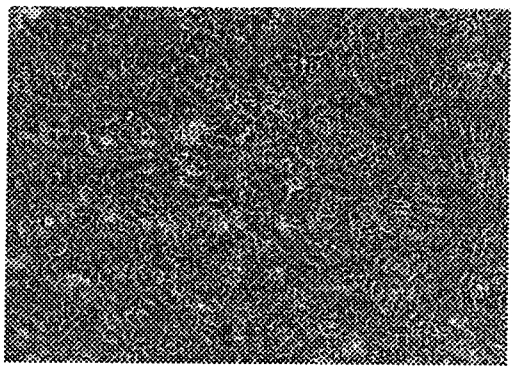


Figure 3. Frequency distribution of chromosomes of pre-chemotherapy early-onset breast cancer cell line

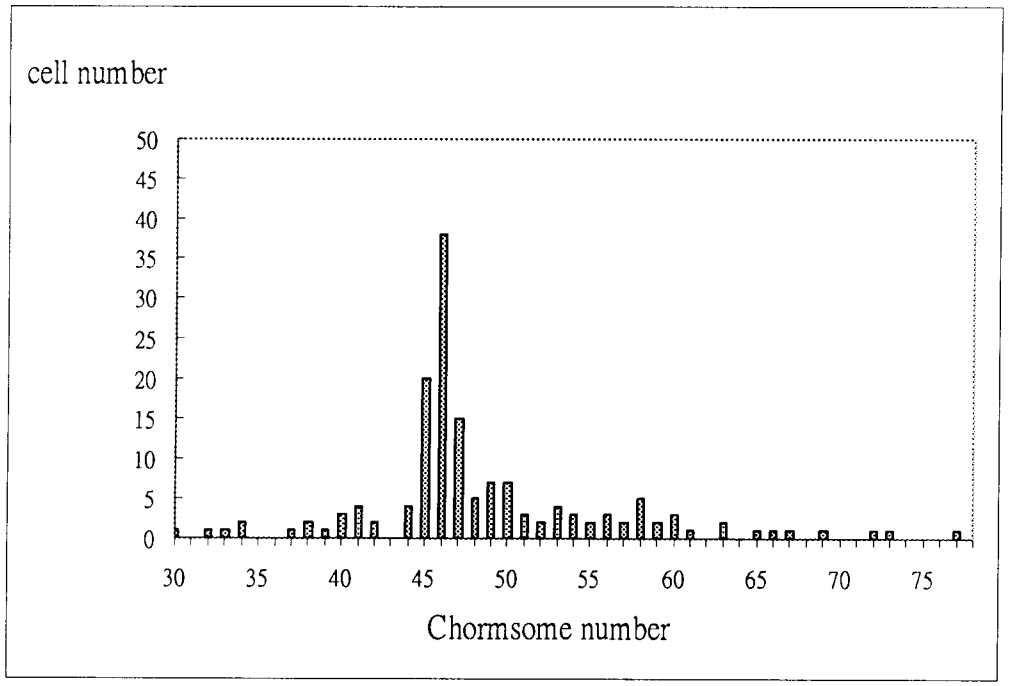
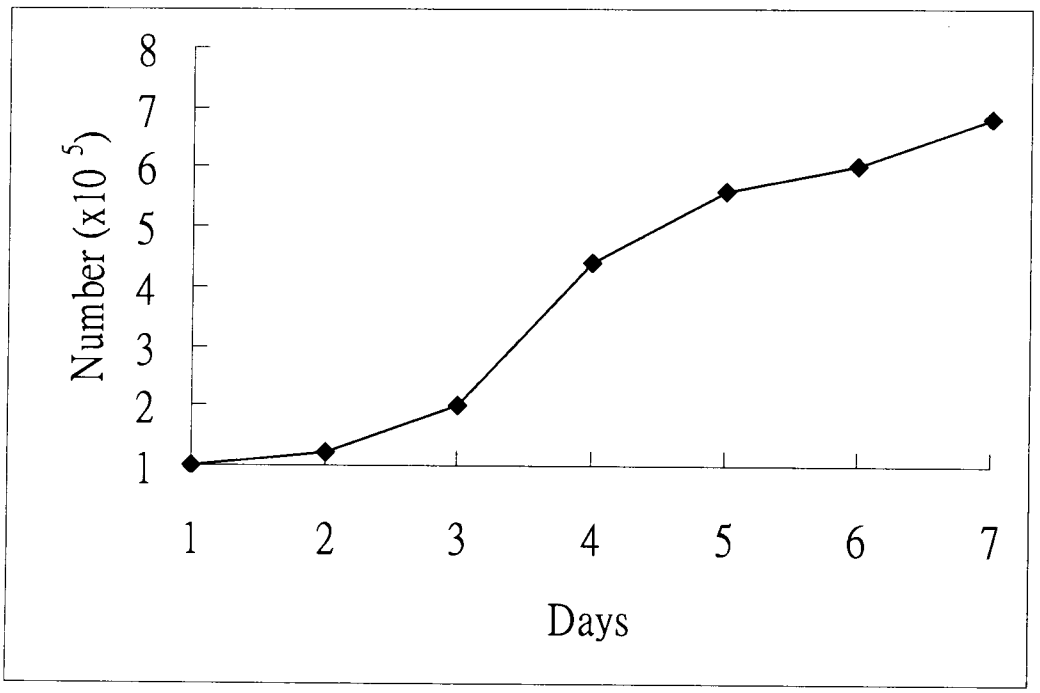


Figure 4. The growth rate of pre-chemotherapy early-onset breast cancer cell line



(3) ***Investigation on the immune responses of breast cancer to chemotherapy***

Our preliminary data concerning **T-cell receptor (TCR) usage** in one advanced breast cancer patients in response to chemotherapy shows exciting findings, which we will introduce here. First we evaluate nine segments of $V_{\alpha/\delta}$ genes that were shown being frequently used by patients with autoimmune disease. We reasoned that these Vs are suitable for TIL studies because to a large degree cancer cell-killing can be regarded also as an autoimmune reaction(see examples in the TCR usage analysis chart). Then the lymphocytes in TIL and peripheral blood were further subclassified into $CD4^+$, $CD8^+$ and Double Negative(DN, $CD4^-CD8^-$) groups.

Before chemotherapy, 1, 4, 3 tested V-segments are seen in $CD4^+$, $CD8^+$, DN cells. The $V_\delta 1$ segment is presented in DN group. It is predictable since $CD8^+$ and DN T-lymphocyte are related to cancer-killing. In peripheral blood lymphocyte (PBL), the presentation of V-segment usage is similar to other breast cancers we have studied previously. After chemotherapy, in TIL, the V-segments presentation remarkably increased in $CD4^+$, $CD8^+$ groups—especially in $CD8^+$ group. The presentation in DN T-lymphocyte, on the other hand, decreased. PBL showed increase in detectable TCR usage after chemotherapy. Even at 5 months after operation, the trend remains the same. Coincidently, there is no detectable presentation in Double negative group(Fig. 5). Whether the decrease in DN group presentation is related to her poor response needs investigation(Table 1). We can't harvest normal tissue before chemotherapy for comparison. However, expression of normal tissue after chemotherapy is much less. It refers that increase in TILs is more than random noise as does the fact that is ***consistent with tumor specific immunity***.

Our next step is to elucidate the possibility of monoclonal expansion in subsets of T-lymphocyte in specific V segment. Sequencing gel can separate PCR products up to a single base pair in difference(Fig. 6). The difference between peripheral blood and TIL is obvious. It is discrete in peripheral blood and concentrated in TILs. Figure 7 is a transformed graph from another V segment. The TILs shows possible monoclonal expansion and the lymphocyte in peripheral blood presents discrete distribution. The last, PCR fragment sequencing showed the expansion of T lymphocyte in tumor is

selected, not random.

In conclusion, T-Lmphocyte reaction *increases* in tumor matrix and peripheral blood after chemotherapy. Increased response is found in *CD4+, CD8+ cells, but not DN cells. Monoclonal or at least oligoclonal expansion* of TILs to specific tumor antigen exists. Lymphocyte reaction *markedly decreases in normal breast tissue.*

TCR usage analysis chart

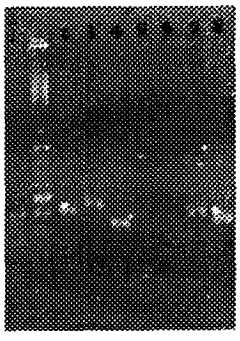
P. #73		Jα -Cα	Jδ -Cδ
Vα 14	PBL	37(-),40,48(5),54,57(-)	1(6,1)
	TIL	15,31(2-),40(-,-),44,52,54(-)	1(1,9)
	T	31,54.	Jδ1(1,3),3(1)
	T+IL2	44(No V),31,48,49	
Vα 15	PBL	4,5(2-,-),15(2),20,30	
	TIL	4(2),9,16(2),22,33,47	
	T		
	T+IL2	10,13(2),29(-),30,31,32,34(-)	
HFR	PBL	13(-),17,22(-),31(2),47,49(1,1),52(-),57	1(1-,1-)
	TIL	10,18,21(2),28,30(1,1),36(-),52	**1(10)(=autoimmune patient')**
	T	15(-),26,34,49	1
	T+IL2	21,24,43,56	
Vδ1	PBL	35,37,40(5),49	1(1,1-),3(4-),4(3-)
	TIL	23,27,32,36,48,49,53	1(11,1,1,1,1-,1-,1-),2(-)
	T	32,40(2=PBL),41,44(-),52	
	T+IL2		1(1,2+,1,1),3(3)
Vδ2	PBL	22(10),23(3)	1(1,1,1,1-),2(1),3(1)
	TIL	15(15-)	1(4,1,1,2,1,1-)
	T	15(10)	
	T+IL2	52(2),54(2),58	1(1,1,1,1,1,1,1-),3(1)
Vδ3	PBL	47(11),48(3)	1(1,1,3-),3(3-)
	TIL	58(13)	1(1,1,1,1,1-,1-,1-),3(2)
	T		
	T+IL2	28(6),53(4),57(3),58(-)	1(10-)
Vδ4	PBL	20,22(2-),23,42(3),49,50(3)	1(3,1),2(5),3(1)
	TIL	10(2),13(5)	1(1,1-),2(3),3(1,1)
	T		
	T+IL2	9(5),15,38,40	1(1,1,1),2(3)
Vδ5	PBL	49(4,3),	1(1,1,1,1,1,1),3(5)
	TIL	30,34,39(-)	1(3,2,1,1-,2-)
	T		1(1,1,1,1,1-),2(1),4(1)
	T+IL2	43,49,50,52,54	

Figure 5. TCR(T-cell receptor) usage before and after chemotherapy

Peripheral Blood Lymphocyte

Before C/T

unsorted

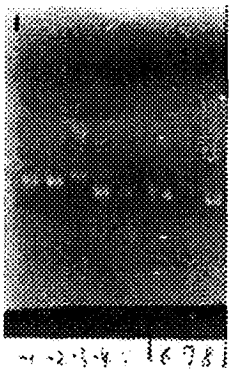


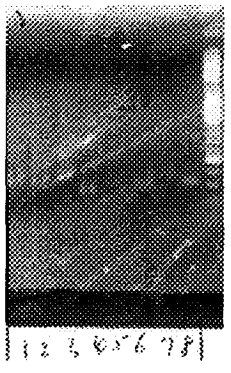
After C/T

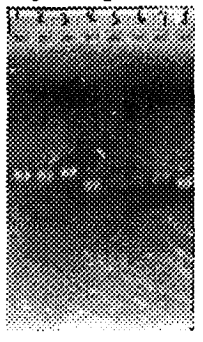
Unsorted

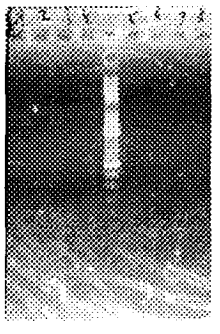
Before completion of C/T

5 months after operation









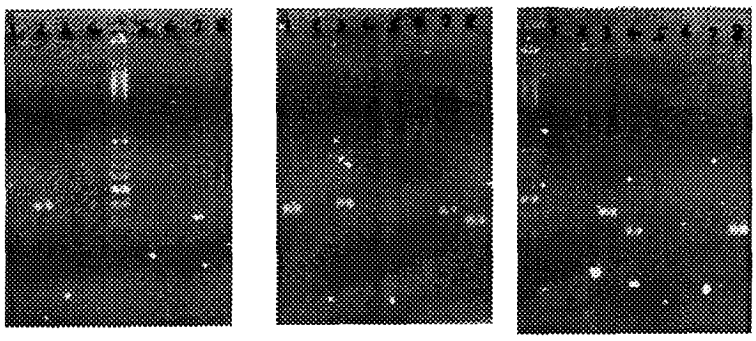
$CD4^+$ $CD8^+$ DN

1	2	3	4	5	6	7	8
$V_\alpha 14$	$V_\alpha 15$	HFR	$V_\delta 1$	$V_\delta 2$	$V_\delta 3$	$V_\delta 4$	$V_\delta 5$

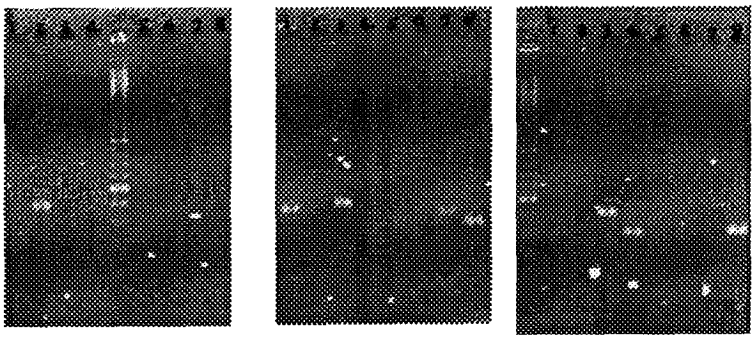
Tumor

CD4$^+$ CD8$^+$ DN

Before C/T



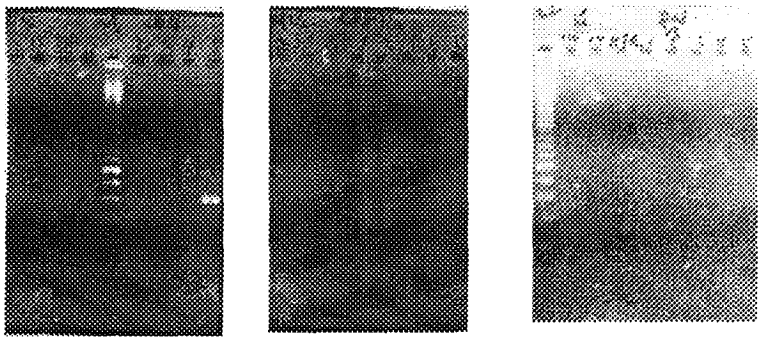
After C/T



Normal

CD4$^+$ CD8$^+$ DN

After C/T

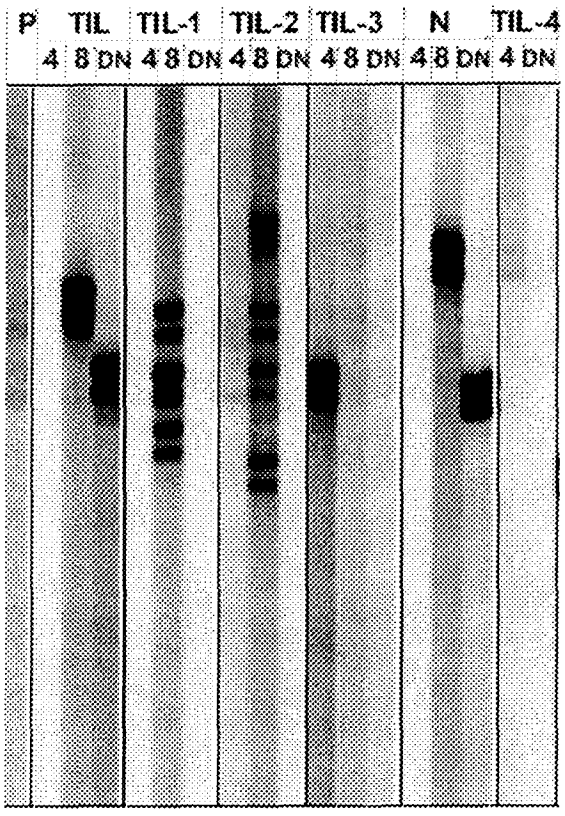


1	2	3	4	5	6	7	8
$V_\alpha 14$	$V_\alpha 15$	HFR	$V_\delta 1$	$V_\delta 2$	$V_\delta 3$	$V_\delta 4$	$V_\delta 5$

Table 1. TCR(T-cell receptor) Usage before and after chemotherapy

Cell origin		$V_\alpha14$	$V_\alpha15$	HFR	$V_\delta1$	$V_\delta2$	$V_\delta3$	$V_\delta4$	$V_\delta5$	$V_\alpha1$
Pre-chemotherapy										
Tumor	$CD4^+$	-	●	-	-	-	-	-	-	-
	$CD8^+$	●	-	●	-	-	-	●	●	●●
	DN	-	-	●	●	-	-	-	●	●
PBL		●	●	●	●	-	-	●	●	●
Post-chemotherapy										
Tumor-1	$CD4^+$	●	-	●	●	-	-	●	●	●
	$CD8^+$	●	●	●	●	-	-	●	●	●●
	DN	-	-	-	-	-	-	-	●	-
Tumor-2	$CD4^+$	●	●	●	●	-	●●	●	●●	●
	$CD8^+$	●●	-	●	-	-	-	●	●●	●
	DN	-	-	-	-	-	-	-	-	-
Tumor-3	$CD4^+$	-	-	●	-	-	-	●	●●	●●
	$CD8^+$	●	●	●	●	-	-	●	●	●
	DN	-	-	-	-	-	-	-	-	-
PBL		●	●	●	●	●	●	●	●	
PBL-5M	$CD4^+$	●	●	●	●	●	●	●	●	
	$CD8^+$	●	●	●	●	-	-	●	●	
	DN	-	-	-	-	-	-	-	-	
Normal	$CD4^+$	-	-	-	-	-	-	-	●●	-
	$CD8^+$	-	-	-	-	-	-	-	-	●●
	DN	-	-	-	-	-	-	-	-	●

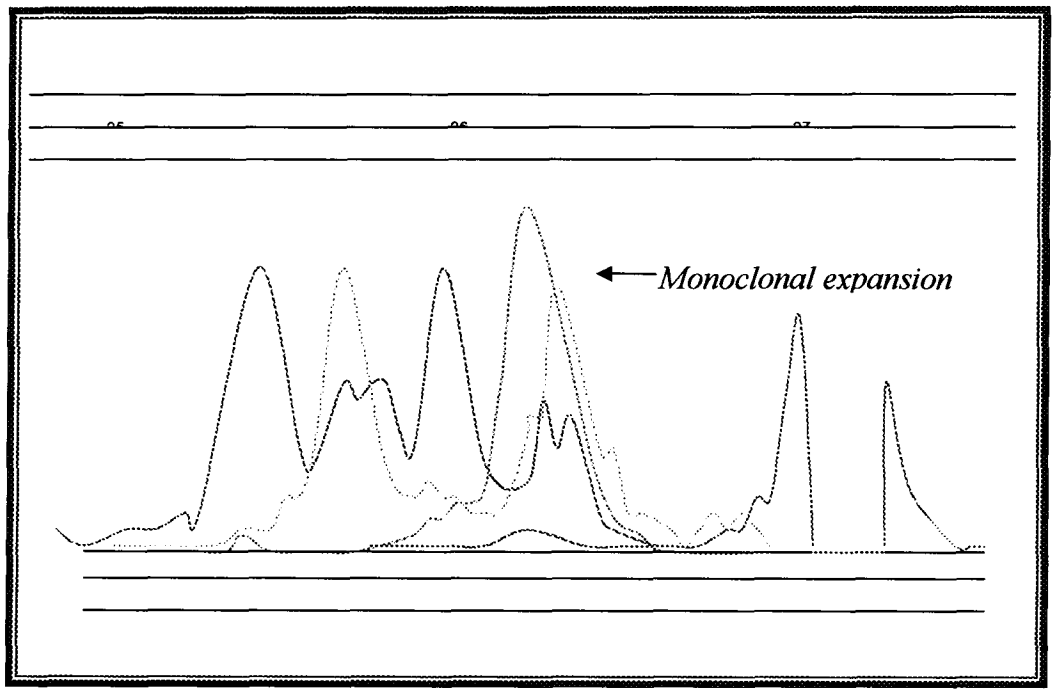
Figure. 6 TCR(T-cell receptor) Usage Sequencing gel



P: Peripheral blood
N: Normal

PBL, Pre-C/T, Mixed
TIL, Pre-C/T, $CD4^+$
TIL, Post-C/T, $CD4^+$
TIL, Post-C/T, $CD8^+$

Figure 7. Evidence of monoclonal expansion in T-cell usage



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(四)計畫成果自評

1. The study results fulfill the specific aims set and pave the way towards the long-term objectives.
2. The results of analyzing change in expression pattern induced by chemotherapy, detecting gene expression signature that related to chemotherapy response, established breast cancer cell line and unveiling the change of T-Lmphocyte reaction during chemotherapy are all suitable to submit for publication .