行政院國家科學委員會專題研究計畫　成果報告

利用 cDNA microarray 分析急性白血病病患的基因表現

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計畫主持人：陳耀昌
共同主持人：唐季祿，姚明

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中文摘要

關鍵詞：急性骨髓性白血病，急性淋巴性白血病，cDNA 微陣列

背景: 急性白血病包含許多不同亞型，表現出不同的形態特徵、細胞表面抗原、細胞染色體異常，臨床表現、治療反應、與預後也有不同。以往我們對其分子機轉所知甚少，近年來 cDNA 微陣列及相關技術的研發，使得大量分析細胞基因轉譯表現型變為可行。

研究目的：

(1). 建立利用 cDNA 微陣列研究急性白血病的模式。

(2). 建立急性白血病各不同亞型初診斷及復發時之基因轉譯表現型模式，以尋找篩選出導致疾病惡化與抗藥性之候選基因。

研究方法：

(1)選擇三組病患: 急性骨髓性白血病併 t(7;11)，急性骨髓性白血病併 inv(16)，及急性淋巴性白血病併 t(9;22)，並收集初診斷及復發時的骨髓細胞。

(2)萃取骨髓細胞 RNA，並進行反轉錄在反轉錄過程中嵌入放射性同位素 ^{33}P。

(3)將嵌入 ^{33}P 的 RNA 和 cDNA 微陣列雜交後以 PhospoImage 系統讀取並以 AtlasImage 系統分析

結果及討論：

在復發時過度表現的基因，多和細胞週期促進或細胞存活有關。例如：在急性骨髓性白血病併 inv(16)中之 c-myc 和 CDKN1A。其他過度表現的基因還包括某些轉錄因子，例如：在急性骨髓性白血病併 t(7;11) 的 basic-leucine zipper transcription factor MAFG 以及 signal transducing adaptor molecule (STAM)。但是也有某些此類基因的表現在復發時被壓抑，例如：急性淋巴性白血病併 t(9;22) 中的 Cell cycle progression restoration protein 8 (CPR8) 以及 elongation factor 1 alpha (EF1 alpha) 。並且我們發現在不同型白血病的復發中，可發現不同的基因表現形式改變，表示它們形成復發及抗藥性的機轉並不相同。

Abstract

Keywords: Acute myeloid leukemia, Acute lymphoblastic leukemia, cDNA microarray

Background: Acute leukemia, a common hematological malignancy, is clinically divided into various subtypes according to morphology, immunophenotypes and cytogenetic changes, and various genetic and molecular abnormalities with significant prognostic implications could be found in many subtypes. However, because of the large-scale data and time-consuming laboratory works needed to illustrate the gene expression patterns, we still know little about the mechanisms of leukemogenesis, disease progression and development of drug resistance in the patients with acute leukemia.

Aims:

1. As a pilot study to evaluate the efficacy of cDNA microarray in exploring the expression gene profiles in acute leukemia.
2. To explore the possible genes differentially expressed in the development of leukemic drug resistance.

Methods:

1. Sample collection: Patients with inv(16) AML, t(7;11) AML and t(9;22) ALL diagnosed in NTUH is selected eligible for study 歐.
2. RNA extraction and RT will be performed, and the cDNAs were labeled with radioisotope ^{33}P at the step of RT.
3. ^{33}P-labeled cDNA probes will be hybridized with designed cDNA microarray. PhospoImage system will be used for reading signals, and AtlasImage system is used for data analysis.

Results and discussion: we find that many over-expressed genes are related to cell cycle progression and cell survival enhancing such as c-myc and CDKN1A in AML with inv(16); some others belong to the family of transcription activation factors, such as basic-leucine zipper transcription factor MAFG and signal transducing adaptor molecule (STAM) in AML with t(7;11). However, this is not always true since several genes related to cell survival and cell cycles also are down-regulated during disease progression, such as Cell cycle progression restoration protein 8 (CPR8) and elongation factor 1 alpha (EF1 alpha) in ALL with t(9;22). Furthermore, different genes are up- or down-regulated in disease relapse in different kinds of acute leukemia. Different kinds of mechanism for drug resistance thus are proposed in various types of leukemia. However, final common results are obtained.

Preface

Acute leukemia is a common hematological malignancy, which is clinically divided into various subtypes according to morphology, immunophenotypes and cytogenetic changes, and various genetic and molecular abnormalities with significant prognostic implications could be found in many subtypes. However, because of the large-scale data and time-consuming laboratory works needed to illustrate the gene expression patterns, we still know little about the mechanisms of leukemogenesis, disease progression and development of drug resistance in acute leukemia. In this project with the assistance of cDNA microarray technology, we try to find the genomic change during the development of drug resistance.

Aims:

1. As a pilot study to evaluate the efficacy of cDNA microarray technology in exploring the expression gene profiles in acute leukemia in Taiwan.
2. To explore the possible genes differentially expressed in the development of leukemic drug resistance.

Background:

(I) Introduction of acute leukemia (Ref 1-6):

In human hematological malignancies, acute leukemia is an important one worth notice because of its rapid course of disease progression and its role as a prototype of a chemo-sensitive malignancy. In the past, two distinct categories are well characterized in acute leukemia, i.e., acute lymphoblastic leukemia (ALL) and acute myelogenous leukemia (AML), and different therapeutic strategies and prognosis for the two groups are described. In adult, AML and ALL both take around 50% of the leukemia patients. In recent years, with the introduction of immunophenotyping, cytogenetics and/or advanced molecular technology, both AML and ALL are found to be heterogeneous in the aspects of clinical presentation, therapeutic response and disease prognosis. They are further classified into a few subtypes defined by MIC-M (Morphology-Immunophenotype-Cytogenetics and Molecular characters) criteria, and each subgroup had its own characters in clinical behaviors and molecular pathogenesis. In the following sections, some of them to be the main study targets in our project will be briefly introduced:

A. AML (Ref 4-5):

For adult AML, several subgroups are characterized below according to their distinct cytogenetic/molecular findings and/or clinical disease risk. In this study, we use a recurrent translocation of low-risk, i.e. inv(16), and a high risk abnormality, t(7;11), as the target.

AML, M4E0 with inv(16) or t(16;16): CBFbeta-MHC fusion protein could be detected in leukemic blasts, and heavy abnormal eosinophil infiltration in initial BM is its clinical characteristics. High-dose Ara-C based consolidation chemotherapy also could enhance the chance of long-term survival.

AML with t(7;11): Relative higher incidence in Chinese and Japanese in literature, and a fusion protein, NUP98-HOXA9, is formed. Clinically, it is a prototype of primary chemotherapy-resistant leukemia while complete remission rate is lower than 50%. Rapid relapse is also ensured in those who achieved CR, and thus aggressive treatment such as allogeneic bone marrow transplantation should be performed as early as possible.

B.ALL (Ref 6)

For ALL, we choose a common high-risk cytogenetic subgroup, t(9;22), as the subject:

ALL with t(9;22): In adult ALL, it could be found in 15-30% patients. Its characteristic fusion protein is BCR-ABL, which is similar to that is found in CML cases, but somewhat differences in protein size in half of the ALL patients could be noted. Clinically, high CR rate could be noted initially, but the disease usually relapses within 2 years and short overall survival could be noted. Aggressive treatment such as allogeneic bone marrow transplantation thus should be performed as early as possible.

(II). Introduction of cDNA microarray:

In the past, the analysis of gene expression level (even in a single gene) is a time- and labor-consuming work; and even when the serial expression level of individual genes could be obtained, the lack of data analysis technology also prohibit the creation of informative conclusions. However in recent years, two major approaches for global gene expression profiling in RNA level had been established with the advance of bio-technology. One is the " serial analysis of gene expression" (SAGE) technique, which is developed by Velculescu et al and mainly relies on the generation of short sequence tags near 3-end of the cDNA; After being ligated into larger molecules, the tags are sequenced and the gene expression level is determined by the frequency of the individual sequence tag in extensive sequencing in a tissue. It is also a laboratory-intensive technique, and thus is not very popular. The other one is the "microarray" technique, which simultaneously performs hybridization in nylon-based or glass-based surface. **(Ref 7-9)**

Currently, several reports had been made about the gene expression pattern in various malignancies by cDNA microarray technique. Foe example, ***van t Veer LJ et al*** successfully applied cDNA microarray technology in the prognosis stratification of breast cancer. With the new "hierarchical" mathematical technique for re-clustering

the samples, they found a gene expression pattern signifying the short survival and rapid distant metastasis ("poor prognosis" signature). The expression signature included genes regulating cell cycles, angiogenesis, disease invasion and metastasis. Besides, they also identified gene expression patterns in the tumors from *BRCA1* carrier. ***Shafter et al*** reported also their works about the *bcl-6*-related gene expression in different lymphoma samples by a specially designed cDNA microarray, lymphochip. Several genes including *blimp-1* (differentiation), *MIP-1alpha* (inflammation), *p27kip1* (cell cycle control) were repressed by *bcl-6* expression, and it suggested that B-cell transformation via *bcl-6* translocation involving inhibition of differentiation and enhanced proliferation capacity. ***Rusiniak et al*** explored the B94 gene as a possible target gene of retinoic acid in acute promyelocytic leukemia by glass-based microarray. ***Lam et al*** recently also discovered a constitutively expressed gene, *Notch-1*, in a erythroleukemic cell line by cDNA microarray; the expression of *Notch-1* were found to block erythroid maturation and thus linked to the pathogenesis of acute erythroid leukemia (AML, M6). ***Cohen et al*** reported 94 CML patients and their cDNA microarray analysis data (By Cloetech^{TM} Human 1.2 Atlas Array). They found 9p deletion was significantly related to disease survival and different gene expression pattern could be noted. ***Ohmine et al*** found that PIASy (protein inhibitor of activated STAT y) was serially over-expressed in the hematopoietic stem cell fraction in CML disease progression by microarray analysis of 3456 genes. These examples proved that cDNA microarray could provide enormous information about the pathogenic mechanism and disease progression of leukemia. **(Ref 10-15)**

Methods

(1). Patient Selection: From August 2001 to July 2002, totally 8 patients with acute leukemia are selected for analysis: 3 are AML with inv(16), 3 are ALL with t(9;22), and 2 are AML with t(7;11). Paired samples (at initial diagnosis and disease relapse) from marrow data bank in NTUH are collected in these patients for RNA extraction and subsequent cDNA microarray analysis

(2). RNA extraction:

A. Mononuclear cells (MNC) were extracted by the acid-guanidine-phenol-chloroform method.

B. Every 1 ug total RNA was reverse transcripted to cDNA by incubation at 42 C for 15 min, heated to 95 C for 5 min, and then soaked at 4 C for 5 min in a total volume of 20 uL containing 50u MMLV reverse transcriptase, 5mM MgCl2, 1mM of each dNTP (2mM biotin-dUTP), and 20U RNase inhibitor. At the step of reverse transcription, biotin-labeled dUTP will be added for

labeling cDNA.

(3). cDNA microarray analysis:

A. Extraction of poly-A tailed mRNA.

B. Reverse transcription with amplification by oligo-dT and random primers.

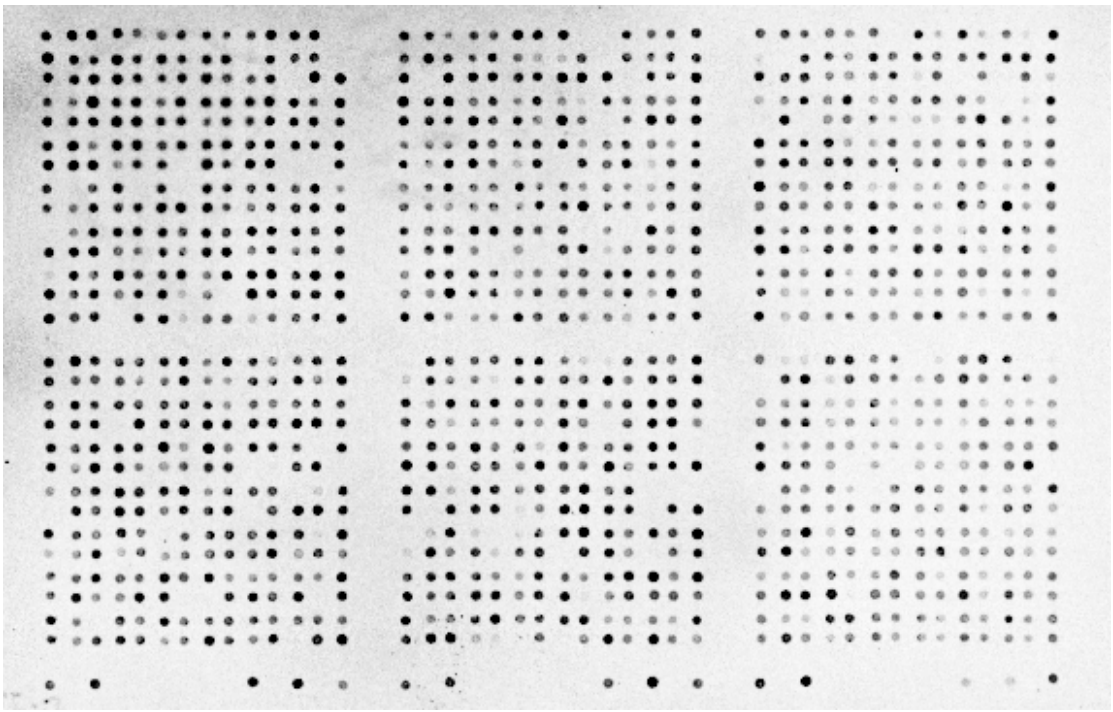
C. Two microgram of extracted cDNA will be labeled with ^{33}P-dNTP during reverse transcription to form ^{33}P-cDNA probes.

D. Atlas human cancer 1.2 membrane (Clontech, USA), which consists of 1176 human genes associated with human cancers, including apoptosis, signaling pathway, growth & differentiation, cytokines, DNA repair, cell cycle, and cytoskeleton genes, etc., is selected as the microarray platform system.

E. After washing off non-specific signals, the image is scanned with PhophoImage and analyzed with AtlasImage 2.01 software (Clontech, USA). The relative expression level for each gene will be normalized by 8 housekeeping genes and the global transcriptional profile of Leukemic cells established for every patient.

Results:

(1). Through this protocol, cDNA microarray is successful performed. The following picture is one cDNA microarray film read by PhosphoImage system:



(2). After performing paired samples from each patient, RNA expression level change is analyzed by AtlasImage 2.01 software. In the same disease group, the change of expression level is averaged. Then the genes of which the expression level change ratio is larger than 3 or smaller than 0.33 are list in every disease group:

A. AML inv(16), Initial vs replase

Down-expressed:

Ratio	Gene Name
0.048387	Hemoglobin alpha subunit
0.072013	High mobility group protein (HMG-I)
0.248000	Tumor suppressor QM; laminin receptor homolog
0.248227	HLA class I histocompatibility antigen C-4 alpha subunit (HLAC)
0.278600	Elongation factor 1 alpha (EF1 alpha)

Over-expressed:

Ratio	Gene Name
3.000000	hEGR1; transcription factor ETR103; KROX24; zinc finger protein 225
3.062500	Type II cytoskeletal 2 epidermal keratin (KRT2E); cytokeratin 2E (CK2E)
3.326087	Ribosomal protein S21 (RPS21)
3.328358	Arginine/serine-rich splicing factor 7; splicing factor 9
3.392857	DNAX activation protein 12
3.642857	VEGFR1; Flt + soluble VEGFR; tyrosine-protein kinase receptor SFLT
4.708333	ERBB-3 RTK precursor;EGFR
6.181818	c-myc oncogene
6.339286	c-myc purine-binding transcription factor puf
6.727273	ADP/ATP carrier protein
7.070313	CDKN1A; CIP1; WAF1

B. AML, t(7;11), Initial vs relapse

Down-expressed

Ratio	Gene Name
0.090822	Immunoglobulin rearranged gamma chain (IgG)
0.227273	58-kDa inhibitor of the RNA-activated protein kinase
0.252199	High mobility group protein (HMG-I)
0.259259	Nuclear pore complex protein 358 (NUP153); 358-kDa nucleoporin
0.269663	Membrane-bound & secreted immunoglobulin gamma heavy chain
0.290323	FRAP-related protein; protein kinase ATR
0.290429	Phosphoribosyl pyrophosphate synthetase subunit
0.315789	Ribosomal protein S6 kinase II alpha 3 (S6KII-alpha 3); ISPK1
0.318182	Wilms' tumor protein (WT33; WT1)
0.323529	Prohibitin (PHB)
0.327273	TGFB2; G-TSF; bsc-1 cell growth inhibitor

Over-expressed

Ratio Gene Name3.000000 Heparin-binding EGF-like growth factor (HBEGF)

3.044944 Ornithine decarboxylase

3.181818 c-jun proto-oncogene; transcription factor AP-1

3.369565 DNAX activation protein

6.000000 cAMP-responsive element modulator 1 alpha protein (HCREM)

C. ALL, t(9;22), Initial vs relapse

Down-expressed

Ratio Gene Name

0.181818 HGF agonist/antagonist

0.189655 Immunoglobulin alpha 1 heavy chain constant region (IgA1; IGHA1)

0.198914 Immunoglobulin rearranged gamma chain (IgG)

0.200000 Membrane-bound & secreted immunoglobulin gamma heavy chain (IgG3)

0.238095 MHC-alpha

0.250000 Zyxin + zyxin-2

0.274510 IgG receptor FC large subunit P51 precursor (FCRN)

0.277778 Osteoclast stimulating factor

0.285714 Purinergic receptor P2Y5

0.300000 Interleukin-7 (IL-7)

0.321429 Competitive hepatocyte growth factor antagonist (HGF antagonist)

0.333333 Cell cycle progression restoration protein 8 (CPR8)

0.333333 Integrin alpha 4 precursor (ITGA4); VLA4; CD49D antigen

0.333333 Interleukin-2 precursor (IL-2); T-cell growth factor (TCGF)

Over-expressed

3.000000 Signal transducing adaptor molecule (STAM)

3.157895 TDGF1

3.333333 IL-17 receptor

3.454545 CDKN1A; CDK-interacting protein 1 (CIP1);

4.000000 Basic-leucine zipper transcription factor MAFG

4.500000 Macrophage inhibitory cytokine 1 (MIC1)

Discussion:

(1). This study is a pilot study in Taiwan to demonstrate the feasibility of cDNA microarray technology in exploring the genomic expression profiles of acute

leukemia. Due to the limitation of study budget, we focus on the expression profile changes after chemotherapy and try to find the mechanism related to the development of drug resistance.

(2). In reviewing the list of genes with expression changes, we find that many over-expressed genes are related to cell cycle progression and cell survival enhancing such as c-myc and CDKN1A in AML with inv(16); some others belong to the family of transcription activation factors, such as basic-leucine zipper transcription factor MAFG and signal transducing adaptor molecule (STAM) in AML with t(7;11). However, this is not always true since several genes related to cell survival and cell cycles also are down-regulated during disease progression, such as Cell cycle progression restoration protein 8 (CPR8) in ALL with t(9;22) and elongation factor 1 alpha (EF1 alpha).

(3). Furthermore, different genes are up- or down-regulated in disease relapse in different kinds of acute leukemia. This may be due to the different treatment protocol in different kind of leukemia so that different defense mechanism is developed, but it may also be the sequence of the specific pathological mechanism related to specific types of acute leukemia. Whether there is a common marker or a common expression profile for identifying disease resistance in acute leukemia may not be able to reach a conclusion until more cases are enrolled for gene analysis.

(4). Horizontal comparison of the gene expression profiles during initial diagnosis of acute leukemia may be done; however, because there are limited cases done in this study, further comparison will be delayed till enough number of patients is enrolled and the difference of expression profiles will be explored between different subtypes of acute leukemia.

(5). For those genes which are identified to be differentially expressed at relapse, real-time RT-PCR examination for their significance in predicting leukemic relapse in specific type of acute leukemia may be the future research topics.

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自我評估:

Due to the limitation of the study budget, we finally focus our study from the global evaluation of cDNA microarray expression in various subtypes of acute leukemia to the development of drug resistance of limited types of acute leukemia, and further evaluation about the detailed genetic analysis with conventional molecular biological methodology such as RT-PCR is omitted also. However, this study still successfully set up the feasibility of cDNA microarray technology in evaluating the genetic expression profiles of acute leukemia in Taiwan, and this will be the basis of further study about the pathogenesis and biological characters in acute leukemia or even other types of malignancy. Even with the limited number of cases tested, we still know something about the expression profile changes during the development of drug resistance: (1). Leukemic cell survival is mostly due to the enhanced expression of cell survival-related genes and transcription factors, at least in several subtypes of acute leukemia, and (2). In different type of acute leukemia, the development of drug resistance is with different mechanism although a common result is obtained. This preliminary data is not enough to be important scientific findings; however, further workups will be based on these findings, and further evaluation of the importance of the genes listed as possible regulators of drug resistance in acute leukemia is highly needed.