行政院國家科學委員會補助專題研究計畫成果報告

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※　**接受造血幹細胞移植前後之微量殘存腫瘤細胞**　※
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計畫主持人：唐季祿

共同主持人：田慧芬, 陳耀昌, 姚明

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□赴國外出差或研習心得報告一份

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□出席國際學術會議心得報告及發表之論文各一份

□國際合作研究計畫國外研究報告書一份

執行單位：國立台灣大學醫學院內科

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行政院國家科學委員會專題研究計畫成果報告
利用定量即時 PCR 法偵測血液腫瘤病人接受造血幹細胞移植前後之微量殘存腫瘤細胞

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一、中文摘要

本研究以最新自動即時 PCR 定量儀器，精確測定白血病患者接受幹細胞移植後殘存微量腫瘤細胞 MRD 值。首先設計慢性骨髓性白血病 (CML) 帶有 bcr-abl 雜合基因及急性骨髓性白血病 (AML) 帶有 AML-ETO 雜合基因之特殊螢光探針，分別以 K562，Kasumi-1 細胞株 RNA 作序列 10 倍稀釋進行單一步驟 RT-PCR 定量建立標準曲線，檢體 RNA 值介於 1000 ng ~ 0.01ng 間時，其最初 RNA 之對數與 PCR 螢光產量達到臨界點所需週期數(C_T)成線性反比，線性關係(r 值)可以達到 0.99 以上，而且同次實驗與不同實驗間之變動性很小，靈敏度達到 10^{-5} (即可以在 10^5 正常細胞中偵測到 1 個異常細胞)。其次選取 29 例 CML 病人系列追蹤分析共 213 個檢體，並與染色體分析及臨床資料對照，發現染色體分析異常偵測臨界點僅為 10^{-2}；20 例緩解病人在移植 3 個月以後 MRD 即小於 10^{-3}，9 例復發病人，其中 4 例 1 年內復發者，MRD 從未能降到 10^{-4} 以下，但是 5 例晚期復發者，部分 MRD 曾經降到 10^{-5} 以下，為何會在多年後再復發，其機轉仍然不甚清楚。至於 10 例 AML 帶有 t(8;21)病例，除 1 例早期死亡，1 例未能得到緩解外。有 4 例移植後半年 MRD 仍大於 10^{-3}，其後 3 例復發，1 例則發生慢性 GVHD 後 MRD 降至 10^{-5} 以下維持緩解，另外 4 例均很快降到 10^{-5} 以下，持續維持緩解。由以上結果顯示此自動即時 PCR 定量法可以用於幹細胞移植後精確定量 MRD 及早期發現白血病復發之高危險群。

關鍵詞：即時 PCR，定量，殘存微量腫瘤，造血幹細胞移植。

Abstract

A novel, real-time quantitative RT-PCR assay was developed for detection of minimal residual disease (MRD) in leukemic patients after hematopoietic stem cell transplantation (HSCT). Specific fluogenic probes acrossing junctional regions of BCR-ABL (for CML) and AML1-ETO (for AML with t(8;21)) fusion genes were designed and used in one-step RT-PCR amplification of serial 10-log dilution of RNA from cell lines (K-562, Kasumi-1) to establish the standard curve. There was excellent log-linear correlation between the threshold cycle number (C_T) and the initial total RNA from 1,000 to 0.01 ng (r > 0.99) with low intra- and inter-assay variability. The sensitivity can reach the level of 10^{-5}, i.e. one leukemic cell in 10^5 normal cells. Sequential monitoring of MRD was then performed in 213 samples from 29 CML patients receiving HSCT. Cytogenetics has low sensitivity (~10^{-2}) in detecting MRD. In 20 patients in continued remission (CCR), the MRD dropped to < 10^{-3} within 3 months; on the contrary, the MRD failed to decline to 10^{-4} in 4 relapse within 1 year. Although in some patients the MRD once dropped to < 10^{-5}, CML eventually relapsed as late as 48

Table 1　Nucleotide sequences of PCR primers and probes

Gene	Primer	Sequences
AML1-ETO	AML1	TCAAAATCACAGTGGATGGGC
	ETO	CAGCCTAGATTGCGTCTTCACA
	Probe	$_{FAM}$-AACCTCGAAATCGTACTGAGAAGCACTCCAC-$_{TAMRA}$
BCR-ABL	BCR	CATTCCGCTGACCATCAATA
	ABL	TCCAGCGAGAAGGTTTTCCT
	Probe	$_{FAM}$-TCAGCGGCCAGTAGCATCTGACTT-$_{TAMRA}$
GAPDH	Forward	GAAGGTGAAGGTCGGAGT
	Reverse	GAAGATGGTGATGGGATTTC
	Probe	$_{JOE}$-CAAGCTTCCCGTTCTCAGCC-$_{TAMRA}$

months after HSCT. The molecular mechanism for this late leukemic relapse is unclear at present. We also monitored MRD changes after HSCTin 10 AML with t(8;21). Except for 1 early death and 1 refractory case, MRD dropped to < 10^{-5} in 4 cases and remained in CCR, while relapse occurred in 3/4 patients whose MRD didn't decline to < 10^{-3} at 6 months. The other patient got molecular CR after occurrence of chronic graft-versus-host disease indicating graft-versus-leukemia effect. These results suggest that this simple and sensitive RT-PCR assay is useful in monitoring MRD and in early detection of high-risk group after HSCT.

Keywords: real-time PCR, quantitation, minimal residual disease, hematopoietic stem cell transplantation.

二、緣由與目的

Hematopoietic stem cell transplantation (HSCT) has become the most successful curative treatment modality in hematological malignancies (1). However, relapse of the primary disease remains the most frequent cause of treatment failure and death after HSCT. Techniques of MRD detection are developed in the past including cytogenetics, multi-color flow cytometry, fluorescence in situ hybridization and PCR. Qualitative PCR was first applied in detecting leukemia-specific fusion gene product with sensitivity down to the level of 10^{-6}, but had limitation in interpretation of results. Quantitative methods such as competitive PCR were more informative but was limited by its complicated procedure and labor consuming. In this study, we apply a novel, real-time quantitative PCR technology (Taqman PCR) for detecting MRD in leukemic patients receiving HSCT. Our results suggest that this MRD quantitation technique is extremely useful clinically in evaluating tretment response, in early detection of leukemic relapse, and in selecting high-risk patients after transplantation.

三、結果與討論

Design of real-time RT-PCR and establishment of standard curve

Two leukemia cell lines were used in this study. K-562 carries b3a2 type fusion transcript of BCR-ABL gene and Kasumi-1 had AML1-ETO gene. GAPDH housekeeping gene was used as internal control of RNA integrity and normalization of loading RNA amount. The PCR primers and TaqMan fluogenic probes was designed with Primer-Express software (Perekin-Elmer) and their sequences were listed in Table 1. Collection of samples, separation of mononuclear cells, and RNA extraction were performed as reported previously. One-step RT-PCR was performed using EZ RT-PCR kit on Taqman PRISM 7700 sequence detector (ABI, Foster city, CA) as manufacturer's recommendation. Briefly, serial 10-fold dilution of total RNA from 1,000 to 0.01 ng K562 or Kasumi-1 was mixed with another negative cell line, KG-1 to keep final RNA at 1,000 ng. Duplicate samples were subjected to RT-PCR in a total volume of 50 μl containing 1x buffer, 200

μmol primers, 200nmol probe, 1,000 ng total RNA and 0.25U rTth DNA polymerase. The RT reaction was done at 50°Cfor 30 min followed by 45 cycles of PCR with 95°C for 15 sec and 62°C for 1 min. All experiments were done in duplicates and including no-template control and negative control RNA to prevent PCR carryover. Fluoresence spectra were continued monitored and analyzed by 7700 with detection software version 1.6.

As shown in Fig. 1, there was an excellent log-linear relationship (correlation of coefficient, r > 0.99) of PCR threshold cycle number (C_T) with wide-range of initial target mRNA concentration between 1,000 ng and 0.01 ng ranges. MRD was calculated as measured target RNA/GAPDH mRNA ratio. The data was qualified as evaluable only if the measured GAPDH RNA > 10 ng. The sensitivity of detecting MRD could reach the level of 10^{-5} with both BCR-ABL AND AML1-ETO genes. Furthemore, there was very little intra-assay and inter-assay variability (Fig.1). The standard error of C_T at 10^{-4} was 1.37 cycles for AML1-ETOgene and 2.11 cycles for BCR-ABL gene. This corresponded to target RNA measured error at ± 2-4 x 10^{-4}. Conventional cytogenetic analysis was found insensitive to detect MRD, its detection limit at 10^{-2} level only (data not shown).

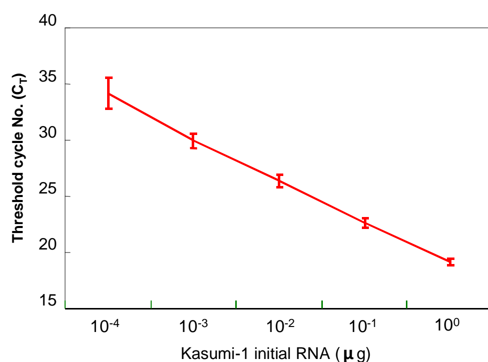

Fig. 1 Standard curve of AML1-ETO real-time RT-PCR by correlation of PCR threshold cycle number (C_T) and log(initial Kasumi-1 RNA amount).

Monitoring of MRD in CML after HSCT

Retrospective analysis of MRD level was performed in 213 samples from 29 CML patients who received HSCT at NTUH between 1994 and 2000. At time of HSCT, 14 were at chronic phase, 9 at accelerated phase, 2 at acute blastic phase, 3 at 2nd HSCT, and 1 2nd CP. After HSCT, 20 patients were at continued complete remission (CCR) with a median follow-up of 33 months (ranges, 3-86+ months). The MRD level was < 10^{-3} at +3 and +12 months and mostly < 10^{-4} after then. Nine patients relapsed between 2 and 48 months. In some of them, the MRD level fluctuated significantly during the first year, but mostly > 10^{-4}. However, late relapse did occurred in some patients whose MRD once dropped to unmeasurable level < 10^{-5}. The molecular mechanism for this late relapse is still unclear currently. In one patient, donor lymphocyte infusion resulted in sustained molecular remission for more than 26 months (Fig. 2).

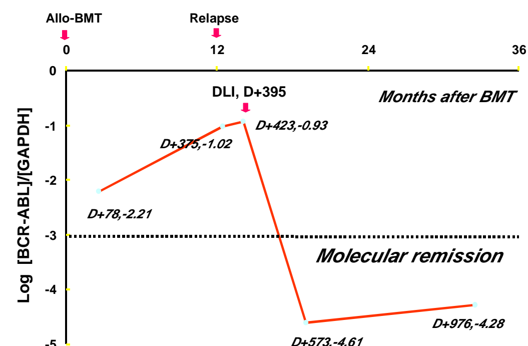

Fig. 2 Molecular CR induced by DLI in a 5 y/o girl with CML whose leukemia relapsed after allo-BMT.

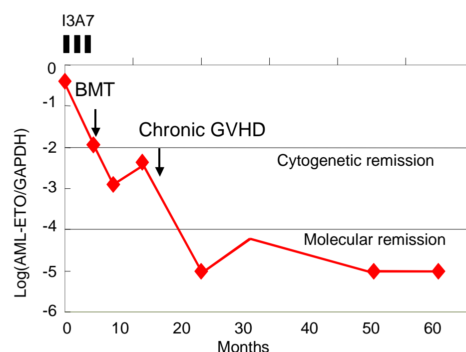

Fig. 3 Molecular CR induced by chronic GVHD in a AML patients with t(8;21).

Monitoring of MRD in AML after HSCT

Sequential monitoring of MRD after HSCT was performed in 10 AML patients with t(8;21). Except for 1 early death and 1 refractory case, MRD dropped to < 10^{-5} in 5 cases and remained in CCR, while relapsed occurred in 3/3 whose MRD persistently > 10^{-3}. In one patient, the MRD was >10^{-3} at 6 months. Molecular CR occurred after chronic graft-versus-host disease (GVHD) occurred and CR maintained for > 60 months (Fig. 3). This demonstrated a significant graft-versus-leukemia effect.

四、計畫成果自評

Our resultsshowed that (1) this real-time RT-PCR is a simple and very sensitive assay useful in quantitating MRD after HSCT; (2) sequential monitoring of MRD is very important within first year after HSCT to detect high-risk patients; (3) MRD monitoring is also very important in following the efficacy of DLI. These assay also can be applied in evaluating the chemotherapy response in AML patients and in evaluating response of CML to interferons or tyrosine kinase inhibitors like STI-571 in future.

五、參考文獻

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[2] Real-time quantitative RT-PCR for detecting minimal residual disease after allogeneic hematopoietic stem cell transplantation in patients with chronic myeloid leukemia. (Manuscript submitted for publication and has been presented in Annual meeting of Hematology Society of Taiwan, March 18, 2001).