行政院國家科學委員會補助專題研究計畫成果報告

兒童急性淋巴性白血病殘留癌細胞之研究----聯合交替使用聚合酶反應與流式細胞儀

Minimal Residual Disease of Childhood Acute Lymphoblastic Leukemia (II)

-- Combined Application of Polymerase Chain Reaction and Flow Cytometry

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Abstract

Although the treatment result of childhood lymphocytic leukemia (ALL) has much improvement in the past decades, there still has 20-25% patients will eventually experience relapses. The level of minimal residual disease (MRD) during clinical remission is one of the most powerful prognostic factors. The most useful methods to monitor the MRD are polymerase chain reaction (PCR) amplifications of antigen-receptor genes and flow cytometric identification of leukemic-associated immunophenotypes. We prospectively use both methods alternatively to follow the fate of the leukemic clone. MRD was examined at time of complete remission and different time-point of treatment protocols. We have enrolled 16 newly diagnosed childhood ALL patients in the past one year. Total 9 patients had individualized markers for their subsequent bone marrow MRD monitoring (56.2%). 7 of these 9 patients had suitable individualized leukemic-associated MoAb combinations and 2 had successful clone-specific primers for MRD monitoring. Fourteen follow-up bone marrow samples from these 9 ALL cases were analyzed for MRD assays. We identified the levels of MRD greater than 0.01% in two samples of a patient after the remission induction (week 11 and 16) and which decreased gradually to less than 0.01% 4 months later (week 32). There was no clinical relapse in all cases during the period of follow-up. In conclusion, alternating with semi-quantitative PCR and flow cytometry methods is a feasible and powerful tool in MRD monitoring and can increase the detection rate up to 56.2% in our study. However, with the limited sample size and follow-up duration, our current study is hard to draw a final conclusion and answer those questions prompted in our proposal. Large-scale study and long-term follow-up is warranted to answer those questions.

Keywords: minimal residual disease, flow cytometry, polymerase chain reaction, childhood acute lymphoblastic leukemia

Introduction

With the advent of contemporary multidrug chemotherapy and supportive care, the survival of childhood acute lymphocytic leukemia (ALL) has been much improved\(^1\). However, at least 20-25% of
those who are regarded as standard risk will still experience relapses. A number of biological and clinical parameters have been used for prediction of these relapses, but none is completely reliable. Sequential monitoring of minimal residual disease (MRD) of bone marrow is thought to be one of the most promising parameter for prediction of relapse. Among the various available techniques for MRD detection in ALL, flow cytometry detection of aberrant immunophenotypes and polymerase chain reaction (PCR) amplification of clone-specific antigen-receptor genes rearrangement have been considered as the most sensitive and reproducible methods. Several retrospective and prospective studies have shown that detection of MRD with these methods during clinical remission appears to be an independent prognostic factor for treatment outcome. However, neither can be applied to all patients.

There are several consensus about MRD in the treatment of childhood ALL. Absence of MRD after remission induction is independently to be a factor of good prognosis. A steady absence of MRD throughout the follow-up duration is associated with a favorable prognosis but persistence of MRD in the course of treatment generally will lead to a dismal relapse outcome. Recently, some studies have shown that the trend rather than the levels of MRD are more significantly related to the treatment outcome. These findings provide us a strong rationale to use the sequential MRD levels as not only for monitoring but for a criteria for risk re-classification in the treatment of childhood ALL. So, we conducted a prospective study to sequentially monitor the MRD in the course of treatment in childhood ALL. To increase the applicability in diagnostic patients, we use semiquantitative PCR and flow cytometry methods alternatively.

Materials and Methods

All patients who were newly diagnosed as ALL according FAB criteria from August 1, 2000 to July 31, 2001 and stuck to the treatment protocols of TPOG were enrolled in this prospective study. Bone marrow (BM) samples were collected at diagnosis, the end of remission, before consolidation, before reinduction, one year, two year and the end of therapy, according to the protocol used. Mononuclear cells (MNC) were separated by centrifugation by a density gradient. All samples were processed within 4 hours. Cells were maintained in RPMI-1640 medium with 10% fetal calf serum, 2mM L-glutamine and antibiotics.

Flow Cytometry

Leukemia-associated immunophenotypes were detected by multiparameter flow cytometry, with various combinations of monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP), and phycoerythrin cyanin (PC). Matched nonreactive fluorochrome-conjugated antibodies served as controls. The staining procedure has been described elsewhere. For each case, marker combinations with the sensitivity of one leukemic cell per 10⁶ normal nucleated bone marrow cells or greater were selected at diagnosis and then applied for subsequent MRD monitoring. We used a FACSort flow cytometer equipped with CellQuest software (Becton Dickinson, San Jose, CA). The staining procedure used for MRD detection has been described. For intracellular staining, cells were permeabilized with 8% solution during the cell-labeling procedure. In all samples, we acquired data from all mononuclear cells in each test tube (more than 1 x 10⁸).

PCR amplification of Immunoglobin Heavy Chain genes

Those who failed to obtain a suitable combination of immunophenotypic markers proceeded to semiquantitative PCR assay. DNA samples were prepared by using QIAamp blood kit. IgH gene rearrangements present at diagnosis were identified with the consensus V-region primer FR1C or FR2B, accompany with a universal JH primer. Reaction mixture of 20 ul contained 1xPCR buffer, 100 ng of genomic DNA, 1.5mM MgCl₂, 200 uM dNTPs, 0.25 uM primers and 1 unit of AmpliTaq Gold (PE Applied Biosystems). The typical amplification conditions consisted of an initial incubation for 10 minutes at 95C, then 45 cycles of 30 sec at 94C and 60 sec at 60C, followed by a final incubation of 5 min at 72 C. PCR products were purified and sequencing. The VH, DΗ and JΗ regions were identified by comparison with sequences in GeneBank using GCG software. For analysis of remission samples, primers complementary to N-D-N and D-N-J nucleotides of the leukemic IgH gene rearrangement were synthesized. A semi-nested PCR assay was used to detect a single copy of the leukemic gene rearrangement among 10⁷ normal genomes. All PCR products were analyzed by electrophoresis on a 3% agarose gel. Specificity was confirmed by using pooled peripheral-blood MNC DNA and sterile water as negative controls. The sensitivity of each assay was established by making serial 10-fold dilutions of leukemic DNA with DNA prepared from pooled PB MNC until no PCR product was observed. MRD was quantified by limiting-dilution analysis using 10 replicates and Poisson statistics.

Results

Total 16 cases of childhood ALL were eligibly enrolled in this study including 7 boys and 9 girls; 14 B-lineage ALL and 2 T-cell ALL. 8 patients were stratified to standard risk group, 4 to high-risk group and 3 to very-high-risk group and 1 to L3 protocol according to the criteria of TPOG guideline. 13 diagnostic samples were examined for MRD markers and seven patients had useful leukemic-associated immunophenotypic markers, which can effectively differentiate leukemic cells from normal BM cells (53.8%). The remaining 9 diagnostic samples were sent for IgH gene rearrangement sequencing. Only 2
patients had successful clone-specific primers, which were suitable for quantification study (22.2%). The reasons for failure were polyclonal amplified Ig heavy-chain genes or more than two amplified Ig heavy-chain genes that could not be readily separated for sequencing in 4 diagnostic samples (33.3%); inability to design a primer owing to too short CDR3 sequence or lack of specificity or sensitivity of the primers in 4 patients (44.4%). Total 9 out of 16 patients had either leukemic-associated immunophenotypic markers or clone-specific primer markers for subsequent MRD analysis (56.2%).

Fourteen follow-up bone marrow samples from these 9 patients were analyzed for MRD. We identified the levels of MRD greater than 0.01% in two samples of the same patient after remission induction (week 11 and week 16, respectively). The percentage of blasts in both samples was all less than 5% by light microscope examinations. Fortunately, the level of MRD decreased from 0.364% to 0.143% and <0.01% in the week 16 and week 32, respectively. No clinical relapse was noted in all 9 patients studied. But, the prognosis of those whose MRD levels converted from positive to negative after remission induction seem to have a relative good outcome.

**Discussion**

Alternating with flow cytometry and PCR techniques for detection of MRD is feasible and can detect leukemic cells down to 10^5 level. The advantage of the three-colored flow cytometry assay is it’s a fast, mature technique and accurate in cell quantitation. The weakness is expensive, skillful, instrument-dependent and immunotyping switch. On the contrary, semiquantitative PCR amplification of IgH gene rearrangement technique is cheaper but low applicability, laborious and time-consuming. With alternating application of both techniques, we have increased the detection rate up to 56.2% in our series. We found that MRD detected during the early clinical course can become undetectable at later time point and that this conversion is associated with a relatively good clinical outcome. These observations also prompt the importance of sequential monitoring of MRD in childhood ALL. Sequential monitoring of MRD can provide a measure of drug sensitivity, indicating the number of leukemic cells remained to be eradicated, and enables early tailor of treatment strategy.

A major limitation of MRD assay was its lack of applicability in a substantial proportion of newly diagnosed cases, especially for cases with B-cell phenotype. Our previous study has shown that with solely semiquantitative PCR technique, only half of the patients had suitable individualized primers for MRD monitoring. Currently, with alternating application of both three-colored flow cytometry and semiquantitative PCR assay, we have been improving this limitation as what was reported by others. We estimate that the suitable MRD markers could ultimately be quantifiable in 70-80% of patients by such approach.

One of the most difficulties in leukemia treatment is to distinguish patients who need more intensive therapy from those who only need less intensive therapy. Sequential MRD monitoring can provide direct measurements of leukemic cell responses to chemotherapy. The MRD at the end of induction remission can be used for risk re-stratification and protocol re-selection. We suggest that those cases with MRD positive at the end of remission induction should be closely monitored for changes in MRD. In our current study, with the limited sample size and follow-up duration, it is too early to say whether it is significant in the prediction of clinical relapse after the remission induction. MRD is protocol-dependent or which time point is critical in clinical course. More cases and longer follow-up duration are needed to answer these questions.

**References**

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【計畫成果自評】

本研究和原提計劃內容相符，本實驗連接上一年度之研究計劃，加入了流式細胞儀之檢驗方法，果然偵測率提高甚多（56.2%），而且比率還在增高中，雖然只是短短一年，但是我們證實了 Flow Cytometry 加上 PCR 是一個可行而且簡單的方法，可以用來偵測孩童急性淋巴性白血病之殘留癌細胞。我們的結論是以流式細胞儀偵測優先，以 PCR 為輔，然而仍有一些病例無法含括在內，殊為可惜，實在有引進新一代四色流式細胞儀之偵測方法之必要。至於 MRD 與臨床之關係，因本研究個案數尚在累積之中，且目前無人有臨床上之復發發生，不過從其中一位起初 MRD 陽性之個案，後來追蹤之後慢慢消失，而病人也沒症狀發生，隱約可見系列追蹤 MRD 的變化，在臨床上之重要性。最後，本 MRD 之研究須要更多的個案，以及時間的追蹤，才可以得到答案，嘉惠病童以及論文發表。