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Research Paper

Detection and clinical significance of multidrug resistance-1 mRNA in bone marrow cells in children with acute lymphoblastic leukemia by real-time fluorescence quantitative RT-PCR☆

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Abstract

Objective: Multidrug resistance(MDR) is one of the most important reasons for treatment failure and recurrence of acute leukemia. Its manifestations are different in children with acute lymphoblastic leukemia(ALL) which may be due to different detection methods. This study was to detect the expression of MDR1 mRNA in bone marrow cells of children with ALL by real-time fluorescence-quantitative reverse transcription polymerase-chain reaction(FQ-RT-PCR), and combine minimal residual desease(MRD) detection by flow cytometry(FCM) and to study their relationship with treatment response and prognosis of ALL. Methods:The MDR1 mRNA levels in bone marrow cells from 67 children with ALL[28 had newly diagnosed disease, 27 had achieved complete remission(CR), 12 recurrent] and 22 children without leukemia were detected by FQ-RT-PCR. MRD was detected by FCM. The patients were observed for 9~101 months, with a median of 64 months. Results:Standard curves of human MDR1 and GAPDH genes were constructed successfully. MDR1 mRNA was detected in all children with a positive rate of 100%. The mRNA level of MDR1 was similar among the newly diagnosed ALL group, CR group, and control group($P > 0.05$), but significantly higher in the recurrence group than that in newly diagnosed disease group and control group(0.50 ± 0.55 vs. 0.09 ± 0.26 and 0.12 ± 0.23, $P < 0.05$). 54 ALL patients were followed up, and it was found that MDR1 mRNA level was significantly higher in ALL patients within 3 years duration than that of ALL patients with 3~6 years and over 6 years duration(0.63 ± 0.56 vs. 0.11 ± 0.12 and 0.04 ± 0.06, $P < 0.01$). For the 28 children with newly diagnosed disease, the MDR1 mRNA level was similar between WBC $> 50 \times 10^9$ group and WBC $< 50 \times 10^9$ group($P > 0.05$). In the 33 CR patients, the MDR1 mRNA level was significantly higher in MRD $> 10^3$ group than that in MRD $< 10^3$ group(0.39 ± 0.47 vs. 0.03 ± 0.03, $P < 0.05$). Conclusion:The sensitivity and specificity of FQ-RT-PCR in detecting MDR1 mRNA in bone marrow cells of children with ALL patients are high. MDR1 mRNA is expressed in children with and without leukemia. MDR1 mRNA is highly expressed in the CR ALL patients with high MRD, recurrence and short duration(within 3 years). Monitoring MRD and the MDR1 mRNA level might be helpful for individual treatment.

Key words: leukemia; children; multidrug resistance; MDR1 gene; minimal residual disease; real-time fluorescence quantitative RT-PCR

INTRODUCTION

Chemotherapy is the main treatment for acute lymphoblastic leukemia(ALL) while the efficacy is individually varied. Some patients take good responses to chemotherapy, while others may develop primary or secondary drug resistance. The reasons for multidrug

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resistance are various, in which over expression of the gene of multidrug resistance(MDR) may implicate a critical role and may be the most frequent form of drug resistance in relapsed acute leukemia. Over the years a number of methods have been developed to measure cellular expression and/or function of MDR1 such as immunohistochemistry[1], flow cytometry[2], drug-or dye-efflux[3] as well as quantitation of MDR1 RNA or messenger RNA(mRNA) byRT-PCR[2]. Real-time RT-PCR assays have been widely used to estimate the expression level of genes of interest.[4] This method is relatively easy to perform with high sensitivity, excellent reproducibility, and accuracy. Several studies have shown that monitoring of minimal residual disease (MRD) in haemopoietic malignant disease predicts clinical outcome. In ALL, MRD detection is useful for evaluating early response to treatment and consequently for improving stratification[5]. This study set out to detect levels of MDR1 mRNA in bone marrow cells of children with ALL by real-time fluorescence quantitative reverse transcription-polymerase chain reaction(FQ-RT-PCR) and combine minimal residual desease(MRD) detection by flow cytometry(FCM) and to study their relationship with treatment response and prognosis of ALL, and to optimize efficacy by the individualized chemotherapy.

MATERIALS AND METHODS

Clinical data

67 children patients with ALL, treated in the Affiliated Hospital of Qingdao University from Jul.1999 to Dec.2006, were recruited, including 40 boys and 27 girls, aged from 4 months to 13 years with a median of 8 years. In 67 ALL patients, 28 had newly diagnosed disease, 27 had achieved complete remission(CR), 12 had recurrence(10 had achieved complete remission again);The immunophenotypes of the patients showed that 18 had early preB-ALL, 41 had preB-ALL, 5 had mature B-ALL, 3 had My-positive ALL. All cases were diagnosed according to diagnosis and treatment proposal for pediatric acute lymphoblastic leukemia.[1] 22 children, including 15 boys and 7 girls, were assigned to the non-leukemia group as controls. In the 22 children, 12 were healthy, 4 had aplastic anemia(AA), and 6 had idiopathic thrombocytopenic purpura(ITP). The distribution of sex and age of both groups were comparable. In the 28 newly diagnosed ALL group, 15 had received CR while 13 had given up treatment for different causes. 54 ALL patients were followed up for 9 to 101 months, with a median of 54 months. In the followed-up 54 ALL patients 41 experienced continous complete remission (CCR) including 3 cases with second complete remission.(Tab 1)

Tab 1 Characteristics of 67 examined children with ALL

Number of patients	67(40 males, 27 females)
Age in years,	8(4 month-13 year)
Median (range)	
Initial WBC	WBC > 50 × 10^9(n = 8)
	WBC < 50 × 10^9(n = 20)
Immunophenotype	early preB-ALL(n = 18), preB-ALL(n = 41),
	mature B-ALL(n = 5), My-positive(n = 3)
Newly diagnosed ALL	with treatment(n = 15), without treatment(n = 13)
Newly diagnosed ALL with treatment	CCR(n = 11), relapsed and died(n = 4)
Follow up	
within 3 years	newly diagnosed ALL(n = 7), recurrence ALL
	(n = 10 in which 2 cases receiving second CCR)
with 3-6 years disease duration	CCR(n = 9), newly diagnosed ALL(n = 8),
	recurrence ALL(n = 2 in which 1 case receiving second CCR)
with 6-9 years disease duration	CCR(n = 18)

Chemotherapy regimens for ALL patients

Right after definite diagnosis of ALL, the patients received 1-week treatment of prednisone and received re-examination of routine blood test and blood smear at the 8th day for blast cells. All patients received VDLD (Vincristin 1.5 mg/m^2, Daunorubincin 30 mg/m^2, L-asparaginase 8 000~10 000 U/m^2, Dexamethasone 6 mg/m^2) regimen as induction chemotherapy, the patients received bone marrow examination on the 19th day after induction chemotherapy and then CAM(CTX 800 mg/m^2;Ara-C 75 mg/(m^2•d);6-MP 50 mg/m^2) regimen as consolidation treatment.HD MTX-CF regimen(high dose of Methotrexate plus Tetrahydrofolic acid calcium) was used to prevent extramedullary leukemia. VDLD regimen plus EA(VM-26 150~300 mg/m^2; Ara-C 200~300 mg/m^2) regimen were used regularly as intensive chemotherapy. 6-MP and MTX were used as maintenance chemotherapy. The average overall treatment

duration was 3 years for the female patients and 3.5 years for the male patients.

Treatment efficacy evaluation

The treatment efficacy was evaluated according to diagnosis and treatment proposal for pediatric acute lymphoblastic leukemia[6].

Detection of MDR1 mRNA in bone marrow cells by FQ-RT-PCR

Total RNA extraction and cDNA synthesis

2 ml fresh bone marrow aspirates with EDTA were collected. RNA were isolated from bone marrow cells with Trizol reagent. Reaction mixture(20 μl) contained 5.0 μl of total RNA, 1.0 μl of arbitrary primer(50 pmol, purchased from Promega Company), 0.5 μl of RNAse inhibitor(DEPC)(40U/μl),0.5 μl of PrimeScript™RTase (200 U/μl,purchased from Promega Company), 1.0 μl of dNTP(10 mmol/L) and 4 μl of 5Ŭ × Script™Buffer and 8 μl of ddH_2O. The prepared samples reacted at 42℃ for 10 min for reverse transcription and at 95℃ for 2 min for inactivation of reverse transcriptase. The obtained cDNA were stored at -20℃.

PCR amplification

The PCR reactions(20 μl) were prepared in 2 μl of cDNA, 0.7 μl of both upstream and downstream primers(6 μmol/L), 10.0 μl of Premix Ex Taq™(2 ×), and 6.6 μl of ddH_2O. The following primer pairs were used for PCR reactions: 5′-CTT AGC ACC CCT GGC CAA G-3′ as the sense primer and 3′-GTC AGG TAC GGT AGT GAC GG-5′ as the antisense primer for GAPDH, 5′-AGC TCT CTG GTG GCC AGA AAC-3′ as the sense primer and 3′-TTC CAA CAG GTT CTT CGG GAC-5′ as the antisense primer for MDR1, conditions for amplification consisted in 10 s of initial denaturalization at 95℃,followed by 40 PCR cycles(5 s at 95℃, 34 s at 60℃).PCR products were analyzed by gel electrophoresis using 2% agarose gel and visualized by ethidium bromide staining. Molecular weight was calculated by a standard DNA ladder. The MDR1 primer set yielded a 131 bp product, and the GAPDH primer set a 150 bp PCR product.

Detection of MDR1 and GAPDH mRNA by FQ-RT-PCR

Gene sequencing confirmed that the cloned fragments were target fragments. The fluorescent probes of MDR1 and GAPDH were synthesized by Shanghai Sangon Biological Technology & Services CO., LTD. The sequences of fluorescent probes were 5′-TGC CAT AGC TCG TGC CCT TGT TAG ACA-3′ for MDR1 and 5′-CAT GCC ATC ACT GCC ACC CAG AAGA-3′ for GAPDH .The components in 20 μl of total FQ-RT-PCR reaction volume were 10 μl of Premix Ex Taq™(2 ×), 0.7 μl of both upstream and downstream primers(6 μmol/L), 0.8 μl of fluorescence probe solution(4 μmol/L), ROX Reference Dye(50 ×) 2.0 μl of template DNA, 5.4 μl of ddH_2O. Cycling condi-tions for MDR1 and GAPDH were as follows:95℃ for 10 s, at 95℃ for 5 s, and 60℃ for 34 s for 40 cycles. Water, instead of template DNA was used as negative control. Standard curves of recombinant plasmids at different concentrations were automatically constructed by SDS software. The relative level of MDR1 mRNA was assessed as comparing its fluorescent curves with standard curves(Fig 1,2).

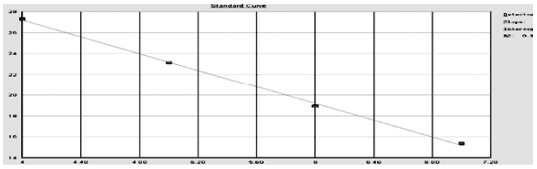

Fig 1 Standard fluorescent intensity curve of MDR1

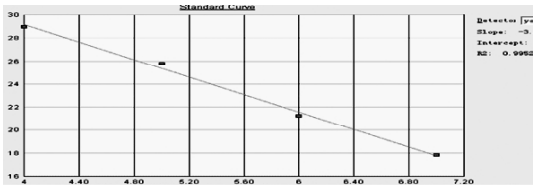

Fig 2 Standard fluorescent intensity curve of GAPDH

Flow cytometry analysis of minimal residual disease

Mononucleated cells from 2 ml fresh heparinized bone marrow samples extracted from 33 ALL with continous complete remission(CCR) for over half an year, including 3 cases with second complete remission(CR_2) were isolated by Ficoll-Hypaque density gradient centrifugation and were washed with cold PBS twice, stained with 10 μl of 3 kinds of fluorescence-marked monoclonal antibodies(anti-CD10-PE, anti-CD19-FITC and anti-CD45-PEcy5 antibodies). The mononucleated cells stained with IgG1-PE, IgG1-FITC and anti-CD45-PEcy5 antibodies were used as controls. After reaction at 4℃ for 30 min, mononucleated cells were washed with cold PBS twice, and analyzed by flow cytometry. Data acquisition and analysis were performed on at least 10 000 viable cells with the Cell Quest software(BD Biosciences).

Statistical analysis

Statistical analyses were performed using SPSS version 10.0. All values were expressed as $\bar{x} \pm s$. The statistical significance of the results were calculated using Student's t test, t′ test, and q test. A value of $P < 0.05$ was regarded as significance.

RESULTS

Standard curves of MDR1 and GAPDH

Standard FQ-RT-PCR curves of recombinant plasmids were constructed with good repeatability. The slope ratios of standard curves were -3.36, -3.25, which were within the theoretical extent, indicating that the standard curves were reliable(Fig 3,4).

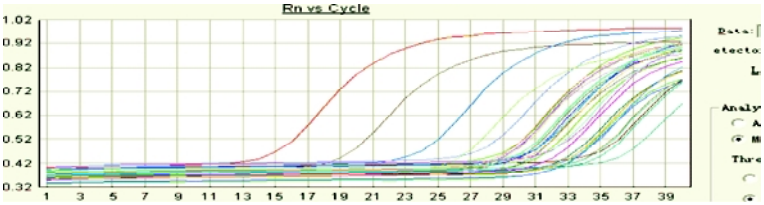

Fig 3 Fluorescent intensity curves of MDR1 in polymerase chain reaction

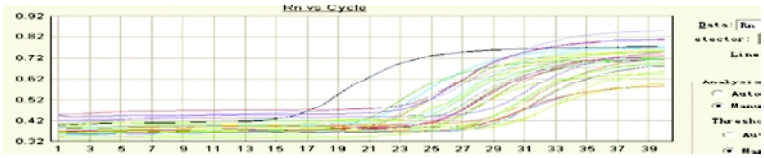

Fig 4 Fluorescent intensity curves of GAPDH in polymerase chain reaction

Result of MDR1mRNA expression in bone marrow cells of all children

Both MDR1 and GAPDH mRNA were expressed in bone marrow cells of all children in ALL group and controls, with a positive rate of 100%; while they were not detected in negative controls(water). MDR1 mRNA as-sessment was shown(Tab 2).

Tab 2 The relative MDR1mRNA levels in different ALL patients and controls

group	The relative MDR1mRNA levels (ratio of MDR1 copies to GAPDH copies)	
	cases(n)	($\bar{x} \pm s$)
controls	22	0.12 ± 0.23
newly diagnosed ALL	28	0.09 ± 0.26
ALL patients with CR	27	0.06 ± 0.10
recurrent ALL	12	0.50 ± 0.55

The relative MDR1mRNA levels(ratio of MDR1 copies to GAPDH copies) in bone marrow cells were 0.12 ± 0.23 in non-leukemia group, 0.09 ± 0.26 in newly diagnosed ALL patients, 0.06 ± 0.10 in ALL patients with CR, and 0.50 ± 0.55 in recurrent ALL patients. There were no significant differences between newly diagnosed ALL patients and controls($t = 0.40$, $P > 0.05$), between ALL patients with CR and controls [$t' = 1.02$, $t'(0.05) = 2.08$, $P > 0.05$], and between newly diagnosed ALL patients and ALL patients with CR [$t' = 0.48$, $t'(0.05) = 2.06$, $P > 0.05$] respectively. While the level of MDR1 mRNA was significantly higher in recurrent ALL patients than that in controls[$t' = 2.28$, $t'(0.05) = 2.19$, $P < 0.05$] and in newly diagnosed ALL patients[$t' = 2.45$, $t'(0.05) = 2.19$, $P < 0.05$] respectively.

When the 28 newly diagnosed ALL patients were stratified according to the amount of white blood cells, the relative MDR1mRNA levels were 0.05 ± 0.02 in the 8 patients with WBC>50×10^9 and 0.12 ± 0.30 in the 20 patients with WBC<50×10^9 respectively, there were no significant differences between them($P > 0.05$).

When the 54 ALL were followed up, they were stratified according to disease duration. The relative MDR1mRNA level was significantly higher in the 16 patients within 3 years disease duration than in the 20 patients with 3~6 years disease duration and the 18 patients with 6~9 years disease duration(0.63 ± 0.56 vs. 0.11 ± 0.12 and 0.04 ± 0.06, $P < 0.01$).

33 ALL patients(including 3 cases with second complete remission) in continous complete remission (CCR) for more than 6 months, were stratified according to MRD. The relative MDR1 mRNA levels were 0.39 ± 0.47 in the patients with MRD>10^{-3} and 0.03 ± 0.03 in the patients with MRD<10^{-3}, respectively. There were significant differences between them($P < 0.05$). The recurrence rate was significantly higher in MRD > 10^{-3} group than in MRD < 10^{-3}, group(50.0% vs. 0.0%, $P < 0.05$).

DISCUSSION

P-glycoprotein(P-gp) encoded by MDR1, is a member of ATP-binding cassette(ABC) superfamily, and possess high avidity for some antineoplastics[7]. It can enhance drug efflux and reduce the intracellular antineoplastic drug content, therefore leading to pre-existing MDR clones and/or treatment-induced acquired resistance.[8] High expression of MDR in acute myeloid leukemia(AML) patients confers cross-resistance to

unrelated drugs such as daunorubicin, adriamycin, etoposide and so on[9] which have been commonly used in treating pediatric ALL. FQ-RT-PCR in measuring MDR1 has advantages of high sensitivity and specificity[4,10]. This study used FQ-RT-PCR to quantitatively detect the MDR1 mRNA levels in bone marrow cells. In accordance with other studies[11-13], we found that MDR1 mRNA was expressed in both ALL group and controls.

Our results showed that there was no significant difference in MDR1 mRNA level between newly diagnosed ALL patients and controls, indicating that MDR1 mRNA is not highly expressed in naïve ALL patients, regardless of high risk factors. We also found that there was no significant difference in MDR1 mRNA level between newly diagnosed ALL patients and ALL patients with CR, which is different from the results in reference[14]. Kourti **et al**[15] had reported a 15-month-old girl with standard risk ALL had relapsed after routine treatment, and the MDR1 mRNA level at recurrence had elevated 5 times than that at diagnosis. Beck **et al**[16] has reported the same up-regulating trends of MDR1 mRNA level in recurrent AML patients just as our findings in ALL patients.

In our study, relapse happened after single hormone irregular treatment and termination of chemotherapy. A patient with the longest treatment duration of 4 years developed testicle leukemia about 1 year after termination of treatment, followed by bone marrow and clinical recurrence. In our opinion, although chemotherapy can induce CR in bone marrow, the leukemia cells in bone marrow and shelters occasionally aren't cleared out, which is the main reason of recurrence. In the patients receiving irregular treatments or inadequate chemotherapy, we found they could not inhibit the proliferation of leukemia cells and even induce secondary drug resistance, therefore, recurrence happened[17]. The occurrence of MDR in recurrent ALL may be related to irregular drug usage. Previous reports[18,19] showed that prednisone can also induce the expression of P-gp and MDR1. Pawlik **et al**[20] reported the down-regulation of the concentrations of interleukin-2(IL-2), interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α) in PBMCs after treatment of Dex. Stein **et al**[21] found that cytokines, such as IL-2, IFN-γ and TNF-α can down-regulate the expression of MDR1 mRNA and P-gp, and enhance the sensitivity of cells to MDR-related drugs. We could deduce that Dex can suppress the leukemia cells sensitivity to chemotherapeutic drugs. Brock **et al**[22] found that increasing the dosage of VP-16 could induce the overexpression of MDR in human small cell lung cancer cell line H69/VP, and lead to drug-resistance. These results indicate that chemotherapeutic drugs can induce acquired drug-resistance, which was manifested as high MDR1 mRNA level in the recurrent patients.

FCM can sensitively detect MRD in ALL patients with CR. It can recongnize leukemia cells of a low quantity from 1×10^{-1} to 1×10^{-4}. High MRD indicates that patients need enhanced chemotherapy or drug resistant clonal develops. MRD combining the MDR1 mRNA level analysis can provide us good predictions. San **et al**[23] found that the higher MRD in CR AML patients, the higher recurrence rate is. We also found that the MDR1 mRNA level was obviously elevated in patients with high MRD. This situation indicates that the proliferation of drug-resistant cells with MDR1 expression result in a high recurrence rate. Once the patients relapse, it is difficult to achieve remission again, or it is likely to recur again after remission; so the mortality is extremely high. In our study, the mortality of the patients with recurrence is 83.3%; Of the 2 patients that still survive and under observation, one had stopped taking medicine for 5 months and had not developed recurrence yet, the other one had achieved CR for 6 months. While in the patients with low MRD, the expression of MDR1 mRNA in bone marrow cells is obviously suppressed, indicating no proliferation of drug-resistant cells and no recurrence. For these patients, we may reduce the intensity and shorten the duration of chemotherapy to improve quality of life.

Initial treatment is important for preventing MDR and reducing recurrence. Comparing the children with different durations of ALL, the expression of MDR1 mRNA is higher in the children with shorter duration (within 3 years) than in those with longer duration(more than 3 years), indicating that MDR1 mRNA of ALL patients is likely to occurr within 3 years. Hence, to detect MDR1 mRNA at the early phase of chemotherapy, early notice and avoiding use of MDR-related drugs, and reversal reagents of MDR1 gene may be helpful for individual treament.

It is well known that the prognosis of leukemia patients with high amount of WBC is poor. While our results showed no significant difference in the expression of MDR1 mRNA between the patients with high and low amount of WBC at initial. There might be some other mechanisms related to the prognosis of ALL with high amount of WBC. MDR1 mRNA may also be a reason. High tumor burden prolongs the duration of early induction. Even if the bone marrow is induced to CR, residual leukemia cells may be adequate to cause MDR. The presence of the MDR phenomenon may be responsible for the poor response to chemotherapy. So detecting MDR1 mRNA combining MRD detecting during treatment would be particularly valuable for the design of new treatment strategies.

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