·Clinical Research·

Minimal residual disease monitoring in chronic myeloid leukemia patients after allogeneic hematopoietic stem cell transplantation using interphase fluorescence in situ hybridization and real-time quantitative reverse transcription PCR

Hong-Qian Zhu, Xiao-Li Liu, Lan-lin Song, Qi-Fa Liu, Fan-Yi Meng, Shu-Yun Zhou

Department of Hematology, Nanfang Hospital, Southern Medical University, Guangzhou, Guangdong 510515, P. R. China

[Abstract] Background and Objective: Interphase fluorescence in situ hybridization (FISH) and real-time quantitative reverse transcription PCR (RQ-PCR) are the common methods for monitoring minimal residual disease (MRD) in chronic myeloid leukemia (CML) patients. This study was to assess the value of monitoring BCR-ABL fusion gene level in CML patients after allogeneic hematopoietic stem cell transplantation (allo-HSCT) using FISH and RQ-PCR. **Methods:** BCR-ABL fusion gene levels were detected in the bone marrow of 31 patients with CML before and 3–48 months after allo-HSCT using FISH and RQ-PCR. **Results:** BCR-ABL positive cells detected by FISH were decreased 3–30 months after allo-HSCT and BCR-ABL/ABL mRNA was reduced by 2 logarithmic units in RQ-PCR ($P < 0.05$). While no BCR-ABL positive cell was detected by FISH 30 months after allo-HSCT, BCR-ABL/ABL mRNA was detected by RQ-PCR and declined by more than 3 logarithmic units, ($P < 0.05$). **Conclusions:** Dynamic monitoring of BCR-ABL gene on molecular level in CML patients after allo-HSCT is useful in the early prediction of susceptibility to recurrence in the patients and in designing intervention, and is thus helpful in improving the overall survival rate after transplantation.

Key words: Chronic myeloid leukemia, BCR/ABL, FISH, RQ-PCR, transplantation

Chronic myeloid leukemia (CML) is a pluripotential hematopoietic stem cell-derived malignant myeloproliferative disease with characteristic cytogenetics (Ph chromosome) and molecular biology (expression of BCR/ABL fusion gene). Blast crisis is the advanced stage of CML when treatment efficacy is generally poor. Allogeneic hematopoietic stem cell transplantation (allo-HSCT) and treatment with tyrosine kinase inhibitor provide a chance of complete molecular response for CML patients, that is, the level of BCR/ABL gene is reduced to an undetectable level. Although the emergence of imatinib has pioneered on the targeting treatment for CML, allo-HSCT is still the only curative method for CML for the time being[1]. However, recurrence after transplantation is one of the major influential factors for the long-term survival in CML patients. Early determination on whether the patients need intervention and dynamic monitoring minimal residual disease (MRD) (BCR/ABL gene) after allo-HSCT is helpful in improving the overall efficacy and safety of HSCT, predicting recurrence and facilitating the implementation of intervention.

Monitoring methods for MRD include fluorescence in situ hybridization (FISH) and real-time quantitative reverse transcription PCR (RQ-PCR). FISH was once used as a diagnostic method for CML and a follow-up and monitoring tool on MRD. RQ-PCR, as a new technique for monitoring MRD in recent years, analyzes BCR-ABL gene quantitatively at mRNA level. Quantitative detection of the level of BCR-ABL gene was performed in 31 CML patients who had received allo-HSCT treatment in our hospital. Herein, we summarized and analyzed the role of FISH and RQ-PCR as clinical monitoring tools on MRD in CML patients.

Co rrespondence to: Xiao-Li Liu; Tel: +86-20-61641615; Fax: 86-20-87280761; Email: lxl2405@126.com

This paper was translated from Chinese into English by Guangzhou Liheng Medical Translation and edited by Jing-Yun Ma on 2009-11-06.

The Chinese version of this paper is avaiable at http://www.cjcsysu.cn/cn/article.asp?id=16163.

Received: 2009-05-07; Accepted: 2009-10-17

Subjects and Methods

Study subjects

A total of 31 CML patients who were diagnosed and treated in

the Nanfang Hospital, Southern Medical University between May 2004 and May 2009 were selected. Of these patients, 26 were male and five female, with a median age of 35 years (age range 12–41 years). All these patients were confirmed and staged by cytomorphology, cell histochemistry, cytogenetics and molecular genetics, and underwent allo-HSCT in Nanfang Hospital within one year after being diagnosed. Informed consent about allo-HSCT was obtained from all the patients. Before transplantation, 22 patients were at chronic phase and nine at blast crisis or acceleration phase. Among them, 24 patients were treated with hydroxyurea and the other seven with imatinib. After the transplantation, FISH and RQ-PCR were performed to detect BCR-ABL fusion gene at varied time points. Follow-up time after transplantation ranged from 3 to 48 months. A total of 159 bone marrow samples were collected at different time points before and after the transplantation.

FISH detection

A dual-color dual-fusion DNA probe provided by Vysis was used for the detection. According to the FISH operation procedures for interphase cells in the hematology laboratory of Nanfang Hospital, the samples were treated, denatured, hybridized and eluted[2]. Hybridization signals were observed under fluorescent microscope Nikon E600 with a tricolor filter (DAPI/TRITC/ FITC); red signal indicated BCR, green signal indicated ABL, while yellow signal indicated BCR-ABL fusion. In the interphasic nucleus of a normal cell, four hybridization signals could be seen, including two red and two green signals, and red signals were well separated from green ones, while in interphasic nucleus with BCR-ABL expression, one red and one green hybridization signals and one yellow fusion signal could be found. Images were taken by a high-resolution CCD (by COHU) and analyzed by a fluorescent image analyzing system (by PSI, England). A total of 1000 cells were counted for each sample. A BCR-ABL positive cell percentage of $\leqslant$ 3% was considered as a background signal[3].

RQ-PCR detection

Extracted bone marrow was anti-coagulated with EDTA and was used for extraction of monocytes with Ficoll separation buffer. Trizol reagent was used to extract total RNA, which was then retro-transcripted into cDNA. Real-time quantitative amplification was performed on the cDNA in a fluorescent quantitative PCR apparatus MJ Opticon2 (Bio-Rad, US). Reaction system (25 μL) included 300 nmol/L upstream and downstream primers, respectively, 200 nmol/L *Taq* man probe, 12.5 μL of 2 × *Taq* man PCR mixed buffer (ABI, US) and 2 μL of template cDNA. Sequences of primers and probes are shown in Table 1. PCR reactions were catalyzed by one cycle of 5 min at 95℃ and 45 cycles of 15 s at 95℃ and 1 min at 58℃. ABL gene was used as an internal control. Sequences of primer and probe were designed and standard curve was generated according to the routine procedures in our laboratory[4]. A standard curve with a correlation coefficient (r) of $\geqslant$ 0.99 was considered robust. The standard curve for the RQ-PCR detection was based on a BCR-ABL positive plasmid with P210 breakpoint sequence (standard sample). The positive plasmid was quantitatively measured and diluted at a ratio of 1 : 10, and underwent quantitative amplification in the PCR reaction system as described above. In this study, we used a relatively quantitative method with standard curve. Using ABL gene as internal control, the standard curve was generated automatically based on the results from the standard sample. The absolute copy numbers of BCR/ABL fusion gene and internal control gene ABL were then measured, respectively. The ratio of fusion gene to internal control gene (copy number of BCR-ABL / copy number of ABL × 100%) was considered as the RQ-PCR result for the patients. Detection results of different patients were calculated using the standard curve.

Table 1 Sequences of primers and probes

Gene	Primer and probe	Sequence
M-BCR/ABL	upstream primer	5′-TCCGCTGACCATCAAYAAGGA-3′
	downstream primer	5′-CACTCAGACCCTGAGGCTCAA-3′
	probe	FAM-CCCTTCAGCGGCCAGTAGCATCTGA-TAMRA
ABL	upstream primer	5′-TGGAGATAACACTCTAAGCATAACTAAAGGT-3′
	downstream primer	5′-GATGTAGTTGCTTGGGACCCA-3′
	probe	FAM-CCATTTTTGGTTTGGGCTTCACACCATT-TAMRA

Statistical analysis

All the data were processed by SPSS13.0 software. FISH results were shown as the percentage of BCR-ABL-positive cells/all the cells counted, and RQ-PCR results as the ratio of BCR-ABL to ABL. Measurement data were presented as mean ± standard deviation (SD). Results of FISH and RQ-PCR detections before transplantation were used as controls. All the detection results from FISH and RQ-PCR after transplantation were compared with the results before transplantation. The differences between the results at various time points after transplantation and those of the controls were analyzed using *t* test. Test level was set at $\alpha = 0.05$.

Results

FISH and RQ-PCR results in CML patients before treatment

In FISH detection among 31 CML patients, the mean percentage of BCR-ABL fusion gene-positive cells was (96.2 ± 3.8)% (Figure 1). The standard curve for RQ-PCR detection of BCR-ABL gene is shown in Figure 2. The mean ratio of BCR-ABL to ABL was 0.74 ± 0.11.

Detection results after allo-HSCT

In one patient, the percentage of BCR-ABL gene-positive cells in FISH detection at three months after transplantation was 10.9%, and the ratio of BCR-ABL to ABL mRNA by RQ-PCR was 0.15; at six months after transplantation, the percentage of positive cells in FISH detection was 54%, and ratio of BCR-ABL to ABL by RQ-PCR was 0.31. At this time point, oral imatinib was given immediately at the dosage of 600 mg, qd. Currently, the patient has maintained in complete hematological remission.

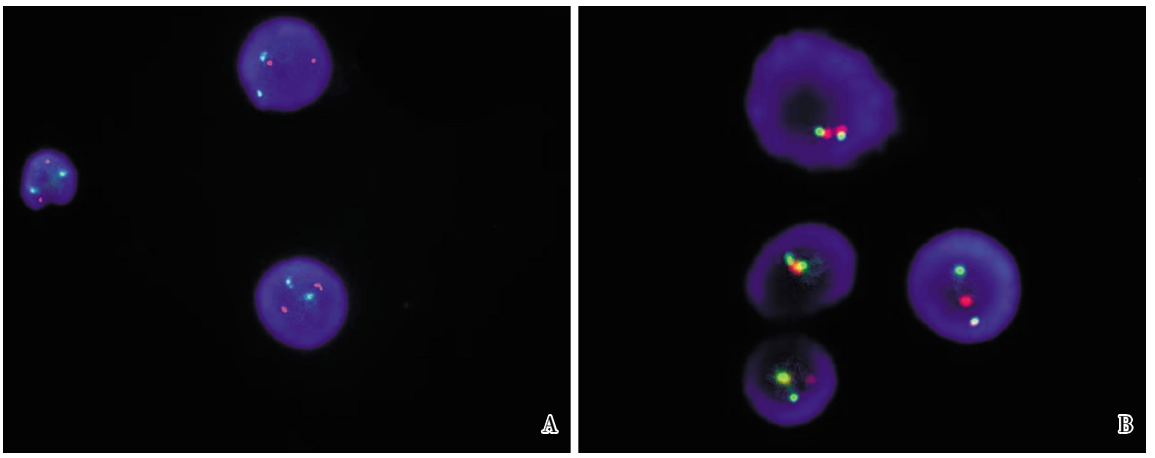

Figure 1 BCR and ABL gene FISH signals

A, in normal interphase nucleus, 2 red signals represent BCR gene and 2 green signals represent ABL gene separate from each other.

B, in interphase nucleus with BCR-ABL fusion gene, 1 yellow fusion signal represents the BCR-ABL fusion gene.

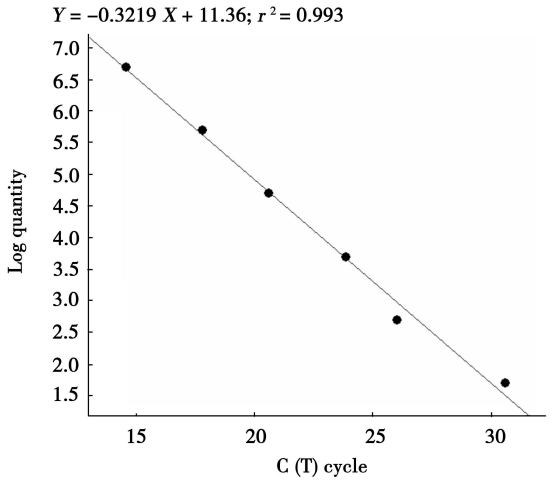

Figure 2 Standard curve of BCR-ABL gene

Another patient had complete hematological remission at six months after transplantation, but FISH detection revealed a positive cell percentage of 13% and RQ-PCR detection revealed a ratio of BCR-ABL to ABL of 0.18, which were far from complete molecular remission. At 12 months after transplantation, FISH detection revealed that positive cell percentage was 65%, while ratio of BCR-ABL to ABL in RQ-PCR was 0.21. At this time point, imatinib was given at the dosage as described above. The patient has maintained in complete hematological remission.

In the rest of the patients, when comparing the results of FISH and RQ-PCR detection at various time points after transplantation with those before transplantation, the detection levels (percentage and ratio) were significantly decreased ($P < 0.05$). At 30 months after transplantation,results from FISH detection were 0, while RQ-PCR still revealed a detectable expression level of BCR-ABL/ABL (Table 2).

Discussion

According to the NCCN guideline 2008, Ph chromosome and/or positive BCR-ABL fusion genes are essential conditions in

Table 2 FISH and RQ-PCR results in CML patients before and after allo-HSCT

Timepoint after allo-HSCT (months)	Patient No.	FISH (%)	RQ-PCR ratio	The log value of BCR-ABL/ABL decline
3	29	0.41±0.22	0.0062±0.0019	2.08
6	29	0.09±0.00	0.0064±0.0011	2.06
9	23	0.02±0.00	0.0037±0.0008	2.30
12	20	0.06±0.05	0.0059±0.0012	2.10
24	17	0.01±0.00	0.0043±0.0005	2.24
30	16	0.00±0.00	0.0007±0.0002	3.03
36	16	0.00±0.00	0.0008±0.0006	3.01
48	9	0.00±0.00	0.0002±0.0001	3.57
Before allo-HSCT	31	96.20±3.80	0.7426±0.1071	0

FISH, fluorescence in situ hybridization; RQ-PCR, real-time quantitative reverse transcription PCR; CML, chronic myeloid leukemia; allo-HSCT, allogeneic hematopoietic stem cell transplantation.

diagnosing CML. During the treatment for CML, it is necessary to conduct a dynamic monitoring on tumor burden, so as to modify treatment scheme and achieve a complete cytogenetic remission (CCR, percentage of Ph^+ cells is 0) and a complete molecular response (CMR, negative BCR-ABL fusion gene mRNA) or a major molecular response (MMR, BCR-ABL mRNA level reduced by $\geqslant$ 3 logarithmic units). Currently, FISH and RQ-PCR have become important approaches in diagnosing CML and monitoring MRD[2].

Cortes *et al*.[5] combined these two methods when detecting 280 patients treated with imatinib and found that results of RQ-PCR and FISH detections were well consistent in FISH-positive samples. Both methods are quantitative monitoring tools for MRD, with different sensitivity and specificity. In FISH, single strand DNA with fluorescence label (probe) and its complementary DNA (sample on the slide) are annealed and hybridized. FISH reflects relevant gene expression by the sites of fluorescent signals in the chromosome as observed under microscope. A total of 1 000 cells were calculated, and the results were shown as the percentage of positive cells. Dual-color dual-fusion FISH has a lower false positive rate than the dual-color single-fusion version, and is a favorable tool for the rapid diagnosis in treatment-naïve patients. However, the sensitivity of FISH detection is dependent on the skill proficiency of the lab technicians in scanning a few hundreds of interphase cells. FISH is not sensitive enough as a monitoring tool on MRD. While in RQ-PCR, fluorescent labels are added to the PCR system. The PCR process is monitored on a real-time basis by the accumulation of fluorescent signals. Finally, unknown template is quantitatively analyzed using a standard curve. In this method, Taq Man probe technique provides an excellent specificity, low false positivity and fine linearity. In the follow-up of CML patients treated with allo-HSCT or imatinib, RQ-PCR is apparently better than FISH and is currently recognized as the golden standard in monitoring the leukemic residue level[6,7].

In the CML patients of this study, with the detection results before transplantation as baseline data, the average percentage of BCR-ABL fusion gene-positive cells in FISH detections at various time points was significantly reduced in 31 patients after transplantation ($P < 0.05$). At 30 months after transplantation, the result of FISH detection was 0. When comparing the BCR-ABL/ABL ratio in RQ-PCR detection to the average baseline level before transplantation, the post-transplantation level was decreased by > 2 logarithmic units within 3-30 months after transplantation and by > 3 logarithmic units at 30 months afterward. Our observation showed that results of RQ-PCR and FISH detections were well correlated. When FISH was negative, RQ-PCR could still reveal the changes in BCR-ABL/ABL ratio, suggesting that RQ-PCR had a better sensitivity than FISH. When positive results were yielded in consecutive RQ-PCRs but FISH revealed negative results, the possibility of recurrence was indicated. At this time point, if imatinib was given, FISH detection could turn negative, while RQ-PCR level might be still decreasing[6,8]. Therefore, RQ-PCR was more accurate in detecting the lower level of BCR-ABL transcript and could be used as a sensitive marker for monitoring MRD.

In conclusion, dynamic monitoring of BCR-ABL gene on molecular level in CML patients after allo-HSCT is useful in the early prediction of susceptibility to recurrence in the patients and in designing intervention, and is thus helpful in improving the overall survival rate after transplantation.

References

[1] Bruno CD, Danilo P. The biology of CML blast crisis [J]. Blood, 2004, 103 (11):4010–4022.

[2] Du QF, Zhou SY, Liu XL, et al. Application of interphase fluorescence in situ hybridization for determination of the tumor load in chronic myeloid leukemia [J]. Ai Zheng, 2003, 22(6):612–615. [in Chinese]

[3] Du QF, Liu XL, Song LL, et al. The application of dual fusion interphase fluorescence in situ hybridization probe in detecting minimal residual disease in chronic myeloid leukemia [J]. Zhonghua Nei Ke Za Zhi, 2002, 41(12):801–804. [in Chinese]

[4] Jiang QL, Meng FY, Jiang S, et al. One plasmid standard for detecting both bcr-abl P210 and abl gene cDNA in real-time quantitative RT-PCR [J]. J Leuk Lymph, 2007, 16(3):161–164. [in Chinese]

[5] Cortes J, Talpaz M, O´Brien S, et al. Molecular responses in patients with chronic myelogenous leukemia in chronic phase treated with imatinib mesylate [J]. Clin Cancer Res, 2005, 11(9): 3425–3432.

[6] B Fei,M Reinhold,L Clarissa,et al. Comparison of FISH and quantitative RT-PCR for the diagnosis and follow-up of BCR-ABL-positive leukemias [J]. Mol Dia Ther, 2007, 11(4):239–245.

[7] Hughes T, Branford S. Molecular monitoring of BCR-ABL as a guide to clinical management in chronic myeloid leukaemia [J]. Blood Rev, 2006, 20(1): 29–41.

[8] Lundán T, Juvonen V, Mueller MC, et al. Comparison of bone marrow high mitotic index metaphase fluorescence in situ hybridization to peripheral blood and bone marrow real time quantitative polymerase chain reaction on the International Scale for detecting residual disease in chronic myeloid leukemia [J]. Haematologica, 2008, 93(2):178–185.