The incidence and distribution characteristics of MLL rearrangements in Chinese acute myeloid leukemia patients by multiplex nested RT-PCR

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Abstract. Occurrence of MLL (Mixed Lineage Leukemia) gene rearrangements indicates poor prognosis in acute myeloid leukemia (AML) patients. This is the first study to report the positive rate and distribution characteristics of MLL rearrangements in AML patients in north China. We used multiplex nested real time PCR (RT-PCR) to screen for incidence of 11 MLL rearrangements in 433 AML patients. Eleven MLL rearrangements included (MLL-PTD, MLL-AF9, MLL-ELL, MLL-AF10, MLL-AF17, MLL-AF6, MLL-ENL, MLL-AFIQ, MLL-CBP, MLL-AF1P, MLL-AFX1). There were 68 AML patients with MLL rearrangements, and the positive rate was 15.7%. MLL-PTD (4.84%) was detected in 21 patients, MLL-AF9 in 15, (3.46%), MLL-ELL in 10 (2.31%), MLL-AF10 in 8 (1.85%), MLL-AFIQ in 2 (0.46%), 3 cases each of MLL-AF17, MLL-AF6, MLL-ENL (0.69% each), a and single case each of MLL-CBP, MLL-AF1P, and MLL-AFX1 (0.23% each). The highest rate of MLL rearrangements was found in 24 patients with M5 subtype AML, occurring in 24 cases (35.3%). MLL rearrangements occurred in 21 patients with M2 subtype AML (30.9%), and in 10 patients with M4 subtype AML (14.7%). Screening fusion genes by multiplex nested RT-PCR is a convenient, fast, economical, and accurate method for diagnosis and predicting prognosis of AML.

Keywords: Acute myeloid leukemia, multiplex nested RT-PCR, MLL rearrangements

1. Introduction

Acute myeloid leukemia (AML) is a malignant clonal disease of hematopoietic stem cells, of which the main symptoms exhibited include infection, bleeding, anemia and extramedullary tissue and organ infiltration. AML has a rapid progression and high mortality rate [1]. Specific gene mutations have been shown to be of great prognostic significance in AML, and are currently being used in risk stratification for treatment.

The multiplex nested real time PCR (RT-PCR) method can screen multiple gene mutations simultaneously and provide important evidence for assessing diagnosis and treatment [2].
The MLL (Mixed Lineage Leukemia) gene is located at Chromosome 11, band 23 (11q23). The fragment size of MLL is 92 kb, including at least 36 exons [3]. MLL rearrangements in AML possess unique clinical and molecular genetic characteristics, and the occurrence of MLL rearrangements indicates a poor prognosis [4]. It has been found that chromosome fusion can occur at 11q23 to produce the corresponding fusion genes, excluding the 13\textsuperscript{th} and the Y chromosome. There were MLL 50–60 fusion rearrangement species [5]. Therefore, understanding the MLL gene in AML patients plays a very important role in diagnosis, prognosis analysis, and micro-residue monitoring.

2. Material and methods

2.1. Patients

This study was conducted from April 2008 to November 2011, and involved 433 AML patients who received treatment at the Department of Hematology Chinese PLA General Hospital. Bone marrow puncture, multiplex nested RT-PCR, immunophenotyping, and karyotype analysis were offered to all patients.

2.2. Reverse transcription-polymerase chain reaction (RT-PCR)

Bone marrow mononuclear cells were purified by density centrifugation using a standard Ficoll-Hypaque method. It is a single-density gradient centrifugation. Ficoll-Hypaque is stratified fluid. In this technique the mononuclear and the polymorphonuclear leucocytes are separated into two distinct bands free from red blood cells. Total RNA was isolated from bone marrow mononuclear cells using TriPure isolation reagent (Roche, Los Angeles, CA, USA), and reverse transcribed to complementary DNA (cDNA) using a kit from Promega (Madison, WI, USA). PCR was carried out using a Veriti\textsuperscript{®} thermal cycler (Applied Biosystems, Foster City, CA, USA).

2.3. Multiplex nested RT-PCR

The experiments included a 110-bp GAPDH mRNA fragment as an internal control. The 25 µL PCR mixture was prepared with 2X GoTaq Green Master Mix (Promega), cDNA, multiplex primers and nuclease-free water. The amplification products from the second round of PCR were analyzed on a 2% agarose gel, and visualized with ethidium bromide (Fig. 1). Each sample was confirmed by individual PCR using specific primers for each transcript under identical cycling conditions and by DNA sequencing [6].

2.4. Immunophenotypic studies

PB (peripheral blood) or BM (bone marrow) blast cells were analyzed by flow cytometry. A panel of monoclonal antibodies against CD45, CD34, CD38, CD33, CD56, CD3, CD2, CD5, CD7, CD10, CD8, CD19, CD20, CD138, CD24, CD22, CD28, kappa, lambda, TdT, HLA-DR and CD79a were used to determine the immunophenotypes of leukemia cells, as described previously [7].
2.5. Karyotype analysis and FISH

Cytogenetic analysis was carried out for bone marrow obtained with the aforementioned diagnostic procedures using a direct method or short-term unstimulated culture. Metaphase chromosomes were karyotyped, and chromosomal abnormalities were reported according to the International System for Human Cytogenetic Nomenclature (ISCN 1995). Fluorescence in situ hybridization (FISH) analysis was also carried out to detect fusion genes.

3. Results

From the cohort of 433 AML patients, 68 with positive results were detected, giving a positive rate of 15.7%. The rates of each of the 11 types of MLL rearrangement were as follows: MLL-PTD, 21 (4.84%), MLL-AF9, 15 (3.46%), MLL-ELL, 102 (23.11%), MLL-AF10, 8 (1.85%), MLL-AF17, 3 (0.69%), MLL-AF6, 3 (0.69%), MLL-ENL, 3 (0.69%), MLL-AF1Q, 2 (0.46%), MLL-CBP, 1 (0.23%), MLL-AF1P, (10.23%), MLL-AFX1, 1 (0.23%). The results are shown in Figs 2A and B. There were 47 male and 21 female patients. The median age was 47 (range 19–75). The diagnosis of patients with MLL rearrangements are shown in Table 1. There were 24 MLL rearrangements occurring in M5, which accounted for the highest percentage of cases (35.3%). Twenty-one cases of MLL rearrangements occurred in M2 (30.9%), and 10 cases of MLL rearrangements occurred in M4 (14.7%).

4. Discussion

There were MLL 50–60 fusion rearrangement species [5,8]. The MLL aberrations by FISH method detected abnormalities at the 11q23 chromosomal region, which were associated with poor leukemia prognosis [9–11].

Multiplex nested PCR combines the advantages of both nested and multiplex PCR. The samples are arranged in parallel groups containing multiple mixed primers for PCR detection of multiple target genes simultaneously. This method is efficient, economical, simple, and accurate. It is superior to karyotype
Table 1

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<th>M3</th>
<th>M4</th>
<th>M5</th>
<th>M6</th>
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Fig. 2. (A). 68 cases of MLL gene-positive patients amongst 433 AML patients. (B). MLL gene rearrangements in 68 MLL gene-positive patients.

analysis and Southern blotting in several ways. Nested multiplex RT-PCR is not affected by chromosome metaphase in the detection of fusion genes, nor in its wide range of other applications. Abnormalities can be detected in low quality samples and small clone numbers, and requires only a tiny fragment of the translocated MLL gene partial tandem repeat. It can be used to identify rare chromosomal translocations, and detect hidden chromosomal abnormalities, providing a technology platform for clinical micro residue (MRD) detection [12].

[13] applied RT-PCR for screening leukemia patient MRD to predict recurrence, and provide opportunities for treatment. Molecular relapse to hematologic relapse time ranged from 8 to 79 days, with an average of 25.5 days. Chemotherapy, biological immunotherapy and monoclonal antibody therapy were applied during this period to enable the patient to achieve molecular remission. This study applied RT-PCR for the screening of MLL rearrangements. After screening out MLL rearrangements, real time quantitative PCR (RQ-PCR) was used to perform quantitative analysis of MLL rearrangements before and after every chemotherapy and hematopoietic stem cell transplantation respectively, to dynamically observe gene levels. RQ-PCR provides the basis for micro-residue monitoring, and is the earliest possible means of detection for the leukemia recurrence, and provides a guide for selecting leukemia treatments.
[14] reported the incidence of MLL genes in AML patients. RT-PCR was used to detect 988 cases of AML; of these, 114 cases (11.54%) were positive for MLL rearrangements. The numbers of patients who tested positive for each type of fusion gene were as follows: 68 patients tested positive for the MLL-PTD fusion gene (6.38%); 14 patients for the MLL-AF9 fusion gene (1.42%); 9 patients for the MLL-AF10 fusion gene (0.91%); 9 patients for the MLL-ELL fusion gene (0.91%); 8 patients for the MLL-AF6 fusion gene (0.81%); 4 patients for the MLL-ENL fusion gene (0.40%); and 1 for each of the MLL-AF1, MLL-AF4, MLL-MSF, MLL-LCX, MLL-LARG, MLL-SEPT6 and MLL-CBL fusion genes (0.10%). Adult MLL-PTD genes were present in significantly higher percentages of adults than in children ($P < 0.05$). RT-PCR can be used to detect all rare and abnormal MLL-related fusion genes [15] studied the MLL gene rearrangements in AML, and found them to have a positive rate of 14.3% (31 out of 217 patients). A study conducted by China Daopei Hospital reported that amongst 494 patients with AML, 36 (7.2%) were positive for MLL gene rearrangement [16]. In our study, the incidence of MLL gene rearrangements was 68 out of 433 patients (15.7%). Twenty-one patients were MLL-PTD fusion gene-positive (4.84%); 15 patients were MLL-AF9 fusion gene-positive (3.46%); 10 patients were MLL-ELL fusion gene-positive (2.31%); and 8 patients were MLL-AF10 fusion gene-positive (1.85%). Our results were different from those of other hospitals. The reason for this may be due to different regional patient distributions.

In China, [17] reported a higher incidence of MLL rearrangements in the M5 subtype of AML than in other types. MLL fusion gene-positive cells possessed typical acute monocytic leukemia morphology, indicating that MLL rearrangements in leukemia cells are linked to monocyte differentiation. In our study, MLL rearrangements occurred in the M5 subtype at the highest rate (35.3%) followed by M2 (30.9%) and M4 subtypes (14.7%). Therefore, before commencing treatment for M5, M2 and M4 types of leukaemia, it would be useful to screen for MLL gene rearrangements. In addition, as reported in previous literature, MLL gene rearrangement is likely to occur after other cancer treatment-related leukemia. Application of etoposide, epirubicin Star, cyclophosphamide and other drugs can precipitate treatment-induced leukemia, particularly M4 and M5 type [18–21]. The prognosis of treatment-related leukemia is poor, and attention should be paid to screening for MLL rearrangements. MLL-PTD occurs predominantly in myeloid dysplasia syndromes, secondary AML (s-AML), and de novo AML. The presence of MLL rearrangement generally confers a poor prognosis. Inhibitors of epigenetic regulators could potentially also prove effective in the treatment of MLL-PTD-related leukemia [22]. The fusion gene of MLL-PTD plays a critical role in the pathogenesis of M4 and M5 AML patients. However, rare chromosome abnormalities have been identified in this type of leukemia [23]. In China, amongst 234 cases of de novo childhood AML, MLL-PTD was detected in 8 cases (4 cases of M5, 2 cases of M4, and one case each of M2 and M6). The total incidence of MLL gene rearrangements was 11.97% (28 out of 234 patients), and most patients (85.7%, 24/28) had M4 or M5 subtype AML [24]. In our study, the total incidence of MLL gene rearrangements was 15.7% (68 out of 433 patients). Thirty-four out of 68 patients (50%) is M4 or M5 subtype AML. MLL-PTD was detected in 21 cases (M5 in 7 cases, M2 in 7 cases, M4 in 4 cases, and M6 in 3 cases).

[25] reported a 10-year follow-up of a patient with MLL-AF9-positive AML. The patient’s natural killer (NK) cells were tested against the K562 cell line, implying that long-term complete remission in this patient could be due to NK cell-mediated disease-suppression [26] found that MLL-AF9-expressing cells are more sensitive to chemotherapeutics such as ATRA and 5-Aza, indicating that different MLL fusion proteins possess different epigenetic properties associated with retinoic acid pathway inactivation. The MLL-AF4 fusion gene was predominant in acute lymphoblastic leukemia (ALL), while the MLL-AF6 and MLL-AF9 fusion genes occurred with the greatest frequency in AML [27]. In our study, MLL-AF9 occurred with the second highest frequency in acute myeloid leukemia, after MLL-PTD which occurred with the highest frequency.
MLL-ELL is the product of the (11;19) (q23p13.1) translocation associated with de novo and therapy-related AML. De Braekeleer et al. reported that there was 21.1% incidence of MLL-ELL in 19 MLL rearrangements in AML patients [28]. In our study, among 68 AML patients with MLL rearrangements, there was a 14.7% incidence of MLL-ELL.

The t (10;11) (p12;q23) translocation and the t (10;11) (p12;q14) translocation encode the MLL-AF10 [29]. The MLL-AF10 fusion gene resulted from the insertion of part of the region that included the 5’ MLL insertion into 10p12; this was concurrent with the deletion of 3’ MLL. Intron 6 of MLL was fused with intron 8 of AF10 on 10p12 in the 5’ to 3’ direction [30]. In our study, there were five MLL-AF10 positive M5 patients and three MLL-AF10 positive M2 patients.

Cytogenetic analysis is of value in the diagnosis and prognosis of leukemia. Conventional banding is not only time-consuming, but also has a low detection rate. Compared to cytogenetic analysis, RT-PCR is a sensitive, rapid PCR technique. [31] reported that using Multiplex nested RT-PCR and karyotype analysis techniques in combination improved the detection rate of clonal chromosomal AML patients. In our study, a total of 40 cases were analysed using combine karyotype analysis technology Karyotype abnormalities in chromosome structure or number were detected in 28 of these 40 cases (70%). Only five cases of chromosome 11 abnormalities were detected (12.5%). The 11q23 detection rate was very low.

In summary, in this study, we report the rate and distribution characteristics of MLL gene rearrangements in AML patients in Northern China. Using multiplex nested RT-PCR to screen MLL gene rearrangements in AML patients can detect multiple target genes simultaneously. This method has advantages in that it is efficient, economical, and accurate compared to other methods. It could be used to identify rare and occult chromosomal translocations. This method was widely used in the diagnosis of AML patients. It provides an important basis for risk assessment of MRD monitoring and prognosis after transplant, which is of very important clinical value.

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Conflict of interest

Authors declare no conflicts of interest.

References


