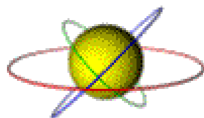

PCR-SSCP分析急性淋巴细胞白血病患者FLT3基因及FLT3/ITD基因突变及意义

Fms-like tyrosine kinase 3 (FLT3), also known as fetal liver kinase 2 (FLK2) or stem cell kinase 1 (STK1), is encoded by the gene located on the chromo- some 13q12 and belongs to the class Ⅲ receptor tyrosine kinase (RTK) family, sharing strong sequence and structural similarities with other class Ⅲ tyrosine kinase members, including the stem cell factor receptor C-kit, macrophage colony-stimulating factor receptor C-fms and platelet-derived growth factor receptor (PDGFR). Structurally, FLT3 possesses much similarity with C-kit and C-fms in that they all contain 5 immunoglobulin-like domains including a transmebrane domain, a juxtamembrane (JM) domain, 2 tyrosine kinase domains (TK1 and TK2) and a C-terminal domain. FLT3 and its ligand play an important role in the growth control of pluripotent hematopoietic cells, early progenitor cells and immature lymphocytes through their signal transduction pathways[1][2]. Recently, some researches show that the internal tandem duplication (ITD) mutation in exon 11, intron 11 and exon 12 of the sequence coding for JM domain, called FLT3/ITD gene mutation, occurs in 17% to 30% of patients with acute myeloid leukaemia (AML) and 3% of those with myelodysplastic syndrome (MDS). The actual location and length of the mutation varied from sample to sample, but the mutation always occurred in a certain reading frame, causing the mutant FLT3 to have an elongated JM domain. FLT3/ITD mutation has been recognized as an important indicator of poor prognosis for acute myeloid leukemia (AML)[3][4]. In this study, PCR was used to detect the FLT3 gene and FLT3/ITD gene mutation in 63 ALL patients for analyzing their implications in the clinical features and prognosis of ALL patients.

MATERIALS AND METHODS

Patients

Sixty-three ALL patients (including 37 male and 26 female patients) with established diagnosis by cell morphology (FAB classification), cytochemistry and flow cytometric immunophenotyping were enrolled in this study. The criteria for immunophenotyping described by Lo Coco et al[5]were adopted. The patients' age ranged from 12 to 43 years with the median age of 21 years. Immunophenotyping identified 45 cases of B-lineage ALL (consisting of 15 cases of pre-pre B-ALL, 18 pre B-ALL, and 12 B-ALL), 14 T-lineage ALL, and 4 both T- and B-lineage ALL. One myeloid antigen expression was detected in 12 of the

63 ALL patients and expressions of two or more myeloid antigens were found in 6 patients. The bone marrow samples from 10 healthy adult donors were used as control.

DNA extraction

Mononuclear cells were isolated from the bone marrow samples by fractionation with Ficoll/Hypaque gradients. High-molecular-weight DNA was extracted by 0.45% nonidet P40-proteinase K technique as described previously[6].

PCR amplification

Because the location of FLT3/ITD was restricted to exons 11 and 12, genomic PCR amplification was performed using the primers 5′-GCA ATT TAG GTA TGA AAG CCA GC-3′ (11F) and 5′-CTT TCA GCA TTT TGA CGG CAA CC-3′ (12R). The primers were synthesized by Shanghai GeneCore BioTechnologies Co, Ltd. PCR was performed in the reaction mixture in total volume of 20 μl containing 2 μl of 2 mmol/L each dNTP, 1 μl of 10×PCR buffer, 20 pmol of the primers 11F and 12R, 0.5 μg of DNA and 1 U of Taq poly- merase (Genda Tech. Corp, Toronto, Canada). The reaction was carried out through 30 cycles of denaturing at 95 ℃ for 30 s, annealing at 62 ℃ for 30 s and extension at 72 ℃ for 30 s using GeneAmp PCR system 480 (Perkin Elmer) after an initial 5-min denaturation at 95 ℃, with a final extension at 72 ℃ for 5 min before termination of the reaction. Six microliters of the PCR products underwent electrophoresis in 2.5% agarose gel with 1×TBE, and stained for 10 min with ethidium bromide (EB) before observation under ultraviolet light.

Statistical analyses

The positivity rates were compared for statistical significance using Chi-square test, and the mean values assuming Gaussian distribution were compared using t test, with those assuming nonnormal distribution examined with Wilcoxon W rank-sum test.

RESULTS

Detection of FLT3 gene in ALL cases

The PCR product of FLT3 gene displayed a distinct band of 329 bp after electrophoresis (Fig.1). Among the 63 ALL cases, 41 (65.1%) were positive for FLT3 gene. The results of FLT3 gene detection in the ALL cases of different subtypes were shown in Tab.1. The positivity rate of FLT3 gene in B-lineage ALL cases was significantly higher than that in T-lineage ALL cases ($P<0.001$). The positivity rate of FLT3 gene in well differentiated B-lineage ALL was lower than that in undifferentiated B-lineage ALL, and ALL cases with myeloid antigen expression had significantly higher positivity rate of FLT3 than those without myeloid antigen expression ($P<0.025$).

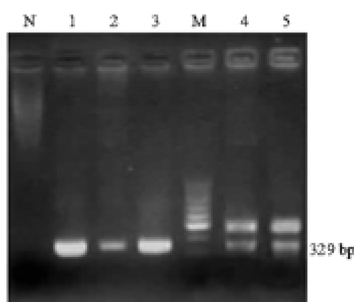

Fig.1 Result of PCR amplification of FLT3/ITD gene mutation

M: DNA marker (100 bp ladder); N: Normal control. A 329-bpband in lanes 1-3 and 5-7 was produced by PCR of the samples from patients with FLT3 gene expression. Two distinct bands in lane 4 were the PCR product of samples from patients with FLT3/ITD gene mutation.

Tab.1 Positivity rate of FLT3 gene in the ALL cases of different subtypes

	n	FLT3 gene positive cases	Positivity rate
B-lineage ALL	45	33	73.3*
pre-pre B-ALL	15	14	93.3%
pre B-ALL	18	14	77.8%
B-ALL	12	5	41.7%
T-lineage ALL	14	4	28.6%
T-B-ALL	4	4	100%
Myeloid antigen expression			
Positive	18	14	77.8%**
Negative	45	27	60%

*P<0.001 *vs* T-lineage ALL group,**P<0.025 *vs* ALL cases negative for myeloid antigen expression

Detection of FLT3/ITD gene mutation in ALL cases

As ITD occurs in the JM domain coding sequence in variable locations with different lengths in FLT3/ITD gene mutation, PCR amplification of samples with FLT3/ITD gene mutation yielded another abnormal longer PCR product in addition to the germline product of 329 bp (Fig.1). Among the 64 ALL cases, only 2 (3.2%) were found to have FLT3/ITD gene mutation, which were positive for expressions of more than two myeloid antigens.

Clinical features of ALL patients with FLT3 gene and their response to treatments

The 41 ALL patients with FLT3 gene included 23 male and 18 female patients with the median age of 26 years, who had peripheral blood white blood cell (WBC) count of (132.43±117.87)$\times 10^9$/L, hemoglobin (Hb) level of (63.92±18.34) g/L and platelet count of (41.68±23.76)$\times 10^9$/L. The peripheral leukemia cell proportion were (46.23±21.71)%. Thirty-two (78.0%) patients were found with splenohepatomegalia. No significant differences in WBC count, Hb level, platelet count, peripheral leukemia cell count or presence of splenohepatomegalia were found between the ALL patients with FLT3 gene and those without (P>0.05). After a 4-week chemotherapy with vincristine, daunorubicin/pirarubicin, asparaginase and prednisone regimen, the complete remission (CR) rate of the 41 patients with FLT3 gene was 85.3% (34/41), as compared with that of 90.1%(20/22) in patients without FLT3 gene (P>0.05).

Clinical features and clinical response of ALL patients with FLT3/ITD gene mutation

The two cases of FLT3/ITD mutation were diagnosed as ALL by morphological examination, but immunophenotyping by three-color flow cytometry with CD34/SSC gating identified the expressions of two myeloid antigens in both cases, and according to the scoring system proposed by the European Group for the Immunological Characterization of Acute Leukemias (EGIL)[7], the diagnosis of acute mixed-lineage leuke- mia was establsihed. Peripheral blood WBC count of the patients was 217$\times 10^9$/L and 261$\times 10^9$/L, Hb level 52.87 g/L and

61.43 g/L, platelet count 21.27×10^9/L and 17.31×10^9/L, and peripheral leukemia cell proportion 71% and 87%, respectively. The 2 cases had no remission after chemotherapy with similar regimen.

DISCUSSION

Proliferation and differentiation of normal hemato- poietic cells are strictly regulated by a number of cell growth factors, acting through binding with their specific receptors. Dysfunction of proliferation and differentiation resulted in malignant proliferation and cloning of leukemic cells. In normal hemopoietic cells, FLT3 was expressed predominantly in primitive hemopoietic cells including $CD34^+$ hematopoietic stem/progenitor cells and pre-B cells, which might regulate the early events in hematopoietic development. Experimental evidence showed that FLT3 mRNA was abnormally expressed in hematological malignancies, and Drexler[2]reported that 80%-100% cases of AML, part of T-ALL, B cell precursor ALL and chronic myeloid leukemia (CML) in blast crisis were found positive for FLT3 receptor expression at the mRNA level. In the present study we investigated the presence of FLT3 gene and FLT3/ITD mutation by PCR in 63 ALL patients with different immunological subtypes. FLT3 gene was detected in 41 (61.5%) cases, and both B-lineage and T-lineage ALL patients were found to have FLT3 gene, but the positivity rate of FLT3 in B-lineage ALL was significantly higher ($P<0.001$). In B-lineage ALL, the positivity rate of FLT3 decreased gradually with the cell maturation, from pre-pre B-ALL to mature B-ALL, with the rates significantly higher in in pre-pre B-ALL and pre B-ALL than in mature B-ALL cases ($P<0.005$), which was consistent with the report by Xu et al[8]that FLT3 gene was expressed more frequently in undifferentiated than in differentiated B-lineage ALL cell lines and FLT3 gene was also expressed in T-lineage ALL cell lines at the mRNA level as detected by RT-PCR. In this study we also found that FLT3 gene positivity rate in ALL cases with expression of myeloid antigens was significantly higher than that in ALL cases without myeloid antigen expression, which supported the presumption that FLT3 gene was more likely to be expressed in acute myelocytic leukemia cells[2]. The absence of significant difference in the clinical features and clinical response between ALL cases with and without FLT3 suggested the possible irrelevance of FLT3 gene with the prognosis of ALL .

In recent years ITD mutation of the FLT3 gene was reported to occur in approximately 17% to 30% of AML patients and 3% of MDS patients. Further studies showed that FLT/ITD mutation had an intimate relationship to the clinical response and prognosis of AML, and the presence of FLT3/ITD was related to high peripheral WBC count as well as peripheral leukemic cell count. AML patients with FLT3/ITD had a lower CR rate and increased risk of relapse, suggesting that FLT3/ITD plays a significant role in the progression of AML[3][4]. In this study, only 2 of the 63 (3.2%) ALL patients were found with FLT3/ITD mutation, and these 2 cases were finally diagnosed as acute mixed-lineage leukemias, further demonstrating that FLT3/ITD mutation is restricted to AML. Both of the 2 cases with FLT3/ITD mutation had high peripheral blood leukocyte count and poor clinical response, which supports FLT3/ITD gene mutation as an important indicator of poor prognosis. Detection of FLT3/ITD gene mutation might be valuable for differential

diagnosis between ALL and AML, diagnosis of acute unclassified leukemia and evaluation of the therapeutic management of acute leukemia.

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