·Clinical Research·

Correlation of Fms-like tyrosine kinase 3 (FLT3) gene expression to FLT3/internal tandem duplication mutation in peripheral blood of acute myeloid leukemia

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[Abstract] **Background and Objective**: Fms-like tyrosine kinase 3 internal tandem duplication (FLT3/ITD) is associated with an unfavorable prognosis in acute myeloid leukemia (AML). However, the role of FLT3 expression as well as its correlation to FLT3/ITD has not sufficiently studied. This study was to evaluate the relationship between FLT3 gene expression and FLT3/ITD mutation in patients with de novo AML. **Methods**: FLT3 gene expression was determined by real-time quantitative polymerase chain reaction (RQ-PCR). FLT3/ITD mutation was detected by PCR in 79 de novo AML patients. **Results**: FLT3/ITD mutations were found in 22.8% (18/79) patients. FLT3 gene expression (range: 0-7320, median: 312) was detected in 92.4% (73/79) patients, but not in normal controls. Compared to AML patients with low FLT3 expressers and without FLT3/ITD mutation, patients with high FLT3 expressers and FLT3/ITD mutation had a significantly higher white blood count as well as a higher ratio of bone marrow blasts. The positive rate of FLT3/ITD mutation was not correlated to the level of FLT3 expression, and no statistical difference of FLT3 expression was found between AML patients with and without FLT3/ITD mutation. The complete remission (CR) rate of AML patients with FLT3/ITD mutation (58.8%) was significance lower than that of those without FLT3/ITD mutation (82.1%). In AML patients without FLT3/ITD mutation, the CR rate was significantly lower in patients with high FLT3 expressers (69.2%) than in those with low FLT3 expressers (93.3%). **Conclusion**: The FLT3 expression is not associated with FLT3/ITD mutation. High-FLT3 expression may be a poor prognostic factor for AML patients without FLT3/ITD mutation.

Key words: acute myeloid leukemia, fms-like tyrosine kinase 3 (FLT3), PCR, real-time quantitative

Acute myeloid leukemia (AML) is a disease with heterogeneity, distinctive efficacy and prognosis among AML patients. Studies on AML have been focusing on investigating biological characteristics and pathogenesis of AML, identifying AML subgroups and exploring new treatments including targeted therapy. At present, using cytogenetics to stratify AML patients into three risk subgroups has been widely accepted and has positive effects on clinical treatment. However, about 50% of AML patients with normal chromosomes are classified into the moderate-risk group,

whose clinical features and prognosis are heterogeneous. Recent studies have found that molecular genetic changes, such as gene mutations or changes in the expression level, can further stratify AML patients with normal chromosomes. Therefore, in-depth study on prognosis-related genes of normal karyotype AML is important.[1]

Fms-like tyrosine kinase 3 (FLT3) gene, which is restrictively expressed in bone marrow CD34 + cells, interacts with FLT3 ligand in bone marrow stromal cells, resulting in the self-renewal, proliferation and differentiation of hematopoietic stem cell.[2] The juxtamembrane sequence internal tandem duplication (ITD) of FLT3 gene is found in about 20%-25% of AML patients, regarding as FLT3/ITD FLT3/ITD mutation, which is an important independent prognostic factors in predicting poor efficacy of AML patients.[3] To further investigate the association of the expression levels of FLT3 with FLT3/ITD mutations in AML patients, we used real-time fluorescent PCR to detect the expression level of FLT3 gene, PCR to detect FLT3/ITD mutation, and explored the correlation of FLT3 expression and FLT3/ITD mutations to prognosis of AML.

Data and Methods

Subjects. In total 79 newly diagnosed AML patients were included in the study, who were diagnosed by cell morphology, histochemical staining and flow cytometric immunophenotyping. Of these patients, 43 were men and 36 were women. The mean age was 33 years (range, 14-61 years). According to the FAB classification, three patients were minimally differentiated AML (M0), six were AML without maturation (M1), 19 were AML with maturation (M2), nine were acute promyelocytic leukemia (M3), 12 were acute myelomonocytic leukemia (M4), 23 were acute monocytic leukemia (M5) and seven were non-categorized AML. Peripheral blood specimens of 33 healthy volunteers were enrolled as normal controls, while the chronic myeloid leukemia K562 cell line was selected as positive control. All patients received induction chemotherapy of anthracyclines (including daunorubicin, idarubicin or THP) and cytosine arabinoside (3 +7) regimen.

Cell DNA, RNA extraction. DNA was extracted from single nuclear cells separated from peripheral blood samples referred to the literature.[4] RNA was extracted using Trizol Kit (Invitrogen Corporation, USA).

Primer and probe design. FLT3 gene primers and probe were designed b using Primer Express 2.0 software: upstream primer 5'-GGTGCAAAGCTGTTCATGTGA-3 ', downstream primer 5'-TCCTCGAGTGCTTT GTTTTCTAATT-3'. The amplified product was 70bp. The probe was 5'-CCATGGATTCGGC CTCACCTGG - 3'. β -actin was used as the internal control: upstream primer 5'-GCGCGG CTACAGCTTCA-3'; downstream 5'-TCTCCT TAATGTCACGCACGAT-3'. The amplified product was 59bp. The probe was 5'-CACCACG GCCGAGCGGGA-3'. Primers and probes were synthesized in high-volume desktop ABI3900 DNA Synthesis. The 5' end of the probe had fluorescence labeled FAM group. The 3' end had fluorescence labeled quenching group TAMRA. FLT3/ITD mutations are mainly located between the exon 11 and exon 12. The primer sequence of the proximal end of exon 11 and distal end of exon 12 were designed as IIF and 12R, respectively. The primer sequence for 11F was 5 '-GCAATTTAGGTATGAAAGCCAGC-3'; while that for 12R was 5'-CTTTCAGCTTTTG ACGGCAACC-3 '.The primers were synthesized by Shanghai GeneCore Company.

PCR detection of FLT3/ITD mutations. This was performed referring to the methods established by our laboratory.[4]

Construction of the fluorescent quantitative-PCR standard template. RNA was extracted from K562 cells. After amplification, the amplified products were purified according to the instruction and connected with the pMD18 vector, which were transformed into E. coliJM109. Subsequently, positive clones were cultured and the plasmids were extracted. After identification and purification by fluorescent quantitative PCR, the template was stored under the temperature of -20℃.

Reverse transcription and fluorescent quantitative-PCR The reverse transcription.

reaction system (20uL) included 5x reverse transcriptase buffer (4uL), 10pmol/uL primer (0.4uL), 25mmol/LdNTPs (0.2uL), 10U/uL reverse transcriptase (1uL), diethyl amine pyrophosphate water (DEPC) (10uL), and RNA template (4Ul). The reaction conditions were 37℃ 1h, 95℃ 3 min. The FQ-PCR reaction system (50uL) contained 5x quantitative buffer (10uL), 10pmol/uL primer (1uL), probe (1uL), 10pmol/uL dNTPs (1uL), 2U/uL Tag enzyme (2uL), cDNA (5uL) and double-distilled water (29uL). The reaction conditions were 93℃ 2min, 93℃ 30s, then 5 ℃ 45s, in total of 40 cycles. Reverse transcription was operated in PE9700 PCR Instrument (PE Corporation, USA), while FQ-PCR reaction was proceeded in PE7300 automatic fluorescence quantitative PCR Instrument (PE Corporation, USA). As the reaction completed, the results were automatically analyzed and calculated by computer.

Statistical methods. β -actin was taken as the internal reference. If the expression level of β -actin was less than $1x10^4$, the samples were regarded disqualified and should be removed. Quantitative data in this study were non-normal distribution. The median of FLT3 gene expression in AML patients was regarded as the cut-off value to divide patients into two subgroups: high and low expression of FLT3 gene groups. The mean of samples were calculated using two independent samples t test, and the rates were compared using χ 2 test. The SPSS11.0 statistical software was used for analysis. $p<0.05$ was taken as statistically significant.

Results

Sensitivity and credibility of real-time fluorescent quantitative PCR. Cryopreserved positive standard recombinant plasmid was taken out, RNA was extracted and was reversely transcribed it into cDNA, with a concentration of $1x10^6$ copies/uL. The product was 10-fold diluted into a concentration of $1x10^2$-$1x10^6$ copies/uL for quantitative PCR amplification. The method achieved a sensitivity of $1x10^2$ copies/uL. Within the detection range of 5 log10, the logarithm and the corresponding cycle threshold (C_t) had a good linear correction, with the correlation coefficient (r) of 0.996 (Fig. 1).

FLT3 mutations and expression of FLT3/ITD gene in patients with newly diagnosed AML. Because ITD mutation is a tandem duplication sequence in the juxtamembrane area of FLT3 gene, two specific bands can be found in positive cases with FLT3/ITD mutation after PCR amplification. Due to the different length of FLT3 internal tandem duplication in AML patients, the locations of the ITD-specific bands were slightly different, but all of which were more than 329bp (Fig.2). Of 79 AML patients, 18 had FLT3/ITD mutation, with the positive rate of 22.7%; 73 patients (92.4%) were detected FLT3 gene

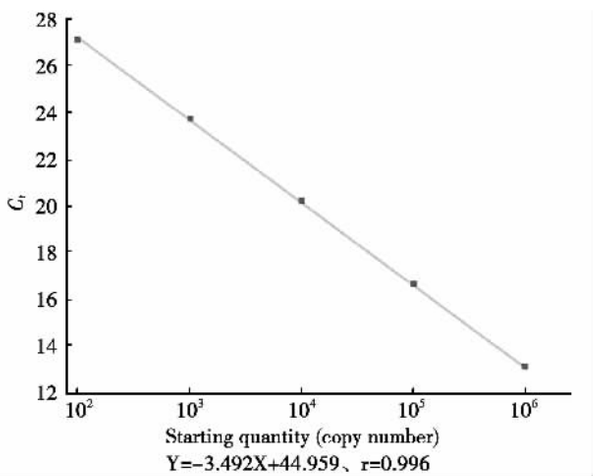

Figure 1 Standard cure for amplification of FLT3 gene using real-time quantitative polymerase chain reaction analysis

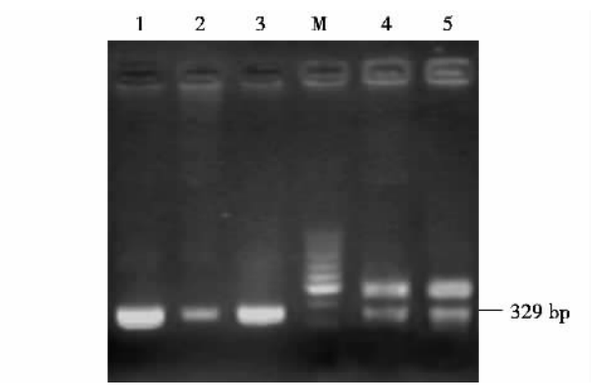

Figure 2 FLT3/ITD gene mutation detected by PCR
Lane M: DNA marker; Lanes 1, 3: patients without FLT3/ITD gene mutation; Lane 2: normal control; Lines 4, 5: patients with FLT3/ITD gene mutation.

Table 1 Correlation of FLT3 gene expression and FLT3/ITD mutation to white blood cells (WBC) count, hemoglobin (Hb), platelet (PLT) and ratio of bone marrow blast cells for acute leukemia patients at diagnosis

	Cases	WBC($\times10^9$/L)	Hb(g/L)	PLT($\times10^9$/L)	Blast ratio(%)
High FLT3 expression	39	19.7±12.1	81.3± 9.8	63.7±36.2	77.6± 9.7
Low FLT3 expression l	40	11.4± 8.9	83.6±11.9	69.7±32.1	48.3±12.8
FLT3/ITD$^+$	18	23.1±13.7	80.3± 8.4	59.6±29.4	79.2±10.3
FLT3/ITD$^-$	61	9.9± 7.2	84.5±10.6	67.8±31.5	46.5±11.7

expression by fluorescent quantitative PCR amplification, with the range of FLT3 gene expression from 0-7320 and a median of 312. The expression of FLT3 gene was not detected in peripheral blood cells of 23 healthy controls.

Association of FLT3 gene expression levels and FLT3/ITD mutation with leukemia cells of AML patients.White blood cell (WBC) count, hemoglobin (Hb), platelet count and the proportion of bone marrow leukemic cells in patients with high expression of FLT3 gene (39), low expression of FLT3 gene (40), FLT3/ITD mutations (18) and unmutated FLT3/ITD (61) are shown in Table 1. The WBC count and the proportion of bone marrow leukemic cells in patients with high expression of FLT3 gene were significantly higher than those of in patients with low expression of FLT3 gene ($p < 0.05$). Furthermore, patients with FLT3/ITD mutation had significantly higher WBC count and higher proportion of bone marrow leukemic cells compared to patients without FLT3/ITD mutation ($p < 0.01$). The levels of Hb level and PLT count were not statistically significant regardless of the expression level of FLT3 and the status of FLT3/ITD mutation ($p > 0.05$)

The relationship of FLT3 gene expression levels and FLT3/ITD mutations in A ML patients. Of 39 patients with high expression of FLT3 gene, 10 (25.6%) had FLT3/ITD mutations, while of 40 patients with low expression of FLT3 gene, eight (20.0%) had FLT3/ITD mutations. The positive rates of FLT3/ITD mutations in two groups with FLT3 expression did not significantly different ($p > 0.05$). The median level of FLT3 expression in 18 patients with FLT3/ITD mutation was 331, while that in 61 patients without FLT3/ITD mutations was 297 ($p > 0.05$).

Relationship of FLT3 gene expression levels and clinical efficacy in AML patients. After receiving two courses of induction chemotherapy, 76.7% of 73 AML patients achieved complete remission (CR). Of those, the CR rate of patients with and without FLT3/ITD mutation was 58.8% (10/17) and 82.1% (46/56), respectively ($p < 0.05$). Furthermore, the CR rate of patients with high and low expression of FLT3 gene was 68.6% (24/35) and 84.2% (32/38), respectively ($p > 0.05$). In 56 patients without FLT 3/ITD mutations, the CR rate of high and low expression of FLT3 gene was 69.2% (18/26) and 93.3% (28/30), respectively; while in the FLT3/ITD mutation-negative group, the CR rate of high FLT3 gene expression group was significantly lower compared to that of the low expression group ($p < 0.05$).

Discussion

Abnormal expression of FLT3 gene can lead to changes in response of blood cells to cell differentiation and signaling molecules, finally resulting in proliferation and colony formation of leukemic cells. Previous studies have revealed that abnormal expression of FLT3 gene exists in the majority of AML, a fraction of acute lymphoblastic leukemia and chronic myeloid leukemia in acute phase, but very rare in chronic phase CML,[2,5] implying that abnormal expression of FLT3 gene is involved with the occurrence and development of acute leukemia. This study established a real-time fluorescent quantitative PCR method to detect FLT3 gene expression, and found that FLT3 gene expression existed in 92.4% of AML patients. Furthermore, compared to low expression, high expression of FLT3 gene is associated with high WBC count in peripheral blood and the proportion of bone marrow

leukemic cells in AML patients, which confirms that FLT3 gene plays an critical role in proliferation of leukemic cells and formation of leukemia.

ITD, a new model of somatic mutation, could extend decisive sequence of proto-oncogene and activate its product. Recent studies showed that FLT3/ITD gene mutation is involved with leukemic development (refractory or relapsed). Other and our previous studies confirmed that FLT3/ITD mutation is a independent factor for poor prognosis. AML is often accompanied by a low CR rate, a high relapse rate and a short disease-free survival and overall survival.[3,4] This study showed the rate of FLT3/ITD mutations was not significantly different in high and low FLT3 expression groups, and FLT3 expression levels in AML patients with FLT3/ITD mutation were not higher than those without mutations. There is no significant correlation between high FLT3 expression and FLT3/ITD mutation, suggesting that they may influence the occurrence and development of AML by different mechanisms. Studies have shown that wild-type FLT3 activates AKT9 (protein kinase B), STAT5 and extracellular signal-regulated enzyme 1/2 (ERK1/2), mainly through the P13K (phosphotidylinositol-3 kinase) and Ras pathways. FLT3/ITD mutation, under the environment lacking of growth factors, results in automatic phosphorylation of the first intracellular domain tyrosine, then leads to activation and proliferation of hematopoietic cells through Ras and STAT5 pathways[6] This may be the reasons that no correlation of the FLT3 gene expression level and FLT3/ITD mutation is found in AML.

To further improve the treatment efficacy for AML, more detailed risk stratification and personalized treatment are needed. FLT3/ITD mutation has been confirmed to be an important indicator of poor prognosis, but the mutation was found in only about 20% of AML. Therefore, for the majority of AML without FLT3/ITD mutation, further stratification is particularly important. This study showed that, in the group without FLT3/ITD mutation, the CR rate of high FLT3 gene expression was significantly lower than that of those with low expression. Kuchenbauer et al.[7] found that high FLT3 expression may be an independent indicator of poor prognosis in AML patients without FLT3/ITD mutations. It is suggested that high FLT3 expression may be a new stratification factor in FLT3/ITD mutation-negative AML. However, due to the limited patient number of this study, we still need to explore reliable conclusions using more study subjects and follow-up resutls.

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