·CLINICAL RESEARCH·

Flt-3/ITD Mutation in Pediatric Leukemia and Its Clinical Significance

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Received: 2006-04-24
Revised: 2006-07-20

[ABSTRACT] **BACKGROUND & OBJECTIVE:** Flt-3 internal tandem duplication (Flt-3/ITD) in transmembrane region is the most frequently identified mutation in Flt-3 gene, which is the most frequently happened in acute myeloid leukemia (AML) and correlated to prognosis. This study was to explore the correlation of Flt-3/ITD mutation to the occurrence of pediatric leukemia, and analyze its clinical significance. **METHODS:** Flt-3/ITD mutation status in bone marrow samples from 302 children with leukemia, including 122 cases of AML, 124 cases of acute lymphoblastic leukemia (ALL), 17 cases of juvenile chronic myelogenous leukemia (JCML), and 39 cases of myelodysplastic syndromes (MDS), were examined by polymerase chain reaction (PCR) and sequencing. **RESULTS:** Of the 122 AML patients, 98 (80.32%) had Flt-3 gene products in bone marrow. Flt-3/ITD mutation was detected in 21 (17.21%) of the 122 patients; the mutation rates were 42.86% (3/7) in M0, 22.22% (2/9) in M1, 12.90% (4/31) in M2, 44.44% (8/18) in M4, and 15.38% (4/26) in M5. Of the 124 ALL patients, 72 (58.06%) had Flt-3 gene products in bone marrow. Flt-3/ITD mutation was found in 2 (1.61%) of the 72 patients. DNA sequencing and Blast alignment revealed that ITD within exon 11 existed in all samples, with various duplication regions and lengths (24-95 bp). No Flt-3/ITD mutation was found in MDS patients and JCML patients. Of the 19 AML patients with Flt3/ITD mutation, the median survival time was 13.5 months (0-47 months), which was significantly shorter than that of the patients without Flt-3/ITD mutation ($P<0.05$). The percentage of neutrophils in peripheral blood was similar in Flt-3/ITD-positive and Flt-3/ITD-negative patients ($P>0.05$). Chromosome karyotype analysis showed chromosome abnormity, including t(11;12)(p15;q13), inv16(q21;q23), and t(6;9)(p23;q23), in 3 AML patients with Flt-3/ITD. **CONCLUSIONS:** Flt-3/ITD mutations are usually detected in AML, seldom in ALL, not in MDS and JCML. Flt-3/ITD mutation is correlated to AML onset and progress. Flt-3/ITD is a significant marker to analyze AML prognosis.

KEYWORDS: Leukemia, children; Flt-3/ITD mutation; Prognosis; Polymerase chain reaction

Introduction

Flt-3 internal tandem duplication (Flt-3/ITD) in transmembrane region is the most frequently identified mutation in Flt-3 gene so far, which is correlated with the onset of acute myeloid leukaemia (AML) and happens most frequently in AML and affect its prognosis [1]. The latest researches demonstrate that Flt-3/ITD mutation happens in 15%-35% of the adults [2], usually between exon11 and exon 12, and the repeated sequences are different in patients[3]. We formerly reported the detection of one mutation of 29bp Flt-3/ITD in 12 Flt-3 positive AMOL cells[4]. In this study, we tested the Flt-3 expression and Flt-3/ITD mutation by PCR and DNA sequencing to explore the correlation of Flt-3/ITD mutation to leukemia onset and its clinical significance in children with different types of leukemia.

Materials and Methods

Samples

All the samples were from the children patients in the Department of Hematology in the Hospitals Affiliated Chinese Medical University and Shenyang Medical College from March, 1998 to October, 2005. Of a total of 302 patients (65 babies), there were 185 males and 117 females, and the medium age was 8 years old (3 months-14 years). Diagnosis was made by immunological, cellular genetic and cell biological examinations. There were 122 AMLs (7 M_0, 9 M_1, 31 M_2, 8 M_3, 18 M_4, 26 M_5, 10 $M_{6/7}$, 13 M_7), 124 acute lymphocytic leukemia (ALL), 17 Juvenile chronic myelogenous leukemia (JCML) and 39 myelodysplastic syndromes (MDS). Ten samples in the control group were non-leukemia patients (lymphoma, allergic purpura, autoimmune thrombocytopenic purpura) in the same term or health donors.

Reagents and instruments

Lymphocyte separation medium was purchased from BioLabs Co., PCR kit was from Promega Co., DNA sequencing box was from Applied Biosystems. PCR primers were synthesized by TaKaRa Bio Co. PCR thermal cycler and DNA sequencer 3130 were purchased from ABI Co, BioPhotometer was from Eppendorf Co.

Methods

Human genomic DNA extraction

Monocytes were separated from bone marrow of patients and the whole peripheral blood of health donors. Genomic DNAs were extracted according to the traditional phenol/chloroform/isopropylalcohol method. DNA samples were store at -20℃ for future use.

PCR primer design

A pair of primers, named as 11F and11R, was designed according to the sequences in the proximal and distal end of exon 11, where Flt-3 mutation usually happens. The amplified product was 133 bp in length[4]. In the control group, a pair of primers named actin F and actin R was also designed according to the sequences of β -actin, whose product was 305 bp in length. All primers were synthesized by TaKaRa Co.

The primer sequences were as follows:
11F: 5-CAATTTAGGTATGAAAGCC-3;
11R:5-CAAACTCTAAATTTTCTCT-3;
actin F:5-CTTCTACAATGAGCTGCGTG-3;
actin R: 5-TCATGAGGTAGTCAGTCAGG-3.

PCR amplification and electrophoresis

The PCR reaction system (50μ l) contained two primers of 0.2 μ mol/L, 0.25 mmol/L dNTPs, 2.5 U DNA polymerase, 200 ng DNA template. The PCR protocol included 35 cycles at 94℃ for 3 min, 94℃ for 1 min, 60℃ for 1 min, 72℃ for 1 min, and 72℃ for 10 min. Then 10 μ l PCR products were examined by 2% agarose electrophoresis.

Sequencing and blast analysis

PCR products were reclaimed and purified by QIAQuick Gel Extraction kit (Qiagen), sequenced in the Department of Forensic Medicine in Chinese Policeman College. All data were analyzed by on-line Blast.

Statistics analysis

All data were input into computer and analyzed by SPSS 11.5 software.

Results

PCR amplification products and electrophoresis results

A specifically amplified 133 bp band was shown in Flt-3 wild type. When Flt-3/ITD mutation happened, another band would appear above the 133bp Flt-3 band. The sites

of ITD bands were different in patients, because of the different lengths of Flt-3/ITD (Figure 1). Ninety-eight samples were Flt-3 positive in 122 AMLs (80.3%). Among them, 21 samples, including 3 M_0, 2 M_1, 4 M_2, 8 M_4, 4 M_5, showed Flt-3/ITD and the mutation rate was 17.2%. Seventy-two samples Of 124 ALLs were Flt-3 positive and the positive rate was 58.1%. Among them, 2 samples showed Flt-3/ITD and the mutation rate was 1.6%. No ITD mutation was detected in 39 MDSs and 17 JCMLs (Table 1).

Figure 1 Flt-3/ITD expression in some leukemia patients detected by polymerase chain reaction (PCR)

Lane M: marker; lane N: normal; lanes 1, 2: M0; lanes 3, 4: M1; lanes 5–7: M2; lanes 8–11: M4; lanes 12, 13: M5; lane 14: ALL.

Table 1 Flt-3 gene expression and Flt-3/ITD mutation frequency in children leukemia patients[case(%)]

Diagnosis	Cases	Flt-3 gene expression	Flt-3/ITD mutation
AML	122	98(80.3)	21(17.2)
M0	7	6(85.7)	3(42.9)
M1	9	7(77.8)	2(22.2)
M2	31	28(90.3)	4(12.9)
M3	8	6(75.0)	0
M4	18	14(77.8)	8(44.4)
M5	26	21(80.8)	4(15.4)
M6/M7	7	6(85.7)	0
M7	16	13(81.2)	0
ALL	124	72(58.1)	2 (1.6)
JCML	17	14(82.4)	0
MDS	39	32(82.0)	0

AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; JCML, juvenile chronic myelogenous leukemia; MDS, myelodysplastic syndromes.

Sequencing of PCR products

PCR products from 23 Flt-3/ITD patients were analyzed by DNA sequencing and Blast. All samples showed ITD at various degrees with different regions and lengths (24-95 bp). Results from sequencing and Blast are shown in Figure 2. Seven insertion sequences existed in 7 samples (ITD and insertion simulation are demonstrate in Figure 3). Repeated sequence, point mutation or insertion was not detected in the samples without Flt-3/ITD.

Clinical characteristics of Flt-3/ITD AML patients

In 21 Flt-3/ITD AML patients, the number of leukocytes in peripheral blood was (31.25± 5.67) × 10^9/l, of hemoglobin (Hb) was (75.97± 1.46) g/l, of platelet (Plt) was (71.38 ± 12.51) × 10^9/l. Among them, 17 (80.95%) patients showed hepatomegaly or splenomegaly at various degrees, with the leukocyte ratio of (79.16± 9.28)% in bone marrow. While in AMLs without Flt-3/ITD, the number of leukocytes, Hb and Plt was (28.98± 7.56)× 10^9/l, (70.16± 8.46) g/l, and (78.59 ± 9.42) × 10^9/l, respectively and the average ratio of granulocytes was (9.28 ± 0.56)%. Statistical analysis showed that the difference of the number of leukocytes in peripheral blood, Hb, leukocyte ratio in bone marrow and hepatomegaly or splenomegaly rate between 21 Flt-3/ITD AML patients and AMLs without Flt-3/ITD were not statistically significant (P> 0.05).

Correlations of Flt-3/ITD to clinical prognosis

The median age of 21 Flt-3/ITD AML patients was 9.5 (0.3-14 years old). Nineteen patients died in short term though they received regular chemotherapy at first diagnosis. The median survival time (MST) was 15 months (ranged from 0.3 to 47 months); the death rate was 90.48%; 2 patients are still alive during follow-up (Table 2). Meanwhile, 299 patients without Flt-3/ITD survived except 3 and the death rate was 1.11%. The difference of death rate between patients with and without Flt-3/ITD was statistically significant (P<0.05). Chromosome karyotype analysis demonstrated that 3 chromosome translocations existed in 21 Flt-3/ITD patients: they were t (11;12)(p15; q13), inv16(q21;q23), t(6;9)(p23;q23). And life span of these 3 patients was 0, 5 and 9 months, which was significantly shorter than those without translocation (P<0.05). Two ALL patients with Flt-3/ITD were both alive during follow-ups.

Discussion

Flt-3 is one of the type Ⅲ receptor tyrosine kinase (RTK), whose gene is located in 13q12 in human beings and encodes 993 amino acids. The gene comprises 5 immunoglobulin-like domains: juxtamembrane (JM), 2 transmembrane tyrosine kinase (TK1,TK2)

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Case 5      GATCTCAAATGGGAGTTTCCAAGAAATTTAGAGTTTGggaaggtactaggatcaggtgcttttggaaaatgggagtttccaagagaaaatt
            ************************************************
Wild  1798- GATCTCAAATGGGAGTTTCCAAGAAATTTAGAGTTTG-------------------------------------------------------

Case  5     tagagtttgGGAAGGTACTAGGATCAGGTGCTTTTGGAAAA
                     ********************************************
Wild  1838- -----------GGAAGGTACTAGGATCAGGTGCTTTTGGAAAA-1869

Case 7      CCTCAGGATAATGAGTACTTCTACGTTGATTTCAGAGAATATGAATATGATCTCAAATGGGAGTTTCCAAG
            ***********************************************************************************************
Wild  1751- CCTCAGGATAATGAGTACTTCTACGTTGATTTCAGAGAATATGAATATGATCTCAAATGGGAGTTTCCAAG

Case 7      AGAAAATTTAGAGTTAGggaaggtactaggatcaggtgc^CA ataatgagtacttctacgttgatttcagagaatatgaatatgatctcaaatgggagtttcc
            ************************
Wild  1827- AGAAAATTTAGAGTTAG-------------------------------------------------------------------------------

Case 7      aagagaaaatttagagttttgGGAAGGTACTAGGATCAGGTGCTTT
                                 ***********************************
Wild  1838- ------------------------GGAAGGTACTAGGATCAGGTGCTTT-1862

Case 11     AGAGAATATGAATATGATCTCAAATGGGAGTTTCC^GAGCCTT tatgaatatgatctcaaatgggagtttccAAGA
            ***********************************************                          *****
Wild  1783- AGAGAATATGAATATGATCTCAAATGGGAGTTTCC-----------------------------------------AAGA-1821

Case 14     GAGTACTTCTACGTTGATTTCAGAGAATATGAATATGATCTCAAATGGGActacgttgatttcagagaatatgaatatgatc
            ******************************************************************
Wild  1762- GAGTACTTCTACGTTGATTTCAGAGAATATGAATATGATCTCAAATGGGA-----------------------------------

Case 14     tcaaatgggaGTTTCCA
                      *********
Wild  1812- -------------GTTTCCA-1818
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Asterisk capital letters represent the identical sequence; small letters represent the duplication sequence in capital letters with cross lines at their 5′ ends; small italic letters in boldface represent the duplication sequence in capital letters with cross line at their 3′ ends; superscript capital letters in boldface represent insertion sequence.

Figure 2 Results of base sequencing and blast alignment analysis in some patients

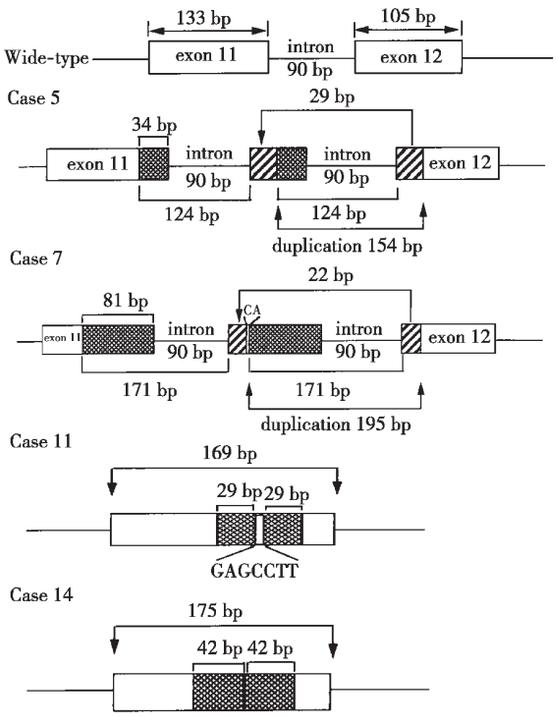

Figure 3 Models of Flt-3 gene duplication and insertion in some patients

and C-terminal intracellular region [5]. Flt-3 gene mutation could destroy the regular proliferation and apoptosis of the blood cells to induce leukemia [5-6].

ITD in JM region is the most frequent mutation of Flt-3 gene, which is named Flt-3/ITD. In 1996, Nakao et al[7] first reported that Flt-3/ITD existed in AML and correlated with its onset. Ever since, researches have revealed that Flt-3/ITD existed in 19.2%-32.0% of AML patients [8], and the rate of Flt-3/ITD patients who had 8 years of event-free survival (EFS) was 7%, suggesting that Flt-3/ITD is correlated with poor prognosis.

In recent years, haematological researchers in China have paid much attention on the function of Flt-3/ITD mutation in the onset of AML [10-13]. But little was reported about the detection of Flt-3/ITD mutation in the children patients [13]. In this study, Flt-3/ITD mutation was detected by PCR and DNA sequencing in 302 children patients. Results demonstrated that 98(80.32%) of 122 AML patients showed positive Flt-3. Among them, 21 (16.80%) samples showed Flt-3/ITD mutation, 3 M_0, 2

Table 2 Clinical data and prognosis of the patients with Flt-3/ITD

Case	Age(years)	Sex	Diagnosis(FAB classification)	Karyotype	Prognosis	Survival time(months)
1	11	Male	AML(M0)	Normal	Died	21
2	9	Male	AML(M0)	Normal	Died	18
3	7	Male	AML(M0)	Unclear	Died	18
4	14	Female	AL(M1)	t(11;12)(p15;q13)	Died	0
5	12	Female	AML(M1)	Unclear	Died	14
6	11	Male	AML(M2)	Unclear	Survival	32+
7	6	Male	AML(M2)	t(6;9)(p23;q23)	Died	9
8	3	Male	AML(M2)	Unclear	Died	42
9	7	Male	AML(M2)	Normal	Died	13
10	14	Male	AML(M4)	Normal	Died	47
11	14	Male	AML(M4)	Normal	Died	13
12	8	Female	AML(M4)	Normal	Died	15
13	10	Male	AML(M4)	Normal	Survival	28+
14	12	Male	AML(M4)	Unclear	Died	23
15	13	Male	AML(M4)	Unclear	Died	13
16	10	Male	AML(M4)	Unclear	Died	16
17	0.3	Male	AML(M4)	inv16(q21;q23)	Died	5
18	12	Female	AML(M5)	Normal	Died	6
19	12	Female	AML(M5)	Normal	Died	12
20	14	Male	AML(M5)	Normal	Died	9
21	13	Male	AML(M5)	Normal	Died	15
22	10	Male	ALL(L1)	Normal	Survival	55+
23	8	Female	ALL(L2)	Unclear	Survival	83+

Unclear: chromosome karyotype examination has not been performed in these cases.

M_1, 4 M_2, 8 M_4, 4 M_5, which was consistent with other reports (20.59%). Latest researches show that Flt-3 genome comprises 24 instead of 21exons, so that ITD in juxtamembrane region usually happens in exon 14 or exon 15 [14].

Our research demonstrated that ITD in juxtamembrane region happened very frequently in exon 11 in children AML patients. This is the main reason of AML onset in children, which was supposed by Xu et al[3]. Besides, Flt-3/ITD has a high incidence in children patients of AML M_0 (42.86%) and M_4 (44.44%) (M_4). In our study, 72 patients (58.06%) showed positive Flt-3 in 124 ALL patients. Among them, 2 patients (1.61%) had Flt-3/ITD mutation. No marrow line antigen was detected in these 2 patients, which suggests that Flt-3/ITD mutation also happens in children ALL, but the reason remains unknown.

Until now, the mechanism of AML onset is not clear. Studies show that the death rate of patients without Flt-3/ITD is greatly lower than that of patients with Flt-3/ITD ($P<0.05$). This suggests that Flt-3/ITD mutation is correlated with the onset and progress of children leukemia, especially AML. Flt-3/ITD mutation may constitutively active Flt-3 kinase, the down-stream STAT5 and RAS/MAPK pathway, promote the proliferation and maintain malignant clones to trigger malignant leukemia [14]. Additionally, TF-ⅡD inhibits the function of transcriptional factors (TF) in the marrow line, and destroys the normal function of Flt-3 [15]. Besides, Flt-3 autophosphorylation caused by the point mutation (PM) in exon 20 in tyrosine kinase domain (TDK) is also the cause of malignant leukemia onset. Flt-3 mutation usually happens with chromosome translocation [16]. Chromosome karyotype analysis in our research showed that the life span of AML patients with Flt-3/ITD and chromosome translocation was much shorter

than that of AML patients with Flt-3/ITD but without chromosome translocation, which was only 0 month, 5 months and 9 months ($P<0.05$). This demonstrates that the synergism of Flt-3/ITD and chromosome translocation may correlate with AML onset as well as its prognosis.

Flt-3 mutation is one of the important signs to evaluate AML prognosis. Research shows that AML children patients with Flt-3 mutation have poor prognosis. Flt-3/ITD in juxtamembrane region exists in 20% of children patients. The induced-release rate and EFS in this positive group are greatly lower than those in the negative group. In our research, 21 patients with Flt-3/ITD received regular chemotherapy at first diagnosis and among them, 19 patients died in short term. The average survival time was only 26.24 months (0-47 months), which was significantly different from that of patients without Flt-3/ITD. This shows that Flt-3/ITD detection is important in evaluating leukemia prognosis. Besides, this also indicates that the research about target inhibition is significant.

[参 考 文 献]

Stirewalt D L, Radich J P. The role of Flt-3 in haematopoietic malignancies [J]. Nat Rev Cancer, 2003,3(9):650-665.

Steudel C, Wermke M, Schaich M, et al. Comparative analysis of MLL partial tandem duplication and Flt-3 internal tandem duplication mutations in 956 adult patients with acute myeloid leukemia [J]. Genes Chromosomes Cancer, 2003,37(3):237-251.

Xu F, Taki T, Yang H W, et al. Tandem duplication of the Flt-3 gene is found in acute lymphoblastic leukaemia as well as acute myeloid leukaemia but not in myelodysplastic syndrome or juvenile chronic myelogenous leukaemia in children [J]. Br J Haematol, 1999,105(1):155-162.

汪亚伦, 王 彤, 许 凤, 等. 急性髓系白血病(AML)细胞 Flt-3 表达及 Flt-3/ITD 突变分析 [J]. 中国实验血液学杂志, 2006,14(3):446-449.

Kiyoi H, Yanada M, Ozekia K. Clinical significance of Flt-3 in leukemia [J]. Int J Hematol, 2005,82(2):85-92.

Yang X, Liu L, Sternberg D, et al. The Flt-3 internal tandem duplication mutation prevents apoptosis in interleukin-3-deprived BaF3 cells due to protein kinase A and ribosomal S6 kinase 1-mediated BAD phosphorylation at serine 112 [J]. Cancer Res, 2005,65(16):7338-7347.

Nakao M, Yokota S, Horiike S, et al. Internal tandem duplication of the Flt-3 gene found in acute myeloid leukemia [J]. Leukemia, 1996,10(12):1911-1918.

Gale R E, Hills R, Kottaridis P D, et al. No evidence that Flt-3 status should be considered as an indicator for transplantation in acute myeloid leukemia (AML): an analysis of 1135 patients, excluding acute promyelocytic leukemia, from the UK MRC AML10 and 12 trials [J]. Blood, 2005,106(10):3658-3665.

Meshinchi S, Woods W G, Stirewalt D L, et al. Prevalence and prognostic significance of Flt-3 internal tandem duplication in pediatric acute myeloid leukemia [J]. Blood, 2001,97(1):89-94.

徐 兵, 周淑芸, 李 冰, 等. PCR 分析急性淋巴细胞白血病患者 Flt-3 基因及 Flt-3/ITD 基因突变 [J]. 中华微生物学和免疫学杂志, 2003,23(12):978-979.

王莉红, 周春林, 张新伟, 等. Flt-3 基因内部串联重复突变与急性白血病的关系及临床意义 [J]. 中华血液学杂志, 2004,25(7):393-396.

徐 兵, 林 榕, 周淑芸. 老年人急性髓系白血病 Flt-3/ITD 基因突变的检测及临床意义[J]. 实用医学杂志, 2003,19(12):1310-1311.

黄 河, 段秀梅, 黄 晶. Flt-3 基因内部串联复制与儿童急性白血病的关系 [J]. 中国妇幼保健, 2006,21(1):105-106.

Zheng R, Friedman A D, Levis M, et al. Internal tandem duplication mutation of Flt-3 blocks myeloid differentiation through suppression of C/EBPalpha expression [J]. Blood, 2004,103(5):1883-1890.

Van Vlierberghe P, Meijerink J P, Stam R W, et al. Activating Flt-3 mutations in $CD4^+/CD8^-$ pediatric T-cell acute lymphoblastic leukemias [J]. Blood, 2005,106(13):4414-4415.

Kottaridis P D, Gale R E, Frew M E, et al. The presence of a Flt-3 internal tandem duplication in patients with acute myeloid leukemia (AML) adds important prognostic information to cytogenetic risk group and response to the first cycle of chemotherapy: analysis of 854 patients from the United Kingdom Medical Research Council AML 10 and 12 trials [J]. Blood, 2001,98(6):1752-1759.

[编辑:张菊;校对:夏宁静]