Leukemia (1997) 11, 1605–1609

RAPID COMMUNICATION

Internal tandem duplication of the FLT3 gene is preferentially seen in acute myeloid leukemia and myelodysplastic syndrome among various hematological malignancies. A study on a large series of patients and cell lines

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In this study, we examined a large number of patients to clarify the distribution and frequency of a recently described FLT3 tandem duplication among hematopoietic malignancies, including 112 acute myelocytic leukemia (AML), 55 acute lymphoblastic leukemia (ALL), 37 myelodysplastic syndrome (MDS), 20 chronic myelogenous leukemia (CML), 30 non-Hodgkin's lymphoma (NHL), 14 adult T cell leukemia, 15 chronic lymphocytic leukemia (CLL) and 38 multiple myeloma (MM). We also evaluated 71 cell lines derived from 11 AML, 31 ALL, two hairy cell leukemia, three acute unclassified leukemia, 10 CML, 12 NHL including six Burkitt's lymphoma, and two MM. Using genomic PCR of exon 11 coding for the juxtamembrane (JM) domain and first amino acids of the 5′-tyrosine kinase (TK) domain, this length mutation was found only in AML (22/112, 20%) and MDS (1/37). According to the FAB subclassification, they were 5/18 (28%) of M1, 4/29 (14%) of M2, 3/17 (18%) of M3, 6/24 (25%) of M4, 4/20 (20%) of M5 and 1/9 of refractory anemia with excess of blast in transformation. In the various cell lines examined, this abnormality was determined in only one derived from AML and never found in other hematological malignancies. The sequence analysis of the abnormal PCR products revealed that 23 of 24 showed internal tandem duplication with or without insertion of nucleotides. In one AML, insertion and deletion without duplication was determined. All 24 lengthened sequences were in-frame. Duplication takes place in the sequence coding for the JM domain and leaves the TK domain intact. In conclusion, we emphasize that the length mutation of FLT3 at JM/TK-I domains were restricted to AML and MDS. Since all these mutations resulted in in-frame, this abnormality might function for the proliferation of leukemic cells.
Keywords: FLT3; tandem duplication; mutation; AML; MDS

Introduction

The FLT3 (fms-like tyrosine kinase, also named FLK2 or STK1) gene encodes a receptor tyrosine kinase (RTK) that is closely related to two well-known receptors, KIT and FMS, that regulate proliferation and differentiation of hematopoietic cells with their respective ligands, stem-cell factor (SCF) and macrophage colony-stimulating factor (M-CSF).[1–4] Initial studies have reported that FLT3 is expressed in the hematopoietic and nervous system, the gonads and the placenta.[3,5] Since FLT3 is expressed predominantly in primitive hematopoietic cells showing CD34 molecules, it might regulate the early events of hematopoietic development.[2,4] In hematological malignancies, expression of FLT3 receptor both at mRNA and protein levels was observed predominantly in precursor B-ALL and AML.[6–9]

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Received 17 February 1997; accepted 25 June 1997

FLT3 gene has some structural similarities including the number of exons, size of exons and exon/intron boundaries with genes RTKs, FMS, KIT, and platelet-derived growth-factor receptor (PDGFR). They are composed of a heavily glycosylated extracellular region with five immunoglobulin-like subdomains, a transmembrane (TM) domain, two parts of intracellular tyrosine kinase (TK) domain divided by a kinase insert (KI), and a juxtamembrane domain (JM) which may regulate the kinase activity of this gene's products.[10,11] Activation of tyrosine kinase is mediated by an allosteric dimerization process through binding with its ligand.[12] The biological function of FLT3 has been well studied. A direct approach to its function by targeted disruption of the FLT3 gene, as well as a number of cellular biological studies clarified the important role of this gene in the differentiation of multipotent stem cells.[13] However, the potential role of FLT3 in tumor genesis was not clear because a somatic mutation of this gene has not been reported.

We recently demonstrated internal tandem duplication within JM/TK-I domains as a somatic mutation of FLT3 found in 17% of patients with acute myelogenous leukemia (AML).[14] Since this mutation was not found in any patients with acute lymphocytic leukemia, we described that this mutation could be specific in myeloid malignancies. To clarify the incidence and distribution of the FLT3 mutation among hematological malignancies, we examined a large number of patients with various hematological diseases.

Materials and methods

Patients and samples

This study included 112 adult *de novo* AML, 37 MDS, 55 adult ALL, 14 adult T cell leukemia (ATL), 30 non-Hodgkin's lymphoma (NHL), 15 chronic lymphocytic leukemia (CLL), 38 multiple myeloma (MM), seven chronic myelogenous leukemia (CML) in chronic phase (CP), 13 CML in blastic phase (BC), and three essential thrombocythemia (ET). All AML and ALL patients were examined at presentation. The diagnoses of patients with AML and MDS were made according to the French–American–British (FAB) classification (Table 1).[15,16]

Cell lines

Seventy-two cell lines established from various hematological malignancies were examined in this study. The origin of these cell lines included 11 AML, 31 ALL, two hairy cell leukemia (HCL), three acute unclassified leukemia (AUL), 11 CML, 12

NHL including six Burkitt's cell lymphoma (BL), and two MM. The names and origin of cell lines were as follows: AML-derived cell lines, MOLM-13, M-07e, HL-60, PL-21, KG-1, ML-1, ML-2, MKB-1, THP-1-0, EoL-1, and ME-1; ALL-derived cell lines, NALM-19, NALM-26, NALM-30, NALM-27, NALM-28, NALM-21, NALM-31, NALM-32, BV-173, HAL-1, OM9;22, NALM-29, NALM-24, NALM-33, KOPN-8, NALM-6, NALM-17, HPB-Null, Reh, Kid-92, RPMI-8402, HPB-ALL, BALL-1, MOLT-3, Jurkat, NALM-16, BALM-1, BALM-6, BALM-16, BALL1 and MOLT-4; those derived from HCL, Hair-M, and JOK-1; those derived from AUL, NALM-20, Tahr-87, and MR-87; those derived from CML, MOLM-6, MOLM-7, MOLM-7, MOLM-11, MOLM-13, JOSK-M, K562, NALM-1, MOLM-1, GDM-1, and KU-812; those derived from NHL, SKW-4, SU-DHL-4, U937, BALM-3, BALM-4, and BALM-5; those derived from BL, BALM-13, BALM-14, BALM-9, BALM-10, BALM-11, and BALM-12; those derived from MM, RPMI-8226, and ARH-77. Cells were grown and maintained by serial passages in appropriate media supplemented with 10–20% (v/v) heat-inactivated fetal bovine serum (PBS).

Screening of the length mutation in TM, JM, and kinase domains of FLT3 gene by genomic PCR

Genomic DNA was extracted from bone marrow samples using a standard procedure.[17] To detect the length mutation in the JM and 5′ TK-I domains by PCR method, we used two sets of primers, 10F/10R and 11F/11R, as described in the previous study.[14] The former set of primers covers the whole exon 10 which encodes the entire TM domain and the first 19 nucleotides of the JM domain. The latter set of primers amplifies the whole exon 11 encoding a remaining JM and the initial 16 nucleotides of the TK-I domains.[14] The products were electrophoresed on 2% agarose gel (FMC, Rockland, ME, USA) and 8% polyacrylamide gel. When an unexpected product was obtained, it was confirmed by a repeated PCR. Since duplication may involve the intron sequence between exons 11 and 12 like case M155 in the previous study, amplification of exons 11 to 12 using the 11F/12R primer combination, based on the genomic sequence of the FLT3 gene, was also carried out to confirm the length abnormality.[11] In order to analyze the intracellular region of the FLT3 receptor, we also analyzed exon 12 to 19 by genomic amplification.[14]

Sequencing

Abnormal PCR fragments were cloned into *pCRII* plasmid vector (Invitrogen, San Diego, CA, USA) and sequencing was carried out using a T7 sequencing kit (Pharmacia LKB, Uppsala, Sweden). Alternatively, a DNA sequencing system (Model 373A; Applied Biosystems, Foster City, CA, USA) was used to sequence cloned fragments.

Results

Length mutations in JM and 5′ TK-I domains of FLT3 gene were completely restricted to AML and MDS

Among 324 patients with hematological malignancies examined by the amplification of genomic DNA, 23 had longer PCR products by amplifying exons 11 and 12 of the FLT3 gene (Table 1). The length abnormality was not found in exon 10 in any case, as we reported previously.[14] Two patients, L184 and L319, showed abnormal bands only after amplification by a 11F/12R primer combination. All 23 patients were restricted only to AML and MDS. In the patients with other hematological malignancies, including ALL, ATL, NHL, CLL, MM, CML, and ET, no length abnormalities were detected.

Among AML patients, the abnormal band was observed in patients with 0/2 of M0, 5/18 (28%) of M1, 4/29 (14%) of M2, 3/17 (18%) of M3, 6/24 (25%) of M4, 4/20 (20%) of M5, 0/1 of M6, and 0/3 of M7 according to the FAB classification. This abnormality was also detected in one patient with MDS diagnosed as refractory anemia with excess of blast in transformation (RAEB-T).

Among 71 cell lines examined, only one, MOLM-13 originated from AML-M5, had a length abnormality (Table 1). The other cell lines derived from either ALL, AUL, HCL, CML, ML or MM showed no abnormality, demonstrating again the restriction of this abnormality to AML. Interestingly, the incidence of FLT3 abnormality in AML cell lines (1/11, 9%) was not higher than that of the patient's (20%).

PCR amplification of exon 11 to exon 19 of the FLT3 gene showed no products of unusual size (data not shown), suggesting the length mutation was restricted to exons 11 and 12 in the FLT3 gene.

Most of the length abnormalities in JM and 5′ TK-I domains of FLT3 gene were originated from internal tandem duplication

We sequenced the PCR products the length of which were larger than the wild-type in 22 AML and one MDS patient and one cell line, MOLM-13. We also deduced the amino acid sequences from the DNA sequences. In 23 of 24 cases examined, the tandem duplication in exon 11 was observed. In one AML patient, L429, insertion of 36 nucleotides and deletion of three nucleotides without duplication within exon 11 was determined. We summarized the characteristics of 24 sequences in the present study (Table 2, Figure 1).

The length of duplicated DNA sequences varied from 17 to 198 bp. Most markedly, the starting and ending sites of duplication were different in each case; therefore, no identical duplication was observed. However, the target regions for duplication seemed to be limited. In 21 of 23 cases with the tandem duplication, the target sequences of duplication were restricted within exon 11. However, one case, L319, included the initial 4-bp intron sequence and another case, L184, including a whole intron and a 5′ part of exon 12. In the former case, the duplicated part of the intron sequence seemed to be involved in the reading frame. In the latter case, a new exon was presumably created by tandem fusion of a 5′ part of exon 12 and a 3′ part of exon 11 like case M155 in our previous study where we confirmed this configuration by RT-PCR.[14]

In 15 cases, simple duplication occurred without any deletion or insertion of nucleotides within exon 11. The numbers of nucleotides duplicated in all these cases were multiples of three. In five (L231, L305, L453, L267, and MOLM-13) of these cases, the duplication was initiated at the first nucleotide of each coding triplet, which could make the altered transcripts strictly in-frame. In the remaining 10 cases, three (L335, L324 and L332) had only a substitution of the first position of the added AA sequence by the other AA (E to D), and seven coincidentally had the same AA sequences despite the nucleotide sequence in the junctional codon being

Table 1 Characteristics of patients and cell lines and incidence of FLT3 length mutation

Diagnosis	*No. of cases examined*	*Length mutation in JM and 5′TK-I of FLT3 gene (%)*
AML (total)	112	22 (20)
M0	2	0
M1	18	5 (28)
M2	29	4 (14)
M3	17	3 (18)
M4	24	6 (25)
M5	20	4 (20)
M6	1	0
M7	1	0
MDS		
RA	5	0
RAEB	6	0
RAEB-T	9	1 (11)
CMML	17	0
Lymphoid malignancies		
ALL	55	0
ATL	14	0
NHL	30	0
CLL	15	0
MM	38	0
Myeloproliferative disorders		
CML (total)	20	0
CP	7	0
BC	13	0
ET	3	0
Cell lines		
AML	11	1 (9)
ALL	31	0
HCL	2	0
AUL	3	0
CML	10	0
NHL	6	0
BL	6	0
MM	2	0

altered. In eight of 23 cases who had the tandem duplication, insertion of nucleotides, ranging from two to 22, was observed between the duplicated regions. Accordingly, the numbers of nucleotides added to the wild-type by duplication and insertion varied from 18 to 204. Interestingly, the insertion of these nucleotides made the total number of increased nucleotides multiples of three. In most cases where the numbers of duplicated sequences were multiples of three, no insertion of nucleotides occurred. In three cases, L179, L428, and L319, the inserted nucleotides could be assigned to nt 1–12 of intron 11, nt 1744–1765, and nt 1785–1793 of exon 11, respectively. In six of nine cases, inserted sequences could not be matched to any part of the wild-type sequences of whole cDNA and intron 11 of FLT3.

By comparing the abnormal sequences with wild-type TM, JM, and TK-I sequences, we determined the extents of the duplicated regions (Table 2, Figure 1). In any case, the deduced AA sequence at the end of the JM domain remained unchanged. Most interestingly, there was no duplication that started downstream of nt 1882, the starting point of the coding region for TK-I. This means that the continuity of a wild-type coding sequence of TK-1 remains intact by this mutation in all cases. If the duplication started in the TK domain, the following wild-type AA sequence would be altered.

Discussion

In the present study of a large number of hematological malignancies, we found that the length mutation was restricted to AML and MDS. We found no length mutation in CML patients by amplification of genomic DNA either in CP or BC. This mutation might not play an important role in aggravating the disease in CML. Among various cell lines of hematological malignancies, we found this mutation only in one cell line established from AML with M5 phenotype. The sequence analyses revealed most of the abnormal amplicons derived from internal tandem duplication within the JM and 5′ TK domains. All of the abnormal products were in-frame and the deduced AA sequence of TK-I domain remained unchanged. In addition, we found the minimum AA regions in the JM domain that were inevitably involved in the duplication.

The expression of FLT3 mRNA was seen in the majority of lymphohemopoietic organs and CD34-positive bone marrow cells, a population greatly enriched with stem/progenitor cells.[3,4] In hematological malignancies, FLT3 expression was observed at a high level in leukemic blasts from most acute leukemias, particularly precursor B-ALL and AML, at both mRNA and protein levels.[18,19] Increased expression of FLT3 was not detected in other hematological neoplasms. Recent studies revealed that FLT3 ligand led to a significant proliferative response in leukemic cells from either fresh samples or cultured cells of AML.[20] In contrast, the leukemic cells from precursor-B-ALL did not respond well to FL even in the cases in which strong expression of the receptor protein was observed.[7,18,21] In normal murine B cell lymphopoiesis, only the most immature progenitor cells can respond to its ligand.[22] These findings might imply that the function of the FLT3 protein as a promoter for the proliferation of hematopoietic cells is limited to the myeloid lineage. Consequently, mutations of the FLT3 gene which might affect the growth of leukemic cells can only occur in myeloid lineage. Acquisition of FLT3 mutation might not influence the growth of leukemic cells in the majority of ALL cases. The finding that the length mutation was not detected in a large number of ALL-derived cell lines seem to support this hypothesis.

The fact that all the tandem duplications resulted in in-frame strongly suggest that the abnormal protein product derived from this mutation functions dominantly and increases the growth of the leukemic cell.

The mechanisms by which the length mutation affect cell growth is of great interest. We have shown that an insertional mutation was not detected in the extracellular part of FLT3.[14] The recent study on the kinase activity of EGF receptor by introducing insertional mutations revealed that insertion of hydrophilic AA sequences on both the extracellular and cytoplasmic sides of the TM domain exhibited dramatically reduced EGF binding but enhanced kinase activity. In contrast, insertion of AA sequence in the juxtamembrane domain of the cytoplasmic side alone affected neither the EGF-binding ability nor the kinase activity of the EGF receptor.[23] Therefore, elongation of the intracellular JM domain alone might not enhance the cell growth.

Another possible mechanism is a gain of a specific AA sequence which binds with SH2-containing cytoplasmic signalling proteins. SH2 domains directly recognize certain phosphorylated tyrosine residues of the RTK protein. It has been

Table 2 Characteristics of the length mutation of FLT3

Case	*Diagnosis*	*Start-end of duplication*[a]	*No. of nt duplicated*	*No. of nt inserted (sequences)*	*Total No. nt increased*	*No. of deduced AA increased*
L179	M1	1745–1837 (ex 11)	93	12 (#1)	105	35
L231	M1	1756–1824 (ex 11)	69	0	69	23
L419	M1	1766–1834 (ex 11)	69	0	69	23
L433	M1	1793–1837 (ex 11)	45	0	45	15
L428	M1	1744–1799 (ex 11)	56	22 (#2)	78	26
L177	M2	1754–1805 (ex 11)	52	2 (#3)	54	18
L319	M2	1800–1837 (ex 11)	38	9 (#4)	51	17
		nt 1–4 (int 11)	4			
L335	M2	1770–1793 (ex 11)	24	0	24	8
L429	M2	No	0	36 (#5)	33[b]	11
L305	M3	1780–1800 (ex 11)	21	0	21	7
L434	M3	1790–1807 (ex 11)	18	0	18	6
L804	M3	1798–1815 (ex 11)	18	0	18	6
L219	M4	1764–1780 (ex 11)	17	4 (#6)	21	7
L453	M4	1759–1833 (ex 11)	75	0	75	25
L770	M4	1767–1801 (ex 11)	35	7 (#7)	42	14
L282	M4	1738–1801 (ex 11)	64	8 (#8)	72	24
L324	M4	1758–1787 (ex 11)	30	0	30	10
L184	M4	1772–1837 (ex 11)	66	6 (#9)	204	38
		nt 1–90 (int 11)	90			
		1838–1879 (ex 12)	42			
E247	M5	1746–1814 (ex 11)	69	0	69	23
L286	M5	1772–1828 (ex 11)	57	0	57	19
L267	M5	1759–1833 (ex 11)	75	0	75	25
L322	M5	1773–1811 (ex 11)	39	0	39	13
L17	RAEBT	1773–1817 (ex 11)	45	0	45	15
MOLM13	M5	1783–1803 (ex 11)	21	0	21	7

[a]Nucleotide numbers correspond to Figure 3 in Ref. 4
[b]Three bases of nucleotides were deleted.
#1, gtaagaatggaa; #2, accggctcctcagataatgagt; #3, cc; #4, agaatatga; #5, atgggaatgggggagaatgtaatcccgggagacaa; #6, gggg; #7, cccgtgg; #8, ctaccagc, #9, taaggg.

Figure 1 The range of duplication within JM and 5′ TK-I domains is matched with the wild-type sequence. Horizontal bars represent the extents of duplication. Adjacent letters indicate amino acids which were added by insertion (underlined) of nucleotides or substitution (bold) of amino acid at the junction of repeated sequences. Shaded and open bars show the minimum amino acid sequences which were duplicated frequently. (YFYV, ▨; YEYDLK, ▩; YEYD, ▩; and YDLK, □).

elucidated that the SH2-binding autophosphorylation residues are clustered within non-catalytic regions of the cytoplasmic domains of the EGF receptor and PDGF receptor.[24] In the JM domain of βPDGF receptor, Y579 and Y581 are implicated in binding to c-Src, a cytoplasmic tyrosine kinase.[25] These tyrosine residues were auto-phosphorylated and composed a SH2-binding motif, YIYV. In comparison with the JM sequence of βPDGF receptor, we found a homologous motif, YFYV (AA number 589–592 in Ref. 4), at the same location of the JM domain of FLT3. Interestingly, this motif was doubled in 12 of 23 cases with the tandem duplication (Figure 1). Another AA sequence, YEYDLK (AA number 597–602 in Ref. 4) which contains either YEYD or YDLK, was frequently involved in the duplicated AA sequences. YEYDLK was seen in 11 cases,

YEYD in five cases and YDLK in three cases. In total, all cases with the tandem duplication had at least one of these minimum AA sequences doubled. Although the autophosphorylation sites in the JM of FLT3 have not been determined yet, our findings suggested that the duplication of the SH2-binding motif in the JM domain might be associated with enhancement of the FLT3 function. Although no direct evidence is obtained so far, these AA sequences could play a crucial role in altering the function of FLT3 kinase and thus reinforcing proliferation of the leukemic cells.

In conclusion, the tandem duplication within the JM/TK-I domains of the FLT3 gene was completely restricted to AML and MDS among a variety of hematological malignancies and might give a growth advantage to leukemic cells.

Acknowledgements

This work was supported in part by a grant-in-aid from the Ministry of Education, Science and Culture in Japan.

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