D-HPLC analysis of the entire *FLT3* gene in *MLL* rearranged and hyperdiploid acute lymphoblastic leukemia

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Acknowledgments: the authors wish to express their gratitude to the members and participating hospitals of the INTERFANT-99 protocol for supporting this study by providing leukemic samples. Members of INTERFANT-99 are: M. Campbell (PINDA), M. Felice, (Argentina), A. Ferster (CLCG), I. Hann and A. Vora (UKCCSG), L. Hovi, (NOPHO), G. Janka-Schaub (COALL), CK. Li, (Hong Kong), G. Mann (BFM-A), F. Mechinaud (FRALLE), R. Pieters (DCOG), G. de Rossi. and A. Biondi (AIEOP), J. Rubnitz (SJCRH), M. Schrappe, (BFM-G), L. Silverman, (DFCI), J. Stary, (CPH), R. Suppiah, (ANZCHOG), T. Szczepanski (PPLLSG), T. Valscecchi and P. de Lorzenzo(CORS).

Funding: this study was financially supported by a grant from the Sophia Foundation for Medical Research (SSWO grant 296).

Manuscript received January 3, 2007.
Accepted September 1, 2007.

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ABSTRACT

MLL rearranged and hyperdiploid acute lymphoblastic leukemia (ALL) are characterized by high-level *FLT3* expression and constitutive FLT3 activation. As known activating *FLT3* mutations are often absent in these patients, we screened the entire FLT3 coding sequence in *MLL* rearranged and hyperdiploid ALL cases for yet unidentified additional genetic alterations using denaturing D-HPLC. Both in *MLL* rearranged and hyperdiploid ALL we found that a small minority of samples, 7% and 10% respectively, carried genetic alterations. Although some of these alterations may induce FLT3 activation, the majority of these patients carry wild-type *FLT3* genes.

Key words: *FLT3*, D-HPLC, MLL rearrangements, hyperdiploid ALL, mutation.

Haematologica 2007; 92:1565-1568. DOI: 10.3324/haematol.11220

Constitutively activated FLT3 as a consequence of mutation has become a hallmark of acute myeloid leukemia (AML) and occurs in approximately 30% of cases.[1] Two well described types of activating *FLT3* mutations are in-frame internal tandem duplications (ITDs) within the juxtamembrane (JM) domain, and point mutations or small deletions/insertions either affecting codon D835 or I836 within the tyrosine kinase domain (TKD). Both types of mutation result in loss of the auto-inhibitory activity of the receptor, giving rise to ligand-independent FLT3 signaling, providing leukemic cells with a growth advantage and transforming capacity.[1] These findings prompted the development of small molecule tyrosine kinase inhibitors to specifically target leukemic cells that are dependent on abnormal *FLT3* activity. These FLT3 inhibitors (including PKC412, CEP-701, SU5416) induce apoptosis *in vitro* in cells expressing different types of activating *FLT3* mutations. The therapeutic potential of these inhibitors has been explored in phase I/II clinical trials with promising results,[2-4] and phase III studies for AML in adults are in progress.[5,6] In pediatric acute lymphoblastic leukemia (ALL), the occurrence of activating *FLT3* mutations seems restricted to *MLL* gene rearranged and hyperdiploid (>50 chromosomes) cases. In these subtypes of ALL, mutations in the TKD of the *FLT3* gene occur in a minority (3-21%) of cases,[7-10] whereas FLT3/ITDs are rarely found.[11] However, in the majority of both *MLL* rearranged and hyperdiploid ALL, a mechanism other than mutation appears to result in constitutive FLT3 activation. We have demonstrated along with others that high-level *FLT3* expression, which is characteristic of *MLL* rearranged[12] and hyperdiploid ALL,[13] in the absence of ITDs and known TKD mutations, is associated with FLT3 phosphorylation (activation) and sensitivity towards FLT3 inhibitors.[8,10]

Until now, most studies have mainly focused on activating ITDs and TKD mutations in *FLT3*, and leukemic samples tested negative for the presence of these mutations are often considered to be wild-type. However, reports on novel *FLT3* mutations also resulting in ligand-independent FLT3 signaling are slowly emerging.[14-16] Therefore, the occurrence of yet unidentified activating *FLT3* mutations may challenge the assumption that constitutive activation of FLT3 as observed in primary *MLL* rearranged and hyperdiploid ALL is only a consequence of wild-type *FLT3* over-expression. We screened the entire *FLT3* coding sequence for genetic abnormalities using Denaturing High Performance Liquid Chromatography (D-HPLC) and subsequent sequence analysis in a large cohort of primary *MLL* rearranged and hyperdiploid pediatric ALL samples.

Design and Methods

Patient samples

Bone marrow and/or peripheral blood samples from untreated infants (<1 year of age) with *MLL* rearranged ALL were collected at diagnosis at the Erasmus MC – Sophia Children's Hospital (Rotterdam, The Netherlands) and other hospitals participating in the INTERFANT-99 collaborative treatment protocol. Pediatric hyperdiploid ALL samples were collected at the Erasmus MC – Sophia Children's Hospital. Within 24 hours after sampling, mononuclear cells were isolated applying density gradient centrifugation using Lymphoprep (Nycomed Pharma, Oslo, Norway). All samples contained at least 90% leukemic cells, as determined morphologically on May-Grünwald-Giemase (Merck, Darmstadt, Germany) stained cytospins. When necessary, contaminating non-malignant cells were removed using immunomagnetic beads as reviously described.[10] Non-leukemic mononuclear cells were isolated from peripheral blood samples obtained from healthy adult volunteers.

RNA extraction and cDNA synthesis

Total cellular RNA was extracted from a minimum of $5x10^6$ leukemic cells using TRIzol reagent (Gibco BRL, Life Technologies) according to the manufacturer's protocol. The integrity of the extracted RNA was assessed on 1% agarose gels and quantified spectrophotometrically at 260 and 280 nm. One μg of RNA was reverse transcribed into single stranded cDNA as previously described.[10]

PCR amplification

With the exception of the first 34 nucleotides the entire *FLT3* coding sequence (NCBI accession number: U02687) was amplified as 9 partially overlapping PCR products, using primer combinations as listed in Table 1. All PCRs were performed in a total reaction volume of 50 μL containing 1x TaqGold buffer II (Applied Biosystems, Foster City, CA, USA), 2 mM MgCl2, 200 μM of each dNTP (Amersham Pharmacia Biotech.), 300 nM forward and reverse primer, 1.25 U AmpliTaq Gold DNA polymerase (Applied Biosystems) and 40 ng of cDNA as a template. PCRs were initiated by a denaturation step at 95°C for 10 minutes, after 40 cycles of 15 seconds at 95°C and 1 minute at 60°C.

Denaturing high performance liquid chromatography (D-HPLC) analysis

PCR products were denatured at 95°C for 5 minutes and slowly cooled down to 4°C to allow heteroduplex formation. Then, 10 μL aliquots of PCR product were injected under temperature and acetonnitrile gradient conditions as listed in Table 1, and analyzed for the presence of genetic alterations by D-HPLC using the WAVE™-system 3500HT (Transgenomic Inc., Omaha, NE, USA). Abnormal PCR products were identified by examination of the WAVE patterns using Navigator software (Transgenomic). PCR products with divergent WAVE patterns were sequenced at least two times. Sequence analysis was performed on a 3100 Genetic Analyser (Applied Biosystems) using the BigDye Terminator v1.1 cycle sequencing protocol (Applied Biosystems). For each PCR, several samples dis-

Table 1. Primer combinations and D-HPLC analysis conditions.

PCR	Sequences†	First nt§	Temp (°C)#	Acetonitrile gradient (%)*
PCR 1				
Forward	5'-CGC TGC TCG TTG TTT TT-3'	92	57	56.7 - 65.7
Reverse	5'-TTG GGT TTC TGT CAT TTT CA-3'	468		
PCR 2				
Forward	5'-GCC CCA GGG AAC ATT T-3'	346	57	58.8 - 67.8
Reverse	5'-AAG GGT TCC CCT ACT TTA AGA-3'	860		
PCR 3				
Forward	5'-CAG GGG GAA AGC TGT AAA-3'	661	56	59.2 - 68.2
Reverse	5'-GAA GGT CCA CGT ACA TCT GA-3'	1212		
PCR 4				
Forward	5'-AAA GCA TCC CAG TCA ATC A-3'	1056	56	57.4 - 66.4
Reverse	5'-TGG GAG ACT TGT CTG AAC AC-3'	1474		
PCR 5				
Forward	5'-AAG CAT CGG CAA GTC AG-3'	1388	58	58.3 - 67.3
Reverse	5'-TCC CAT TTG AGA TCA TAT TCA-3'	1868		
PCR 6				
Forward	5'-ATG AAA GCC AGC TAC AGA TG-3'	1772	56	58.1 - 67.1
Reverse	5'-GGG GTA AAA ACT GAA ATT GTG-3'	2238		
PCR 7				
Forward	5'-GGG CGT GCA CAC TGT-3'	2093	57	58.1 - 67.1
Reverse	5'-AGC CAA TCC AAA GTC ACA TA-3'	2555		
PCR 8				
Forward	5'-AAG TCG TGT GTT CAC AGA GAC-3'	2470	58	56.8 - 65.8
Reverse	5'-GGC CGT TTC CTT GAG TC-3'	2858		
PCR 9				
Forward	5'-TTC CGG TTG ATG CTA ACT T-3'	2732	57	57.5 - 66.5
Reverse	5'-TGA AGC AGC AGT TGA TAA TAG AT-3'	3155		

*†All oligonucleotides were designed using the OLIGO 6.22 software (Molecular Biology Insights, Cascade, CA, USA); §the position of the first nucleotide (nt) given for each primer is based on the complete coding sequence for FLT3 with NCBI accession number U02687; #temperatures used for D-HPLC analysis for indicated PCRs. *% WAVE Optimised TEAA buffer B (Transgenomic) from time 0.5–5 minutes.*

playing WAVE patterns that were considered to be wild-type were also sequenced to confirm the absence of genetic variation associated with this pattern.

Results and Discussion

Using 9 partially overlapping PCRs, we amplified the coding sequence of the *FLT3* gene in leukemic samples from 45 *MLL* rearranged infant ALL patients and 30 pediatric hyperdiploid ALL patients, as well as in mononuclear cell samples from 23 healthy adults. All PCR products were screened for the presence of genetic variations using D-HPLC. A total of 8 different divergent D-HPLC patterns was observed, all of which corresponded to genetic alterations as determined by sequence analysis. One of the genetic abnormalities was a known C→T single nucleotide polymorphism (SNP) (NCBI: rs1933437). With the exception of this SNP, none of these genetic variants were observed in mononuclear cell samples from healthy individuals.

MLL rearranged infant ALL

Within the *MLL* rearranged infant ALL group (n=45), 6 out of 45 (~13%) patients carried genetic alteration (Table 2). Three patients carried an A→G substitution at codon L561, residing within the transmembrane (TM) domain. This proved to be a synonymous variant that had been described before.[17] In the three remaining patients (~7%), the alteration actually resulted in an amino acid change. The first of these 3 patients carried an A→T change that resulted in the substitution of the basic amino acid arginine (R) for a polar (uncharged) serine (S) at codon 391 within the extracellular Ig-like domain of the FLT3 receptor. The extracellular Ig-like domains of receptor tyrosine kinases are responsible for ligand binding, and therefore a conformational change as a result of this amino acid substitution may affect binding of FLT3 ligand (FLT3L) to its receptor. For example, point mutations within the Ig-like region of the fibroblast growth factor receptor 2 have been reported to affect ligand binding specificity.[18] Therefore, point mutations within the extracellular region may also influence ligand binding of FLT3, provided that these mutations occur at critical amino acids. Nevertheless, it seems unlikely that such mutations are involved in ligand-independent FLT3 signaling.

The second *MLL* rearranged infant ALL patient carried a G→A substitution, replacing valine (V) for isoleucine (I), both of which are non-polar, hydrophobic amino acids, at codon 557 within the TM domain (Table 2). The fact that this alteration resides within the TM domain, suggests that it may not affect domain that appeared critical in the regulation of the FLT3 receptor. However, Akin *et al.* recently described a patient diagnosed with mast cell disease as carrying a germ line F522C mutation within the TM region of the Kit receptor protein, a class III receptor tyrosine kinase family member of FLT3. Interestingly, introducing c-kit carrying this mutation in Cos-7 cells induced ligand-independent activation of Kit which could be inhibited by imatinib mesylate.[19] Similarly, although extremely rare, an activating mutation within the TM region of the non-tyrosine kinase granulocyte colony-stimulating factor (G-CSF) receptor occurs in patients with AML.[20] Both of these examples suggest a significant role for the TM domain in receptor activation. By contrast, however, 3 described point mutations occurring in the TM region of the fibroblast growth factor receptor 3 (FGF3), another receptor tyrosine kinase, do not seem to influence receptor activation directly, but induced increased sensitivity of FGF3 to its natural ligand.[21] A third *MLL* rearranged infant ALL patient was found to harbor a known *FLT3* activating Δ836 mutation, which is a three base pair (CAT) deletion affecting codon I836 within the activation loop of the TKD.[10, 22]

Table 2. Novel and known *FLT3* mutations identified in primary *MLL* rearranged infant ALL samples.

Mutation	Frequency*	Exon	Amino acid change	Location	Described before
A→T	2% (1/45)	9	R391S	Ig-like domain 4	No
G→A	2% (1/45)	13	V557I	TM domain	No
A→G	7% (3/45)	13	Synonymous at codon L561	TM domain	Yes, in AML[25]
CAT del	2% (1/45)	20	Δ836	JM domain	Yes, in MLL+ ALL[7]

**6/45 (~13%) MLL rearranged infant ALL patients carried a mutation. In 3/45 (~7%) cases the mutation resulted in an amino acid change.*

Table 3. Novel and known *FLT3* mutations identified in primary childhood hyperdiploid ALL samples.

Mutation	Frequency*	Exon	Amino acid change	Location	Described before
A→G	7% (2/30)	13	Synonymous at codon L561	TM domain	Yes, in AML[25]
A→T	3% (1/30)	14	K567I	JM domain	No
T→A	3% (1/30)	14	V579Q	JM domain	No
Insertion	3% (1/30)	14	7 aa insert at D586	JM domain	No

**5/30 (~17%) childhood hyperdiploid ALL patients carried a mutation. In 3/30 (~10%) cases the mutation resulted in an amino acid change.*

Pediatric hyperdiploid ALL

Within the childhood hyperdiploid ALL group, 5 out of 30 (~17%) patients carried a genetic alteration (Table 3). Two patients appeared to harbor the synonymous variant also found in three *MLL* rearranged ALL cases (see above). The remaining three patients (~10%) carried genetic alterations that resulted in amino acid changes. Two of these samples harbored single nucleotide substitutions, both located within the juxtamembrane (JM) domain of FLT3. One patient carried an A→T alteration resulting in the substitution of basic amino acid lysine (K) for a non-polar

(hydrophobic) isoleucine (I) at codon 567. In the second patient, a T→A substitution was detected changing the non-polar (hydrophobic) amino acid valine (V) into a polar (uncharged) glutamine (Q) at codon 579 (Table 3). In a third hyperdiploid ALL patient, a 7 amino acid insertion was identified at codon 586 within the JM domain, in the vicinity of the insertion site at which most activating FLT3/ITDs have been identified. The JM domain is believed to play an important role in the auto-inhibitory activity of the receptor, and disruption of this domain by ITDs for example, releases its repressive conformation allowing ligand-independent activation.[23] However, whether the observed single amino acid substitutions, or the seven amino acid insertion are sufficient to significantly disrupt the JM domain must still be explored.

In conclusion, some of the observed leukemia-specific alterations which lead to actual amino acid changes may potentially result in constitutive FLT3 activation. Unfortunately, due to a lack of sufficient primary patient samples, we were not able to evaluate the phosphorylation status of FLT3 in samples in which we identified these genetic alterations. However, these alterations only occurred in a small minority (~7-10%) of the cases studied. Considering that *FLT3* is consistently highly expressed in the majority of *MLL* rearranged and hyperdiploid ALL cases, and that high-level *FLT3* expression is associated with FLT3 phosphorylation,[8,10] these alterations do not explain FLT3 phosphorylation in the majority of these patients. We therefore conclude that constitutive FLT3 activation as a consequence of mutation occurs in a small minority of *MLL* rearranged and childhood hyperdiploid ALL patients, but that in the majority of these patients the constitutively activated FLT3 signal is a consequence of high-level expression of wild-type *FLT3*.

Authors' contributions

RWS: conception and design, data acquisistion, analysis and interpretation, drafting article; MLdB: data interpretation, and revising article for important intellectual content; PS: conception and design, data acquisition and analysis, revising article; MM: data analysis and interpretation, revising article; HBB: revising article for important intellectual content and data acquisition; RB: revising article for important intellectual content, and final approval of the version to be published.

Conflicts of Interest

The authors reported no potential conflicts of interest.

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