Review

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FLT3 Length Mutations as Marker for Follow-Up Studies in Acute Myeloid Leukaemia

Susanne Schnittger Claudia Schoch Wolfgang Kern Wolfgang Hiddemann
Torsten Haferlach

Laboratory for Leukaemia Diagnostics, Department of Internal Medicine III, Ludwig Maximilians University of Munich, University Hospital Grosshadern, Munich, Germany

Key Words
FLT3-LM · Minimal residual disease · AML · FLT3-ITD · Follow-up studies

Abstract
Length mutations within the FLT3 gene (FLT3-LM) can be found in 23% of acute myeloid leukaemia (AML) and thus are the most frequent mutations in AML. FLT3-LM are highly correlated with AML with normal karyotype and other cytogenetic aberrations of the prognostically intermediate group. This group is supposed to be a mixed group of AML with differences in the underlying pathogenesis. For more individualized treatment options it would be helpful to better characterize this large AML group not only by molecular mutations but also use these markers for the definition of minimal residual disease (MRD). However, so far the cytogenetically intermediate AML has been lacking suitable markers for PCR-based MRD detection like the fusion genes in the prognostically favourable subgroups. The suitability of the FLT3-LM as a target for PCR-based MRD was discussed controversially as it seemed to be a rather unstable marker. Thus, we aimed at the evaluation of FLT3-LM as a marker for residual disease in a large cohort of AML. Paired samples of 97 patients with AML at diagnosis and at relapse were analyzed. It could be shown that in only four cases a loss of the length mutation was detected. This is in the range of other well-characterized AML relapsing with a different geno- and/or phenotype. In contrast, a change in the ratio of the mutated allele in comparison to the wild-type allele was frequently observed. In detail, the FLT3-LM showed a tendency to accumulate during disease progression and was found more frequently at relapse than at diagnosis. In addition, 45 patients were analyzed at different time points during and after therapy. Using conventional PCR it clearly could be shown that for most of the patients positive at presentation FLT3-LM is a reliable PCR marker for monitoring treatment response. Even an early detection of relapse was possible in some cases.

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Dr. rer. nat. Susanne Schnittger, Laboratory for Leukaemia Diagnostics
Department of Internal Medicine III, University Hospital Grosshadern
Ludwig Maximilians University, Marchioninistrasse 15
DE–81377 München (Germany), Tel. +49 89 7095 4970, Fax +49 89 7095 4971
E-Mail susanne.schnittger@med3.med.uni-muenchen.de

Introduction

FLT3 is a member of fms-like receptor tyrosine kinases and is expressed in early haematopoietic stem cells [1, 2]. The gene coding for this receptor is targeted by mutations in the juxtamembrane domain in 20–23% of all unselected acute myeloid leukaemias (AML) [3–8]. In addition, point mutations in the activation domain have been described in an additional 6–7% of AML [9–11]. Thus, FLT3 is the gene most commonly targeted by mutations in AML. The mutations in exon 14 and/or exon 15, the part of the gene that is coding for the juxtamembrane domain, have been described to be internal tandem duplications (ITD) resulting in the term FLT3-ITD. However, as we have found that not all of these mutations are simple ITDs but about 30% have insertions of extranucleotides between the duplicated stretches and some cases are even more complicated, we will refer to this mutations as FLT3 length mutations (FLT3-LM) [8]. The elongation of the juxtamembrane domain results in a conformation change that leads to autoactivation of the receptor through a constitutive phosphorylation [12] and can induce IL-3-independent growth in model systems [13, 14]. The FLT3-LM has been shown to be strongly correlated with AML and myelodysplastic syndromes of the RAEB and RAEBt subtypes [8, 15]. In many different study groups the FLT3-LM has been shown to be strongly correlated with the prognostically intermediate karyotype group. This large group of AML is supposed to be a pool of insufficiently characterized AML with different molecular mutations. Now it is becoming clear that the FLT3-LM defines a prognostically worse subset within this group [3, 5–8, 16, 17]. Today the karyotype of the leukemic cells is considered the most important parameter indicating the prognosis in patients with AML [18, 19]. Although further pretherapeutically defined prognostic parameters have been identified such as age of the patient and AML occurring as a secondary disease the prognosis of patients within the respective subgroups defined by these parameters is still heterogeneous. As a consequence, the implementation of therapy-dependent parameters into stratification systems has been approached. Along this line, the degree of reduction of the leukemic cell mass following the first course of induction therapy as well as the time to achieve complete remission have been demonstrated to independently impact the prognosis [20–22]. These studies thus have proved the concept of prognostication based on therapy-dependent factors.

The most prominent therapy-related factor is the level of minimal residual disease (MRD). Beside the conventional control of therapy response by cytomorphology, assessment of MRD by flow cytometry is of growing importance [23]. However, polymerase chain reaction (PCR)-based methods are still the most sensitive ones for the detection of residual leukaemic cells. The application of PCR to quantify MRD so far was based on the presence of fusion genes or their respective fusion transcripts, which occur exclusively in the respective subentities of AML but not in normal bone marrow. In patients with AML the detection of MRD by quantitative PCR has been shown to be feasible and prognostically relevant [24–32]. However, in AML with normal karyotype or other prognostically intermediate aberrations like trisomies 8 or 11 and del(9q) PCR was not applicable due to the lack of a target suitable for PCR. The portion of cases assessable may now be extended by the inclusion of cases with FLT3-LM.

To analyze whether FLT3-LM is a valuable marker for follow-up controls two different analyses were performed: (1) the stability was assessed by paired analysis of samples from diagnosis and relapse and (2) FLT3 status from samples during and after therapy was compared to the status of remission, fluorescence in situ hybridization (FISH) and cytogenetics.

Patients, Materials and Methods

Patient Samples

Bone marrow or peripheral blood samples were sent by overnight service to our lab. All were diagnosed as having AML according to standard French-American-British (FAB) criteria [33–38] and were referred between July 1997 and December 2002 for cytomorphological, cytogenetic, molecular genetic and multiparameter flow cytometry analysis.

Cytogenetic and FISH Analysis

Cytogenetic G-banding analysis was performed with standard methods [39]. The definition of a cytogenetic clone and descriptions of karyotypes followed the International System for Human Cytogenetic Nomenclature [40].

For FISH the commercially available probes CEP#8SG, LSID7S486/CEP7, LSIPMLRARA, LSICBFB, and LSIAML1/ETO were used according to the manufacturer's instructions (Vysis, Bergisch Gladbach, Germany).

Sample Preparation

Mononucleated bone marrow cells were obtained by Ficoll-Hypaque density gradient centrifugation. Total RNA was extracted from 10^7 cells with RNeasy (Qiagen, Hilden, Germany) (1997–2000) or mRNA with the MagnaPureLC mRNA Kit I (Roche Diagnostics, Mannheim, Germany) (since January 2001). The cDNA synthesis of 1–2 μg total RNA or mRNA from an equivalent of 10^7 cells was performed using 300 U Superscript II (GibcoBRL/Invitrogen, Karlsruhe, Germany) and random hexamer primers (Pharmacia, Freiburg,

Germany). DNA was extracted using a simple salting-out procedure [41].

PCR at Diagnosis

PCR and semiquantitative estimation of the FLT3-LM status was performed as has been described [8]. Strict precautions were taken to prevent contamination. Water instead of cDNA was included as a blank sample in each experiment. Amplification products were analyzed on 2% agarose gels stained with ethidium bromide.

Sequencing

For direct sequencing of the length mutations the amplified fragments were cut from the agarose gels and isolated with Quiaex II (Qiagen) following the manufacturer's instructions. Approximately 100 ng of purified PCR products were directly sequenced with 3.3 pmol of primers as described above with the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Weiterstadt, Germany). After initial denaturation at 95°C for 5 min, 25 cycles at 94°C for 15 s and 60°C for 4 min were performed. Cases with a relatively small length of the mutations or those with status 1 or 5 mutations were subcloned using the TOPO II cloning system (Invitrogen, Karlsruhe, Germany) prior to sequence analysis. Sequence detection was performed on an ABI 310 sequence detection system (Applied Biosystems).

Efficiencies, Reproducibility and Sensitivities of the PCR Assays

The sensitivity of the PCR assays was assessed by performing limited dilution series of DNA and cDNA of diagnostic patient samples with a different status of FLT3-LM into cDNA of samples without FLT3-LM.

Results

Frequency of FLT3-LM at Presentation and in Relapse

In total 2,338 samples were analyzed, 2,135 were from newly diagnosed AML and 203 from relapsed AML. FLT3-LM were found in 461 (21.6%) of the diagnostic samples at first diagnosis and in 62 (30.5%) of the relapses. Thus the frequency is higher in relapsed AML (table 1) ($p = 0.005$).

Semiquantitative Analysis of the Mutations

Analysis of the amplification fragments on agarose gels revealed that the band representing the mutation was not always of the same intensity as the wild-type allele. Thus, we divided the FLT3-LM into five categories: (1) mutant fragment less intense than wild-type band (status 1), (2) mutant fragment with the same intensity as wild-type band (status 2), (3) mutant fragment more intense than wild-type band (status 3), (4) only mutant fragment and loss of wild-type band (status 4), and (5) presence of more than one mutant fragment (status 5) (fig. 1). Type 1 has to be interpreted as subclone in an otherwise FLT3-LM-negative population, type 2 as an equal amount of wild-type and mutated allele, type 3 as loss of the wild type in a subclone, and type 4 as complete loss of the wild type. In type 5 there were two different FLT3-LM in different clones.

Table 1. FLT3-LM status according to a semiquantitative estimation in a cohort of 2,135 AML patients at diagnosis and 203 at relapse

Mutation status	Diagnoses (461/2,135; 21.6%), %	Relapse (62/203; 30.5%), %
1	10	–
2	60	16
3	11	32
4	6	52
5	13	–

At diagnosis 70% of the patients with FLT3-LM have status 1 and 2 mutations, whereas a loss or partial loss of the wild-type allele was found in only 17%. In contrast, in relapses status 1 and 5 was never detected and status 2 in only 16%. Most of the relapsed FLT3-LM-positive cases had a partial (32%) or complete (52%) loss of the WT-FLT3.

Use of DNA and cDNA for PCR Diagnostics

The PCR screening for FLT3-LM was done at the genomic and cDNA level in parallel for most of the cases. For some cases with limited material the analysis was restricted to the cDNA level. No major differences concerning the intensities of the mutated allele in comparison to the wild-type allele were observed, suggesting that there were no major expression differences between wild-type and mutated alleles.

At diagnosis it was possible to detect an FLT3 expression in every sample. In contrast, in follow-up samples of patients in complete clinical remission, it was not always possible to obtain an amplification product of FLT3 by PCR. This is in accordance with previous studies that have shown FLT3 expression on early haematological and leukaemic cells but not on differentiated haematological cells [2]. Thus for follow-up controls DNA is highly recommended as diagnostic material.

Paired Samples from Diagnosis and Relapse

In total the FLT3 status was assessed in 97 paired samples at diagnosis and at relapse (table 2). Fifty-one patients were negative at both time points. Thirty-eight

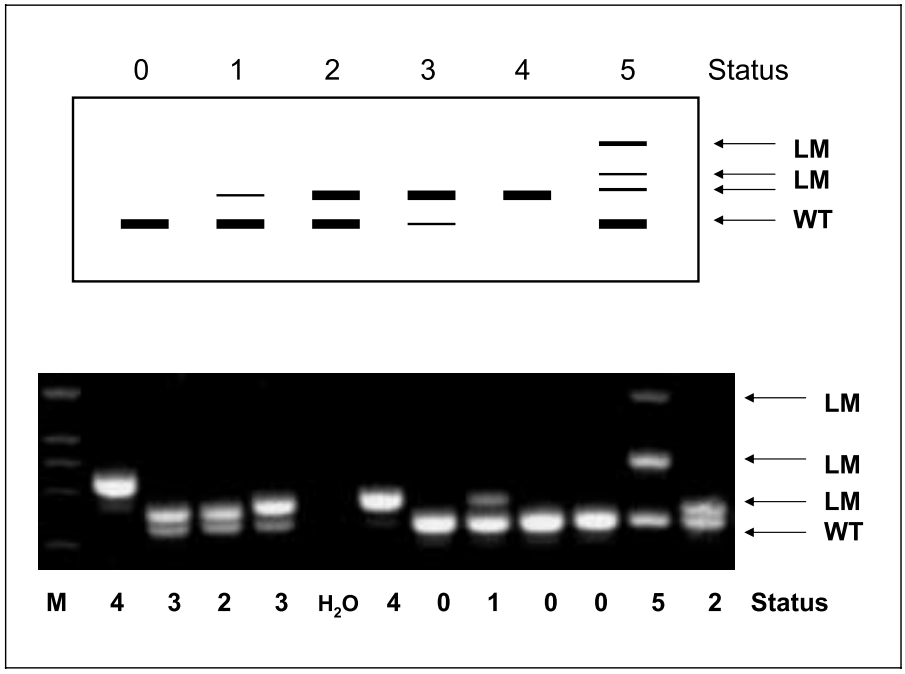

Fig. 1. Assessment of the FLT3-LM status according to mutation/wild-type relation.

Table 2. FLT3-LM in paired presentation and relapse samples: present study and review of the literature

	Patients	FLT3 status at presentation/relapse			
		–/–	–/+	+/–	+/+
Nakano et al. [48]	28	16	6	1	5*
Kottaridis et al. [16]	44	20	4	5	15*
Hovland et al. [42]	2	–	1	–	1
Shih et al. [44]	108	83	8	1	16*
Present study	97	51	4	4	38
Total	278	170 (61.2%)	23 (8.3%)	11 (4%)	75 (27%)

cases were positive for an FLT3-LM at both time points. All 3 cases with a second relapse retained the FLT3-LM at that time point. A gain of an FLT3-LM was found in 4 cases. A loss of the mutation was detected in only 4 cases (IDs 2, 41, 42, 43), three of whom had only a status 1 mutation at presentation (table 3), meaning that at diagnosis only a subclone of all leukemic cells had the mutation. Of the 4 cases with a gain of the FLT3 mutation, in two (IDs 44 and 47) also a change of the FAB from M4 to M2 and M6 to M1, a change of the immunophenotype and in case 47 also a different karyotype were observed raising the question of whether these two might be secondary AML rather than relapsed AML. In addition, in case 46 the same FAB but a complete change of the karyotype was observed.

Focussing on the FLT3 status (relation of the mutated to the WT-FLT3) in more detail (table 4 and 5) we found stability of the status in 12 cases (24.5%); however, a progression from lower to higher status was detected in 30 cases (63%) and a regression to lower status or loss of the FLT3-LM in only 6 cases (12.2%). Thus the overall maintenance of an FLT3-LM in relapse was relatively stable with (45/49; 91.8%). However the mutation had a tendency to accumulate during progression of leukaemia.

Table 3. Paired samples of patients with FLT3-LM at diagnosis and/or relapse (n = 47)

ID	FAB	FLT3-LM status	Karyotype
1 PD	M0	3	46,XX [24]
1 Rel	M0	3	46,XX [5]
2 PD	M1	2	46,XX [25]
2 Rel	M1	–	46,XX
3 PD	M1	2	46,XX [25]
3 Rel	M1	3	46,XX [8]
4 PD	M1	2	46,XX [25]
4 Rel	M1	3	46,X,del(X)(q11),t(7;18)(q22;p11.2)[8]/ 46,XX [7]
5 PD	M1	4	48,XY,+8,+10 [10]
5 Rel	M1	2	46,XY [25]
6 PD	M1	4	46,XY [20]
6 Rel	M1	4	n.d.
7 PD	M1/M2	2	46,XY [6]
7 Rel	?	4	n.d.
8 PD	M2/M4	1	46,XX [12]
8 Rel	M2	3	n.d.
9 PD	M2	4	46,XX [25]
9 Rel	M2	4	46,XX [6]
10 PD	M2	1	46,XY [25]
10 Rel	M2	4	46,XY [21]
11 PD	M2	2	46,XY [22]
11 Rel	M2.	3	46,XY [25]
12 PD	M2	2	46,XY [25]
12 Rel	M2	4	49,XY,+8,+13,+19 [20]
13 PD	M2	2	46,XX [24]
13 1. Rel	M2	2	46,XX,t(1;19;3)(p32;q13?;p21)[25]/ 46,XX [2]
13 2. Rel	M2	2	46,XX,t(1;19;3)(p32;q13?;p21)[14]/ 46,XX [1]
14 PD	M4	5	46,XX [25]
14 Rel	?	5	46,XX [25]
15 PD	M4	2	46,XX [25]
15 Rel	M4	3	46,XX [25]
16 PD	M4	3	46,XX [25]
16 Rel	M4	3	46,XX [25]
17 PD	M4	2	46,XX [25]
17 Rel	M4	3	46,XX [15]
18 PD	M4/M5	2	46,XY [25]
18 Rel	M4/M5	2	48,XY,t(2;3)(q31;p21),+8,+8,t(10;17) (q22;q22), del(13)(q14q31)[13]/46,XY [12]
19 PD	M4	3	46,XX [25]
19 Rel	M4Rez	4	46,XX [25]
20 PD	M5b	3	46,XX [20]
20 1. Rel	M5b	4	46,XX [20]
20 2. Rel	M5b	4	46,XX [20]
21 PD	M5b	3	46,XX [25]
21 Rel	M5bRez	4	46,XX [25]
22 PD	M5b	1	46,XX [25]
22 Rel	M1	4	46,XX [25]
23 PD	AML	2	46,XY [15]
23 1. Rel	AML	2	46,XY,t(10;12)(q22;p13)[6]/46,XY [6]
23 2. Rel	AML	2	46,XY,t(10;12)(q22;p13)[14]/46,XY [1]
24 PD	M2	2	46,XY,t(8;21)(q22;q22) [25]
24 Rel	M2	2	46,XY,t(1;5)(q21;q33),t(8;21)(q22;q22)[6]/ 46,XY,t(1;5)(q21;q33),t(8;21)(q22;q22), del(11)(p13) [4]
25 PD	M0/M1	2	46,XY,der(5)t(5;11)(q22;?)[9]/46,XY [11]
25 Rel	M0	3	46,XY [20]
26 PD	M0	2	46,XY,t(11;19)(q13;p13)[17]/46,XY [3]
26 Rel	M0	2	46,XY,t(11;19)(q13;p13)[9]/46,XY [1]
27 PD	M0 (Chlorom)	2	48,XX,+8,+22[4]/46,XX [20]
27 Rel	M0	3	50,XX,+X,+8,+10,+13 [3]
28 PD	M2	2	47,XX,del(5)(q15q33)+11 [20]
28 Rel	M2	3	47,XX,del(5)(q15q33)+11[9]/ 47,idem,del(17)(q23)[9]/ 47,idem,add(7)(q3?4)[2]
29 PD	M4	2	46,XX,inv(3)(q21q26)[17]/46,XX [8]
29 Rel	M4	3	46,XX,inv(3)(q21q26)[7]/46,XX [13]
30 PD	tM4	5	46,X,i(X)(q10)[4] 46,XX [19]
30 Rel	M1	2	46,XX [20]
31 PD	M5a	1	46,XY,del(11)(q13q21) or del(11)(q21q23)[6]/47,idem,+8[13]/ 46,XY [3]
31 Rel	M5a	2	46,XY,del(11)(q13q21) or del(11)(q21q23)[1]/47,idem,+8 [19]
32 PD	M5b	2	47,XY,+8[16]/46,XY [4]
32 Rel	M5b	2	47,XY,+8 [15] 47,XY,t(1;18)(p22;q23),+8,del(11) (p11.2p15) [5]
33 PD	M1	1	46,XX [25]
33 Rel	M1	3	46,XX [15]
34 PD	M1	1	46,XX [22]
34 Rel	M1	4	46,XX [25]
35 PD	M2	2	46,XY,der(7)t(1;7)(q31;q32)[8]/46,XY [8]
35 Rel	M2	4	46,XY,t(3;6)(q21;p12),der(7)t(1;7) (q31;q32),der(10)t(8;10)(q22;q26),t(12;15) (q13;q22)[13]/46,XY [2]
36 PD	M1	2	46,XX [21]
36 Rel	M2	4	46,XX [20]
37 PD	M5b	2	46,XX [25]
37 Rel	M5b	2	46,XX [25]
38 PD	M3v	1	46,XY,t(15;17)(q22;q12)[3]46,XY, der(15)t(15;17)(q22;q12),ider(17)(q10) t(15;17) (q22;q12) [17]
38 Rel	M3v	3	46,XY,t(15;17)(q22;q21)[18]/46,XY [2]
39 PD	M5a	1	46,XX [25]
39 Rel	M5a	4	46,XX [25]
40 PD	M1	1	46,XY [21]
40 Rel	M1	4	46,XY [18]
41 PD	M2	1	46,XX,t(8;21)(q22;q22)[19]/46,XX [1]
41 Rel	M2	0	46,XX,t(8;21)(q22;q22)[11]/46,XX [4]
42 PD	sAML	1	47,XY,+8 [15]
42 Rel	sAML	0	47,XY,+8[4]/46,XY [16]
43 PD	M4eo	1	46,XY,inv(16)(p13q22)[2]/ 47,XY,+8,inv(16)(p13q22)[1]49,XY,del(1) (q21),+8,+13,inv(16)(p13q22),+21 [4]
43 Rel	M4eo	0	46,XY,inv(16)(p13q22) [1]\n47,XY,+8,inv(16)(p13q22)
44 PD	M4	0	46,XX [25]
44 Rel	M2	3	46,XX [20]
45 PD	M1	0	46,XX [26]
45 Rel	M1	1	46,XX [20]
46 PD	M1	0	46,XY,del(7)(q21)[5]/46,XY [13]
46 Rel	M1	2	47,XY,+13[2]/47,XY,+15[2]/46,XY [7]
47 PD	M6	0	47,XY,+8[12]/46,XY [8]
47 Rel	M1	4	46,XY,t(1;17)(q21;q23),inv(6)(p21q27), t(9;13)(q21;q14)[5]/46,XY [16]

PD = Primary diagnosis; Rel = relapse; n.d. = not done.

Table 4. FLT3-LM status in paired samples at presentation and relapse (n = 49)

FLT3 status at diagnosis	1	2	3	4	5	0	0	0	0	1	1	1	2	2	3	1	2	4	5
FLT3 status at relapse	1	2	3	4	5	1	2	3	4	2	3	4	3	4	4	0	0	2	2
Patients	–	6	2	3	1	1	1	1	1	1	3	5	9	5	3	3	1	1	1
Total patients	constant: n = 12					progression: n = 30										regression: n = 6			

Table 5. Karyotype in paired samples at presentation and at relapses in patients with FLT3-LM

	Diagnosis	Relapse	Total (n = 44)
Constant (n = 26)	normal karyotype	normal karyotype	23
	aberrant karyotype	same aberrant karyotype	4
Progression (n = 9)	normal karyotype	aberrant karyotype	5
	aberrant karyotype	same aberrant karyotype with additional aberrations	4
Regression (n = 6)	aberrant karyotype	normal karyotype	3
	aberrant karyotype	less aberrant karyotype	3
Change (n = 3)	aberrant karyotyp	regression + progression	3

Comparison of FLT3 Status and Karyotype

Karyotype at diagnosis and relapse was available in 44 paired samples. Most of the cases (n = 23) had a normal karyotype at both time points and 4 cases had equal aberrant karyotypes of the prognostically intermediate karyotype group. Thus 27/44 cases (61.4%) had stable karyotypes. However, of these 27 cases with stable karyotype 18 had a progression of the FLT3-LM. Five cases with normal karyotype had a chromosomal aberration at relapse. In addition, 5 cases with aberrations had additional aberrations at relapse. All these aberrations were of the prognostically intermediate group as is characteristic for cases with FLT3-LM; thus there was no change with regard to the cytogenetically defined risk group but only gains of a so-called additional chromosomal aberration in the sense of progression. Five of the 10 cases with karyotype progression in parallel showed progression of the FLT3 status. In 6 cases a karyotype regression was detected, 3 of these had a regression and a progression in combination. One (ID 43) also revealed regression of FLT3-LM; however, 5 of these cases revealed a progression of the FLT3-LM. Thus cytogenetic regression can be replaced by a molecular progression. Four cases (ID 5, 30, 46, 47) had both a cytogenetic as well as a molecular regression.

Evaluation of FLT3-LM as Follow-Up Marker Using Conventional PCR

The sensitivity as estimated by the limited dilution series of FLT3-LM-positive patients' RNA or DNA from the time of diagnosis in samples negative for the mutation was dependent on the strength of the initial mutation status and was between 1:100 and 1:1,000 (fig. 2). The course of the disease could be followed according to the wild-type/mutation ratio of the amplification product.

In total 174 bone marrow samples of 45 patients were analyzed during or after therapy. The median sample number per patient was 4 and the median follow-up time of sampling was 12 months.

In 6 cases it was possible to detect FLT3-LM positivity after previous PCR negativity 1–3 months before cytomorphological relapse. In additional 4 cases a pending relapse was detectable due to persistent PCR positivity of the FLT3-LM (examples are depicted in fig. 3).

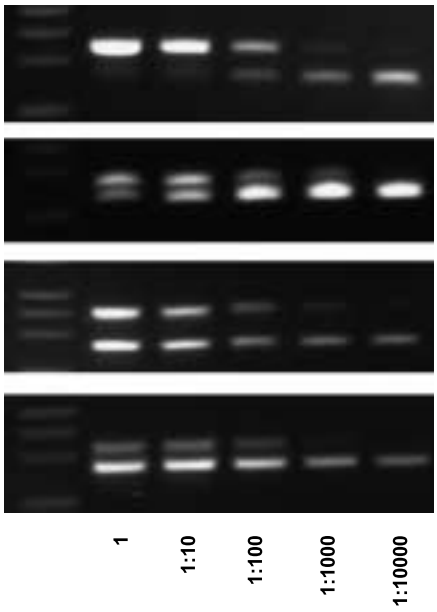

Fig. 2. Limited dilution assays of FLT-LM-positive DNA of patients with different status at diagnosis into FLT3-LM-negative DNA showing the detection limits of FLT3-LM by conventional PCR.

FISH and PCR

In some cases it was possible to perform FISH analysis or PCR using a second marker in parallel to FLT3-LM PCR. For 11 patients with chromosome aberrations at diagnosis a FISH marker was available for follow-up. Four patients with reciprocal translocations could be analyzed with respective colocalization probes [AML1-ETO (n = 2), PML-RARA and CBFB-MYH11]. Four cases had a trisomy 8 that was followed by a centromere 8 probe. One had a 5q- and 2 a 7q- that were analyzed with probes from the respective deletion region. In the 4 cases with AML1-ETO, PML-RARA, and CBFB-MYH11 fusion

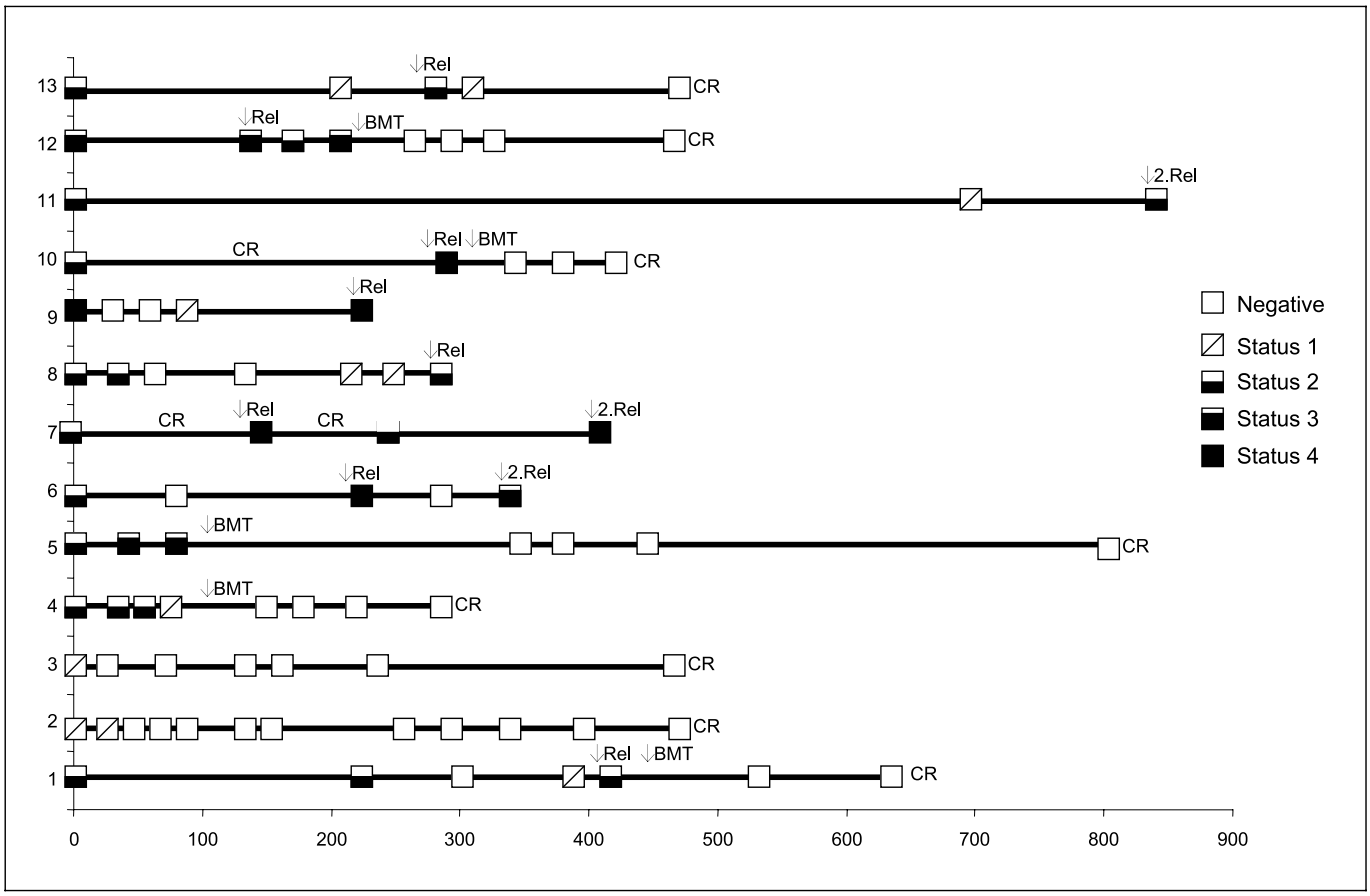

Fig. 3. Follow-up analyses in some exemplary patients showing the possibility of controlling therapy response and of detecting relapses early. Rel = Relapse; CR = complete remission; BMT = bone marrow transplantation.

gene-specific PCR and FISH were done in parallel to FLT3-PCR. In each of these cases fusion gene-specific PCR was the most sensitive method. In 6 cases (1 × AML1-ETO, 1 × PML-RARA, 2 × +8, 1 × 7q-, 1 × 5q-) FISH and FLT3 revealed comparable results during follow-up. One case with AML1-ETO and the one with CBFB-MYH11 and low FLT3-LM at diagnosis lost their FLT3-LM at relapse. Two of the cases with trisomy 8, 1 with 7q- and the 1 with 5q-, lost these chromosomal aberrations at relapse but retained the FLT3-LM or even revealed a progression of the FLT3-LM. Thus, for patients with fusion genes, fusion gene-specific PCR or even FISH clearly are superior to FLT3-PCR. However, in cases with other aberrations like trisomy 8, 5q- or 7q- FLT3-LM PCR seems to be the more reliable follow-up marker.

Discussion

FLT3-LM is the most frequent genetic marker in AML. It is most commonly found in the intermediate risk group, a subgroup of AML where PCR markers for follow-up controls have been missing so far. Therefore, it was suggested that an FLT3-LM should be used as a follow-up marker [8]. However, many studies reported on the instability of this marker during the course of the disease [42–45]. Here we performed a comprehensive analysis regarding the applicability of the FLT3-LM as a follow-up marker.

We and others have previously shown that the amount of FLT3-LM in comparison to the wild-type allele is heterogeneous [7, 8]. In the present study in 17% of the patients at diagnosis and in 84% at relapse partial or complete loss of the wild-type allele was found. Although the clinical significance of FLT3-LM per se is still discussed controversely the loss of the wild-type allele was reported to be clearly associated with a worse outcome [7, 46]. These patients have a higher white blood cell count, a higher percentage of bone marrow blasts, and a shorter overall and disease-free survival [7, 16, 46]. The loss of WT-FLT3 is of interest with respect to functional aspects. As a mutant FLT3 in a mutant/wild-type heterodimer can *trans*-phosphorylate the wild-type chain [47]; it implies that a mutant homodimer has some gain of function more than simply activating the kinase. Alternatively, formation of the homodimer may reflect an underlying mechanism of genetic instability that has other unknown genomic consequences that may, in turn, influence clinical outcome.

Different incidences of the patients with loss of the wild type were described in different studies. Whereas Whitman et al. [46] found in 8/23 (35%) of their patients a level of mutant greater than the wild-type allele (WT), Fröhling et al. [17] found it in only 1/71 (1.4%). To some extent, this may reflect differences in the cut-off value for loss of wt allele and stresses the need for a truly quantitative assessment of the FLT3 mutation status even at diagnosis. We found a higher FLT3-LM ratio in comparison to the wt in 11% at diagnosis, in 32% at relapse, and complete loss of the WT-FLT3 in 6% at diagnosis and 52% at relapse. Thus a loss of the WT-FLT3 is not only associated with a worse prognosis but, in addition, accumulates during the course of the disease and thus seems to be a marker for more progressed disease.

Several studies have been published investigating FLT3 mutations in paired presentation and relapse samples [8, 42–44, 48]. In some of these studies 7–15% of all AML revealed a gain of the FLT3-LM at relapse [43, 44]. Like in the study presented many of the patients lost their wt at relapse. This accumulation of the FLT3-LM implies a role of FLT3-LM in leukaemia progression and onset of relapse.

Taking the results of these previous studies together with those of the study presented only 88% of the analyzed patients maintained the same FLT3 status (FLT3-LM positive or negative) at both time points. Consequently, as was suggested previously [45] the FLT3-LM should be regarded with caution with respect to its usefulness as a follow-up marker. Patients that were positive for an FLT3-LM at presentation often showed an increased mutant level at relapse, usually with evidence of the loss of wild-type alleles. Where more than one mutation had been detected at presentation, usually only one was dominant at relapse [8, 43, 44]. Thus accumulation in the sense of progression seems to be the most common direction of the instability of the FLT3-LM and does not interfere with the applicability for MRD diagnostics. However, in some studies a significant proportion of patients either gained (8.3%) or lost (4%) an FLT3-LM at relapse, and some patients have been reported with a loss of the presentation LM and a gain of a completely new one at relapse [42–44]. These data imply that FLT3-LM are secondary events, arising in an already transformed clone, which induce the outgrowth of a subclone as a result of an additional proliferative advantage conferred by the FLT3-LM. Further support for the secondary etiology of the FLT3-LM is given by those cases that carry the FLT3-LM in only a subset of their leukaemic cells (status 1 mutation in the study presented). In our study all cases

with a loss of the FLT3-LM at relapse revealed only a low FLT3-LM/wt ratio at diagnosis. One of the cases had a t(8;21) and one an inv(16) which are subtypes with a rare incidence of FLT3-LM. Also in these cases there seems to be only an FLT3-LM-positive subclone at diagnosis which got lost at relapse. Also these cases had cytogenetically normal clones at diagnosis and not at relapse. Thus in cases with only a low mutation ratio at diagnosis the results of the follow-up analysis should be regarded with special care.

Detailed cytomorphological, cytogenetic, and immunophenotypic analysis of our cases with a loss of the FLT3-LM at relapse revealed a shift of the FAB type and immunophenotype in 2 cases and a complete change of the karyotype in another 2 cases, suggesting that these AML might be secondary AML instead of relapses. Taking together our data and data published in the literature (table 2), only 4% have a loss of the FLT3-LM. This would be the subgroup that would escape early detection of relapse. However, from our experience this is in the same range as other leukaemias (for example, those followed up with AML1-ETO or CBFB-MYH11) 'relapse' with a different AML. In all of these cases an impending relapse cannot be detected with molecular methods. In addition, therapy response in the early course of the disease can also be applied in these 4% of cases.

For individual patients it was possible to apply different methods of follow-up controls in parallel. It was shown that in patients with fusion genes, fusion gene-specific PCR was clearly superior to FLT3-PCR because it is quantitative instead of semiquantitative and more sensitive. Even the fusion gene-specific FISH analysis was more sensitive in these cases, because all these cases had a very low FLT3-LM status at diagnosis. In contrast, in cases with other aberrations like trisomy 8, 5q- or 7q- FLT3-LM was more reliable than FISH specific for the respective chromosome aberrations, because all these chromosome aberrations were unstable at relapse and FLT3-PCR although being not highly sensitive with a sensitivity of 1:1,000 is more sensitive than FISH.

To make the FLT3-LM assessment truly semiquantitative, we suggest that GeneScan analysis should be used at diagnosis for better prognostication [7]. During follow-up this method would also improve the estimation of the reduction of the leukemic cells in comparison to standard PCR and gel electrophoresis. It could be shown that real-time quantification with patient-specific primers for individual FLT3-LM is applicable and highly specific and sensitive. Thus in the future a highly sensitive and quantitative PCR may still improve the use of FLT3-LM as a follow-up marker. However, this approach is time consuming and expensive and for prospective assessment of the FLT3-LM in clinical studies it does not seem to be feasible for most of the diagnostic labs.

As FLT3-LM characterizes an unfavourable subset of the intermediate group with an increased risk for relapse it is of high importance to monitor especially this group. With the present study it could be shown that FLT3-LM indeed is a reliable marker to assess therapy response and it was even possible to detect relapse early up to 3 months before a clinical relapse. In 94% of cases with FLT3-LM at diagnosis it is also present at relapse. However, the amount of the mutation in relation to the wild-type allele is increasing in 63% of the cases. It seems to be a common signature of the FLT-LM that the status is increasing in the course of unfavourable disease.

Although the applicability of FLT3-LM as marker for follow-up controls was regarded quite critically [43, 45] the results of the presented study are encouraging. This study using conventional qualitative PCR shows that FLT3 is a reliable marker for follow-up controls in most of the patients carrying an FLT3-LM at diagnosis.

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