Trisomy 13 is strongly associated with *AML1/RUNX1* mutations and increased *FLT3* expression in acute myeloid leukemia

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AML1/RUNX1 **is implicated in leukemogenesis on the basis of the *AML1-ETO* fusion transcript as well as somatic mutations in its DNA-binding domain. Somatic mutations in *RUNX1* are preferentially detected in acute myeloid leukemia (AML) M0, myeloid malignancies with acquired trisomy 21, and certain myelodysplastic syndrome (MDS) cases. By correlating the presence of *RUNX1* mutations with cytogenetic and molecular aberration in a large cohort of AML M0 (N = 90) at diagnosis, we detected *RUNX1* mutations in 46%** **of cases, with all trisomy 13 cases (n = 18) being affected. No mutations of *NRAS* or *KIT* were detected in the *RUNX1*-mutated group and *FLT3* mutations were equally distributed between *RUNX1*-mutated and unmutated samples. Likewise, a high incidence of *RUNX1* mutations (80%) was detected in cases with trisomy 13 from other French-American-British (FAB) subgroups (n = 20). As *FLT3* is localized on chromosome 13, we hypothesized that *RUNX1* mutations might cooperate with trisomy 13 in leukemogenesis by increas-** **ing *FLT3* transcript levels. Quantitation of *FLT3* transcript levels revealed a highly significant (*P* < .001) about 5-fold increase in AML with *RUNX1* mutations and trisomy 13 compared with samples without trisomy 13. The results of the present study indicate that in the absence of *FLT3* mutations, *FLT3* overexpression might be a mechanism for *FLT3* activation, which cooperates with *RUNX1* mutations in leukemogenesis. (Blood. 2007;110:1308-1316)**

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Introduction

Acute myeloid leukemia (AML) is the consequence of a multistep process with the accumulation of multiple recurring genetic mutations in hematopoietic stem cells. Mutation targets in this process can be subdivided into two complementation groups.[1] The first group confers a proliferation/survival advantage and is represented mainly by members of the *RAS* and the receptor tyrosine kinase families. Fms-like tyrosine kinase 3 (*FLT3*)-length mutations (*FLT3*-LMs or frequently called *FLT3*-internal tandem duplications [*FLT3*-ITDs]) in the juxtamembrane domain as well as mutations in the second tyrosine kinase domain of *FLT3* (*FLT3*-TKD) confer a ligand-independent constitutive activation of downstream signaling pathways.[2,3]

Hematopoietic transcription factors belong to the second complementation group.[1] Members of this group include *AML1/RUNX1* and *CBFB* coding for the 2 components of the heterodimeric core binding factor (CBF).[4] Both genes are involved in chromosomal translocations associated with acute leukemia.[5] Approximately 8% to 15% of adult AML patients are affected by the translocation t(8;21)(q22;q22), which generates the *AML1-ETO* (*RUNX1-RUNX1T1*) fusion transcript.[6] Less frequent are variant translocations of *RUNX1* on chromosome 21q22 with other partners like *EVI1* on 3q26[7] or *ETV6* on 12p13.[8] In transgenic mice, conditional expression of the *AML1-ETO* fusion gene per se was shown not to cause overt leukemia. However, the treatment of such mice with N-ethyl-N-nitrosourea induced hematopoietic neoplasms including AML, indicating that collaborating mutations that can be induced by this agent are necessary.[9] Respectively, *AML1-ETO* in combination with a *FLT3*-LM/ITD, which is suspected to be one of the most frequent collaborating mutations in AML, induced aggressive acute leukemia in a murine bone marrow transplantation model.[10]

In addition to deregulated expression by translocations, *RUNX1* gene function was found to be impaired by point mutations.[11,12] Recently, haploinsufficiency of *RUNX1* has been identified as the reason for autosomal familial platelet disorder (FPD), which predisposes to the development of AML.[13] At the same time, somatic mutations of RUNX1 have been identified in various AML subtypes and myelodysplastic syndromes (MDSs).[11] Somatic mutations in the N-terminal DNA-binding domain, the so-called Runt domain, of *RUNX1* were reported to occur with the highest incidence in AML M0 as well as in myeloid malignancies displaying acquired trisomy 21.[14-16] In MDSs, somatic mutations were found to be located in the N-terminal Runt domain as well as in the C-terminal transactivation domain of *RUNX1* and were detected in refractory anemia with excess blasts (RAEB), RAEB in transformation (RAEBt), and AML following MDS (the 3 categories were collectively termed MDS/AML). In these cases, *RUNX1* mutations were associated with a significantly worse prognosis compared with cases with wild-type *RUNX1*.[17] A significant association of *RUNX1* mutations with loss of the long arm of chromosome 7 was reported in therapy-related MDS (t-MDS) cases. Here, *RUNX1* mutations were correlated with a predisposition to leukemic transformation.[18]

Consistent with the model of cooperation between mutations of genes of the two complementation groups, a recent report detected a significant association of inactivating *RUNX1* point mutations with activating *FLT3* mutations in the AML M0 subtype.[16] Later this finding was extended to cooperation of *RUNX1* mutations with activating mutations in the ras/receptor tyrosine kinase (rtk) signaling pathway in AML M0[19] but also in MDS/AML.[20] An alternative to *FLT3* activation by mutation, overexpression of *FLT3*

Table 1. Patients' characteristics

AML patients	N = 156
Median age, y (range)	63.3 (20–86)
Sex, M/F, n	98/58
AML M0	115
AML M1	9
AML M2	15
AML M4	12
AML M5	1
s-AML M2	1
AML (not further FAB classified)	3
Median WBC, /μL (range)	13 300 (49–330 000)
Median platelet count, 10^9/L (range)	65 000 (49–330 000)
Median hemoglobin level, g/dL (range)	8.9 (3.0–13.8)
Median blast count, % (range)	82 (21–99)

WBC indicates white blood cell count.

was recently proposed to be an additional mechanism that was associated with unfavorable prognosis.[21,22] Gene amplification was suggested as one possible mechanism for *FLT3* overexpression.[22,23]

In this study we analyzed the incidence of *RUNX1* mutations in a large cohort of M0 AML (N = 90) and selected AML of other subtypes (n = 20) and correlated the results to cytogenetic aberrations as well as to *FLT3* mutations of the samples. A significant association of *RUNX1* mutation was detected to trisomy 13 but, in contrast to previous studies, not to *FLT3* mutations. The presence of trisomy 13 was also found to be significantly associated with an *AML1* mutation in other AML French-American-British (FAB) subtypes (n = 20). *FLT3* expression was significantly increased in trisomy 13 samples, indicating that *FLT3* expression levels might be the factor on chromosome 13 that collaborates with *AML1* mutation in leukemia development.

Patients, materials, and methods

Patients

Fresh blood or bone marrow from 156 patients was analyzed after informed written consent was obtained in accordance with the Declaration of Helsinki. Diagnosis of AML was according to standard FAB and World Health Organization (WHO) criteria.[24-26] Patients were referred to our laboratory for cytomorphologic, immunophenotypic, cytogenetic, and molecular analyses. Of the 156 patients, 115 were diagnosed as AML M0, 9 as AML M1, 15 as AML M2, 12 as AML M4, 1 as AML M5, and 1 was a secondary AML M2 after chronic myeloproliferative disorder (CMPD). Three AML cases were not further classified according to FAB criteria. The AML M0 cohort was unselected, whereas the samples of the other FAB subgroups were specifically selected for the presence of trisomy 13 or as the control group for the respective *FLT3* analysis. Clinical sample characteristics are given in Table 1. Approval for this study was obtained from the Bayerische Landesärztekammer (Bavarian Medical Association).

Mutation analysis of RUNX1

Mononucleated bone marrow cells were obtained by Ficoll density gradient centrifugation. The preparation of mRNA and cDNA was as previously

described.[27] The entire coding region of the *RUNX1* gene was amplified from cDNA using 4 separate polymerase chain reactions (PCRs). For AML M0, amplicons 1 and 2 were analyzed surrounding the runt homology domain (RHD), whereas for the other FAB subtypes the analysis was extended to the entire coding region of *RUNX1*. Primer sequences are as previously described,[16] with some modifications (Table 2). The PCR amplification of amplicons 1-3 (Table 2) was performed using the proofreading enzyme Optimase polymerase (Transgenomic, Omaha, NE) according to the instructions of the manufacturer. Amplicon 4 (Table 2) was amplified using the Taq PCR Master Mix Kit (Qiagen GmbH, Hilden, Germany). A touch-down protocol was applied for all PCR reactions, with preheating of the samples at 97°C for 2 minutes followed by 15 touch-down cycles, 20 cycles at constant annealing temperature, and a final elongation step at 72°C for 5 minutes. The touch-down cycles started with denaturation for 30 seconds at 97°C, annealing for 1 minute at 63°C, and elongation for 1 minute at 72°C, where the annealing temperature was decreased by 0.5°C after each cycle. The residual 20 PCR cycles were performed at 58°C annealing temperature.

DHPLC and DNA sequencing

The denaturing high-performance liquid chromatography (DHPLC) analysis was performed on a WAVE 3500 HT system (Transgenomic) with a DNASep Cartridge (Transgenomic) and Navigator Software 1.6.4 (Transgenomic). DNA was bound to the cartridge by 0.1 M triethylammonium acetate (TEAA) in H_2O (pH 7.0; buffer A; Transgenomic) in the mobile phase at a flow rate of 1.5 mL/min. Increasing amounts of buffer B (0.1 M TEAA, 25% acetonitrile in H_2O; pH 7.0; Transgenomic) were used to elute the DNA off the cartridge. DNA was detected by UV absorption (260 nm).

PCR products for amplicons 1-3 of the *RUNX1* cDNA were obtained as described in "Mutation analysis of *RUNX1*." For heteroduplex formation, the PCR products of the amplicons of each patient sample were mixed with equal amounts of the respective PCR product derived from a healthy control, denatured at 95°C for 5 minutes, followed by cooling to 4°C at a rate of 0.1°C/s. DHPLC analyses were done at the appropriate temperatures. Mutations were detected as aberrant elution profile of the PCR product from the cartridge and were verified by direct sequencing using BigDye chemistry (Applied Biosystems, Weiterstadt, Germany). To evaluate the sensitivity of mutation detection by DHPLC, we diluted PCR products of 2 homozygously mutated cases (case 34: R139X, 415C>T; case 40: R174Q, 521G>A) with increasing amounts of wild-type (wt) PCR products. While a dilution of the mutations down to 1:32 could be detected by DHPLC analysis, a 1:8 dilution already resulted in a background signal-to-noise ratio by direct sequencing. However, all mutations detected by DHPLC in this study could be verified by direct sequencing.

Screening of FLT3, NRAS, and KIT mutations and quantification of FLT3 transcripts

Mutation screening was performed as described previously.[6,28,29] *FLT3* and *ABL* transcripts were analyzed by real-time PCR, as reported,[21] using the method involving absolute quantification of copy numbers.

Cytogenetic analysis

Cytogenetic G-banding analysis was performed according to standard methods.[30] The definition of a cytogenetic clone and description of karyotypes followed the International System for Human Cytogenetic Nomenclature (ISCN).[31] The criteria for classification of a complex aberrant karyotype were as previously described.[32]

Table 2. Sequences of primers used for PCR amplification and sequencing of *RUNX1*

Amplicon	Forward primer	Reverse primer	Nucleotide position*
1	5′-tgcagggtcctaactcaatc-3′	5′-cattgccagccatcacagtgac-3′	1548-1906
2	5′-tttcaaggtggtggcccta-3′	5′-ctgagggttaaaggcagtggagt-3′	1842-2283
3	5′-ccgggagcttgtccttttcc-3′	5′-cggcaggtaggtgtggtag-3′	2144-2643
4	5′-caggcgccttcacctactc-3′	5′-tgacctacagcgagatcctg-3′	2546-3100

*Nucleotide positions are according to GenBank entry D43968 of the human *RUNX1* transcript.

Statistical analysis

The correlation between *RUNX1* mutation status and cytogenetics was assessed by chi-square test. The significance of the differences in *FLT3* expression was evaluated by *t* test. For statistical analysis, SPSS (version 14.0) software (SPSS, Chicago, IL) was used.

Results

Frequency of *RUNX1* mutations in the AML M0 cohort

A total of 90 AML M0 samples were tested at diagnosis for *RUNX1* mutations in the 227 N-terminal amino acids (aa's) of the *RUNX1β* transcript including the DNA-binding runt homology domain (RHD; aa's 50-177). *RUNX1* mutations were detected in 41 (46%) of 90 cases and were mostly located inside the DNA-binding RHD (aa's 50-177), whereas only 6 were outside the RHD, displaying a missense mutation at Leu29 and frame-shift insertions/deletions at Arg178 (n = 1), Pro189 (n = 1), Arg197 (n = 1), and Ser199 (n = 2; Figure 1A; Table 3). The spectrum of mutations as summarized in Table 3 was composed of 21 missense mutations, 19 frame/stop mutations, and 3 in-frame deletions/insertions. Analysis of these mutations by direct sequencing identified a homozygous pattern in at least 19 of these cases (44%). In all cases referred to as heterozygous, homozygosity cannot be excluded due to the possibility of contaminated nonleukemic cells that may simulate a heterozygous pattern. In 2 patients, the combination of

Figure 1. Schematic presentation of the location of RUNX1 mutations. The horizontal bars represent the full linear polypeptide sequence from amino acid 1 to 455 of RUNX1β. Amino acid numbering is given above the bars. The DNA-binding RHD (amino acids 50-177) and the TA domain (amino acids 291-371) are indicated in gray. The location of mutations is indicated by thin vertical lines with the type of mutation on the protein level given in text boxes for (A) the AML M0 cohort and for (B) the non-AML M0 cases.

2 heterozygous missense mutations was detected (Asn69Ser/Arg80His and Lys144Asn/Gly172Ala). Interestingly, amino acid residues that are involved in direct DNA binding[34] were affected by 11 of 21 missense mutations (Arg80, n = 4; Asn171, n = 5; Arg174, n = 1; and Arg177, n = 1). Additional missense mutations targeted amino acids known to undergo conformational changes upon *CBFB* binding (Asn69; n = 1) or to be involved in protein-protein interaction between *RUNX1* and *CBFB* (Ser114, n = 2).[34]

Cytogenetic aberrations and molecular mutations in the AML M0 cohort

The AML M0 cohort (N = 90) was separated into 2 groups according to their *RUNX1* mutation status. The results of the metaphase cytogenetic analysis in both groups are summarized in Tables 3 and 4.

RUNX1 status was correlated to cytogenetics as summarized in Table 4. The distribution of *RUNX1*-mutated versus wt samples was not significantly different in AML M0 patients with normal karyotype (*P* = .114) or in samples with other abnormalities (classified as karyotype "others" in Table 4) including structural and numeric abnormalities not covered by the groups mentioned in Table 4 (*P* = .476; Table 4). In contrast, a complex aberrant karyotype was more likely to be associated with *RUNX1*-wt (*P* = .031), whereas trisomy 13 (+13) as the sole aberration (n = 18) was exclusively associated with a mutation in the *RUNX1* gene (*P* < .001). However, only one case with +13 in the context of a complex aberrant karyotype was not *RUNX1* mutated. Due to the size of some cohorts, the significance of distribution of the *RUNX1* mutation was not calculated for the residual cytogenetic subgroups.

In addition, *FLT3*, *NRAS* (codons 12/1361), and *KIT* were analyzed for mutations (Table 3). In total, 38 (93%) of 41 *RUNX1*-mutated cases and 38 (78%) of 49 *RUNX1*-wt cases were available for analysis of *FLT3*-LMs as well as for mutations in the *FLT3*-TKD. We detected *FLT3* mutations in 6 *RUNX1*-mutated cases (4 *FLT3*-LMs, 1 *FLT3*-TKD, 1 *FLT3*-LM + TKD). This number was not significantly different from the cohort with *RUNX1*-wt (7 *FLT3*-LMs and 1 *FLT3*-TKD). In addition, mutations in *NRAS* (codons 12/1361) and *KIT* (D816) were screened in *RUNX1*-mutated (n = 23) and *RUNX1*-wt cases (n = 29). Of the *RUNX1*-wt cases, 3 (10.3%) of 29 had mutations in *NRAS* in contrast to *RUNX1*-mutated cases that did not reveal any *NRAS* mutation. There was no overlap of *NRAS* with *FLT3* mutations. *KIT* mutations were not detected in this cohort of 23 patients analyzed.

RUNX1 mutations in cases with trisomy 13 independent of FAB subtype

In the next step, we selected 20 further samples based primarily on the presence of trisomy 13 and independent of FAB AML subtypes and analyzed *RUNX1* mutations within the entire coding region (Figure 1B; Table 5). Of these, 3 were diagnosed as AML M1, 7 as AML M2, 5 as AML M4, and 1 as AML M5. Three cases were not further morphologically classified according to FAB due to lacking bone marrow smears (1 acute leukemia, 2 AML). One case had a secondary AML (s-AML) after polycythemia vera. In this cohort, 17 samples carried +13 as the sole primary aberration, whereas 2 cases had 1 additional aberration and only 1 case had 2 additional cytogenetic aberrations, including +21. A *RUNX1* mutation was detected in 16 (80%) of 20 and a *FLT3* mutation in 3 (15%) of 20 (2 *FLT3*-LMs and 1 *FLT3*-TKD) of these samples (Table 5). Interestingly, 2 (1 *FLT3*-LM, 1 TKD) of the 3 *FLT3* mutations were
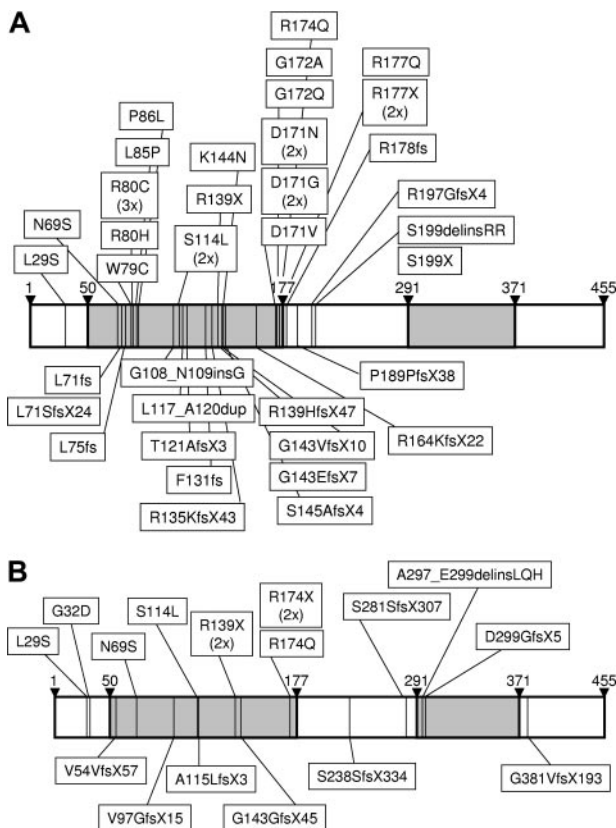
Table 3. Molecular and cytogenetic characterization of the AML M0 cohort

Patient	Diagnosis	Karyotype	*RUNX1* mutation*	*RUNX1* allele status	*FLT3* mutation	*NRAS* mutation	*KIT* D816
1	AML M0	46,XY [25]	Ser114Leu	mut/wt	*FLT3*-TKD	ND	ND
2	AML M0	46,XY,der(5)t(5;11)(q22;?) [9],46,XY [11]	Leu71fs	mut/wt	*FLT3*-LM	wt	wt
3	AML M0	46,XY [25]	[Asn69Ser(+)Arg80His]	mut/wt	*FLT3*-LM	wt	wt
4	AML M0	92,XXYY,+1,dic(1;5)(p21;q13),der(2)t(2;3)(q31;?),+8,-17 [5],46,XY [20]	Asp171Asn	mut/wt	*FLT3*-LM/TKD	wt	wt
5	AML M0	47,XX,+14 [6],46,XX [10]	Pro86Leu	mut/mut	ND	ND	ND
6	AML M0/M2	46,XX,del(5)(q15q31) [22]	Pro189ProfsX38	mut/wt	ND	ND	ND
7	AML M0	92,XXYY [15]	Leu117_Ala120dup	mut/wt	wt	ND	ND
8	AML M0	46,XY [24]	Leu75fs	mut/mut	wt	ND	ND
9	AML M0	46,XX [20]	Asp171Gly	mut/wt	wt	wt	wt
10	AML M0	46,XX [25]	Arg80Cys	mut/wt	wt	ND	ND
11	AML M0	46,XX [25]	Leu71SerfsX24	mut/wt	wt	wt	wt
12	AML M0	46,XY [22]	Gly108_Asn109insGly	mut/mut	wt	wt	wt
13	AML M0	46,XY [15]	Asn112LysfsX5	mut/wt	wt	wt	wt
14	AML M0	46,XY [25]	Arg80Cys	mut/mut	wt	ND	ND
15	AML M0	47,XY,+21 [2],48,XY,+21,+21 [14],46,XY [1]	Asp171Asn	mut/wt	wt	wt	wt
16	AML M0	46,XX [25]	Ser199delinsArgArg	mut/wt	wt	wt	wt
17	AML M0	46,XY [25]	Arg80Cys	mut/mut	wt	wt	wt
18	AML M0	46,XY,der(2)t(2;5)(p23;q15)ins(2;12)(q23;q21q24), der(4)ins(4;17)(p14;??)t(4;5)(p15;?),del(5)(q15), der(12)t(2;12)(p23;p13)del(12)(q21), der(17)t(4;17)(p15;q11), del(17)(p13) [cp15],46,XY [5]	[Lys144Asn(+)Gly172Ala]	mut/wt	wt	wt	wt
19	AML M0	45,X,-Y [8],46,XY [17]	Arg177X	mut/wt	wt	wt	wt
20	AML M0	46,XX,del(7)(q11.2) [2],46,XX [23]	Arg139HisfsX47	mut/wt	wt	wt	wt
21	AML M0	46,XY,i(17)(q10) [17]	Gly143ValfsX10	mut/mut	wt	wt	wt
22	AML M0	46,XX,r(7)(p13q11.2) [19],46,XX [1]	Asp171Gly	mut/mut	wt	wt	wt
23†	AML M0	46,XY.ish der(11)(MLL3′),der(10)(MLL5′) [24]	Leu29Ser	mut/wt	wt	ND	ND
24	AML M0	47,XY,+13 [11],46,XY [9]	Gly172Gln	mut/mut	*FLT3*-LM	ND	ND
25	AML M0	46,XX,der(19)t(13;19)(q11;p13) [6],46,XX [5]	Arg135LysfsX43	mut/wt	*FLT3*-LM	ND	ND
26	AML M0	47,XY,+13 [16],46,XY [4]	Thr121AlafsX3	mut/wt	ND	ND	ND
27	AML M0	94,XXYY,+13,+13 [13]46,XY [9]	Ser199X	mut/mut	wt	ND	ND
28	AML M0	47,XY,+13 [7],46,XY [8]	Arg197GlyfsX4	mut/wt	wt	ND	ND
29	AML M0	47,XY,+13 [10],46,XY [2]	Phe131fs	mut/wt	wt	wt	wt
30	AML M0	47,XY,+13 [7],46,XY [5]	Ser114Leu	mut/wt	wt	wt	wt
31	AML M0	47,XY,+13 [5],45,X,-Y [8],46,XY [12]	Leu85Pro	mut/mut	wt	ND	ND
32	AML M0	47,XY,+13 [9],47,XY,del(7)(q22),+13 [2],46,XY [6]	Arg177Gln	mut/wt	wt	wt	wt
33	AML M0	47,XY,+13 [6],94,XXYY,+13,+13 [2], 46,XY [6]	Arg178fs	mut/wt	wt	wt	wt
34	AML M0	47,XX,+13 [1],46,XX [2]	Arg139X	mut/mut	wt	wt	wt
35	AML M0	47,XY,+13 [10],94,XXYY,+13,+13 [1], 46,XY [9]	Gly143GlufsX7	mut/mut	wt	ND	ND
36	AML M0	47,XY,+13 [2],46,XY [12]	Trp79Cys	mut/mut	wt	wt	wt
37	AML M0	47,XY,+13 [18],46,XY [3]	Asp171Val	mut/mut	wt	wt	wt
38	AML M0	47,XY,+13 [6],46,XY [14]	Ser145AlafsX4	mut/mut	wt	wt	wt
39	AML M0	47,XY,+13 [7],45,XY,-7 [5],46,XY [8]	Arg164LysfsX22	mut/mut	wt	ND	ND
40	AML M0	47,XY,+13 [16],46,XY [2]	Arg174Gln	mut/wt	wt	ND	ND
41	AML M0	46,XX,-7,+13 [3],46,XX [17]	Arg177X	mut/mut	wt	ND	ND
42	AML M0	46,XY [11]	—	wt/wt	*FLT3*-TKD	wt	wt
43	AML M0	46,XY,t(11;19)(q13;p13) [17],46,XY [3]	—	wt/wt	*FLT3*-LM	ND	ND
44	AML M0	46,XY [25]	—	wt/wt	*FLT3*-LM	wt	wt
45	AML M0	46,XX [25]	—	wt/wt	*FLT3*-LM	wt	wt
46	AML M0	46,XX [20]	—	wt/wt	*FLT3*-LM	wt	wt
47	AML M0	46,XX,t(10;17)(p15;q21) [2],47,XX,+7, t(10;17)(p15;q21) [7],46,XX [16]	—	wt/wt	*FLT3*-LM	wt	wt
48	AML M0	47,XY,+8 [5],46,XY [15]	—	wt/wt	*FLT3*-LM	wt	wt
49	AML M0	43,XY,-3,der(3)t(3;7)(q11;q11),-5,der(7) t(5;7)(p11;q11), der(12)t(12;15)(p13;q22),-15, der(17)t(3;17)(q11;p13) [9],42,idem,-18 [7]	—	wt/wt	*FLT3*-LM	wt	wt
50	AML M0	55,XY,+X,+4,+5,+8,+10,+13,+14, +17,+18 [4],47,XY,+X [8]	—	wt/wt	*FLT3*-LM	ND	ND
51	AML M0	46,XY,del(11)(q13q21) [10],46,XY [15]	—	wt/wt	ND	ND	ND
52	AML M0	47,XY,+8,i(17)(q10) [20]	—	wt/wt	ND	ND	ND

mut indicates mutated; ND, not determined; and —, not applicable.

*Nomenclature of the mutations is according to den Dunnen and Antonarakis.[33]

†A cytogenetically cryptic MLL rearrangement; MLL-AF10 rearrangement was proven by RT-PCR.

‡A monosomy 13 detected by FISH.

Table 3. Continued

Patient	Diagnosis	Karyotype	RUNX1 mutation*	RUNX1 allele status	FLT3 mutation	NRAS mutation	KIT D816
53	AMLM0	46,XX [25]	—	wt/wt	ND	ND	ND
54	AMLM0	46,XX [7]	—	wt/wt	ND	ND	ND
55	AMLM0	46,XX [12]	—	wt/wt	ND	ND	ND
56	AMLM0	44,XY,add(3)(p13),-5,dic(11;?)(q14;?), add(11)(p15),-15,add(17)(p11) [13], 50–51,idem,+4,+5,+6,+6,+2–3mar [cp4],46,XY [3]	—	wt/wt	ND	ND	ND
57	AMLM0	47,XX,+14 [19],46,XX [1]	—	wt/wt	ND	ND	ND
58‡	AMLM0	46,XY [25],FISH-Screening: 65% Monosomie 13 oder grosse Deletion	—	wt/wt	ND	ND	ND
59	AMLM0	47,XY,+22 [13],46,XY [20]	—	wt/wt	ND	ND	ND
60	AMLM0	46,XX [15]	—	wt/wt	ND	ND	ND
61	AMLM0	46,XY [25]	—	wt/wt	ND	ND	ND
62	AMLM0	46,XY [20]	—	wt/wt	wt	mut	wt
63	AMLM0	46,XX,-7,+19 [10],46,XX [14]	—	wt/wt	wt	ND	ND
64	AMLM0	45,X,-Y,t(11;19)(q23;p13.3) [19],46,XY [1]	—	wt/wt	wt	wt	wt
65	AMLM0	46,XX,t(9;11)(p22;q23) [12],46,XX [13]	—	wt/wt	wt	wt	wt
66	AMLM0	45,XX,inv(3)(q21q26),-7 [20]	—	wt/wt	wt	wt	wt
67	AMLM0	47,XY,+9 [2],54,XY,+4,+6,+8,+9, +11,+13,+13,+19 [4],46,XY [14]	—	wt/wt	wt	wt	wt
68	AMLM0	46,XY [25]	—	wt/wt	wt	wt	wt
69	AMLM0	46,XY [25]	—	wt/wt	wt	wt	wt
70	AMLM0	46,XX [21]	—	wt/wt	wt	wt	wt
71	AMLM0	46,XY [20]	—	wt/wt	wt	wt	wt
72	AMLM0	46,XY [22]	—	wt/wt	wt	wt	wt
73	AMLM0	42,XX,del(3)(p13),der(5)t(5;12)(q13;q15), dic(7;18)(p11;q11),i(8)(q10), dup(9) (q32q34),-12,der(13;15)(q10;q10), der(14) t(12;14)(p11;p10)t(1;14)(?;q32),-17 [15]	—	wt/wt	wt	wt	wt
74	AMLM0	46,XY [20]	—	wt/wt	wt	wt	wt
75	AMLM0	46,XY [21]	—	wt/wt	wt	mut	wt
76	AMLM0	46,XX [25]	—	wt/wt	wt	ND	ND
77	AMLM0	46,XY [20]	—	wt/wt	wt	wt	wt
78	AMLM0	46,XY [22]	—	wt/wt	wt	wt	wt
79	AMLM0	44–45,XX,del(4)(q23),der(4)t(4;7)(q23;?), del(5)(q13q31), der(7)del(7)(p15)t(4;7) (q23;q21),der(10)t(10;11)(q21;q13),-11, dic(12;17)(p13;p13),der(19)t(11;19)(?;q13) ins(11;19)(?;?)t(10;11) [cp12], 46,XX [2]	—	wt/wt	wt	wt	wt
80	AMLM0	46,XX,del(5)(q13q31) [1],46,XX,t(1;3;9)(p11;q26;q34),del(5) (q13q31) [14]	—	wt/wt	wt	wt	wt
81	AMLM0	46,XX,del(7)(q22q31) [19],46,XX [2]	—	wt/wt	wt	wt	wt
82	AMLM0	45,XY,-7 [15]	—	wt/wt	wt	mut	wt
83	AMLM0	45,XY,t(3;3)(q21;q26),-7 [11],46,XY [3]	—	wt/wt	wt	wt	wt
84	AMLM0	46,XX,del(9)(q22) [4],46,XX [13]	—	wt/wt	wt	wt	wt
85	AMLM0	46,XX,t(2;11)(p21;q23),del(5)(q13q33) [16],46,XX [6]	—	wt/wt	wt	ND	ND
86	AMLM0	46,XY [15]	—	wt/wt	wt	ND	ND
87	AMLM0	40–42,XY,-1,-5,-7,-8,-11,del(12)(p11), der(14)t(1;14)(p13;p11),-17, der(17)t(5;?;17)(q?;?;p12),-18,+2–4mar [cp16],46,XY [1],40,XY,-1,-5, add(7)(q11),der(8)t(1;8)(p13;p23), der(11)?del(11)(p13)del(11)(q21), del(12)(p11),add(14)(p11),der(17),add(1)	—	wt/wt	wt	ND	ND
88	AMLM0	46,XY,t(3;4)(q25;q31),del(5)(q15),der(7)t(5;7) (q?;q11),del(15)(q15) [20]	—	wt/wt	wt	ND	ND
89	AMLM0	46,XX,del(5)(q13q31),r(7)(p11q11),del(18)(q11) [20]	—	wt/wt	wt	wt	wt
90	AMLM0	40–43,XY,del(5)(q13q31),der(8)t(8;12)(q21;q13), ins(11)(q13;?),-12, del(13)(q14),i(17)(q10) [cp6],46,XY [20]	—	wt/wt	wt	ND	ND

mut indicates mutated; ND, not determined; and —, not applicable.

*Nomenclature of the mutations is according to den Dunnen and Antonarakis.[33]

†A cytogenetically cryptic MLL rearrangement; MLL-AF10 rearrangement was proven by RT-PCR.

‡A monosomy 13 detected by FISH.

Table 4. *RUNX1* mutation status is correlated to cytogenetic aberrations in the AML M0 cohort

M0	*RUNX1* mutation status		
Karyotype	*RUNX1*-wt, n=49	*RUNX1* mutated, n=41	*P*
Normal	21	11	.114
Complex	10	2	.031
11q23/MLL	3	1	ND
inv(3)t(3;3)	2	0	ND
-5/5q-	0	1	ND
-7/7q-	2	2	ND
+8	1	0	ND
Others	10	5	.476
+13	0	18*	<.001
+21	0	1	ND

ND indicates not determined.

*In contrast to all other cases, the trisomy 13 subgroup was not entirely derived from a randomly selected cohort but was expanded from 9 (from the randomly selected group) to 18 samples (after the initial analysis) selected on the basis of a +13.

detected in cases with *RUNX1*-wt. The *RUNX1* mutations could be divided into 5 missense and 12 out of frame/stop mutations (1 patient carried Gly32Asp/Ser238SerfsX334 in parallel). Three of the missense mutations were located inside the RHD and were already described for the AML M0 cohort (Asn69Ser, Ser114Leu, Arg174Gln), whereas two were located outside with Leu29Ser and Gly32Asp. Most of the 12 out of frame/stop mutations were also located inside the RHD (n = 8). The other 4 mutations, however, were N-terminal to or inside the transactivation (TA) domain (aa's 291-371), except for one, which was C-terminal to the TA domain (Gly381ValfsX193). The data on the non-AML M0 cases are summarized in Table 5.

Of note is also the lack of *RUNX1* mutations in 15 of 15 cases with +13, all seen in the context of a complex aberrant karyotype, indicating that these cases have completely different underlying molecular mechanisms (not shown).

Correlation of +13 to *FLT3* expression

As there was a highly significant correlation of trisomy 13 with *RUNX1* mutation independent of the AML FAB subtype, we suspected that factors on chromosome 13 might collaborate with *RUNX1* mutations during development of myeloid leukemia. *FLT3* is located on chromosome 13; however, there was no significant correlation of *FLT3* mutation with +13 or with *RUNX1* mutation. We hypothesized that trisomy 13 might be a mechanism to increase *FLT3* expression in order to activate *FLT3*-mediated signal transduction.[22] *FLT3* transcript levels of 33 AML M0 cases were analyzed by quantitative real-time PCR (RQ-PCR) with a LightCycler instrument using *ABL* as a reference gene. This group consisted of 8 cases with trisomy 13 as the sole cytogenetic abnormality and *RUNX1* mutation, and these cases were compared with 25 consecutive AML M0 cases without +13. The mean *FLT3* expression level of the +13 cases with *RUNX1* mutation (n = 8) was 3332 ± 1101 (mean $\pm$ SEM), which was significantly higher than in cases without +13 (mean value of 522 ± 109; $P < .001$, t test; Figure 2A). This increase was also significant ($P = .024$) when *FLT3* expression of the AML M0 +13 cases (n = 8) was compared with AML M0 with normal karyotype (n = 9) in the same cohort with mean $\pm$ SEM values of 3332 ± 1101 and 659 ± 248, respectively.

To permit comparison of *FLT3* expression of +13 samples to samples without +13 independent of FAB subtype, *FLT3* expression was evaluated in FAB subtypes other than AML M0 (n = 35). This group consisted of 14 cases from various FAB subgroups with +13 and *RUNX1* mutation as characterized in "Results," "*RUNX1* mutations in cases with trisomy 13 independent of FAB subtype," and 21 AML cases with normal karyotype, which reflected the same FAB subtype distribution. Again, a highly significant increase of FLT3 expression of the +13/RUNX1-mutated cases versus the cases with normal karyotype was detected with 1612 ± 556 and 369 ± 78, respectively (mean $\pm$ SEM, $P = .011$; Figure 2B). These cases were combined with the previously analyzed AML M0

Table 5. Characterization of non–AML M0 cases according to karyotype and *RUNX1* and *FLT3* mutation

Patient	Diagnosis	Karyotype	*RUNX1* mutation*	*RUNX1* allele status	*FLT3* mutation
1	AL	47,XX,+13 [20]	[Gly32Asp(+)Ser238SerfsX334]	mut/mut	wt
2	AML	47,XX,+13 [10],46,XX [14]	R139X	mut/mut	wt
3	AML	47,XX,+13 [3],48,XX,+13,+13 [6],46,XX [11]	Val54ValfsX57	mut/wt	ND
4	AML M1	47,XX,+13 [2],46,XX [19]	wt	wt/wt	*FLT3*-LM
5	AML M1	47,XY,+13 [8],48,XY,+13,+13 [13],46,XY [8]	Asn69Ser	mut/wt	wt
6	AML M1	47,XY,+13 [5],46,XY [12]	Gly143GlyfsX45	mut/mut	wt
7	AML M2	47,XY,+13 [20]	Arg174X	mut/mut	wt
8	AML M2	47,XY,+13 [20]	Leu29Ser	mut/wt	wt
9	AML M2	47,XY,+13 [4],46,XY [16]	wt	wt/wt	wt
10	AML M2	46,XY,+13,+15 [15],46,XY [2]	Val97GlyfsX15	mut/wt	wt
11	AML M2	47,XY,+13 [2],47,XY,+13,i(17)(q10) [13],46,XY [5]	wt	wt/wt	ND
12	AML M2	94,XXYY,+13,+13 [2],93,XXYY,-7,+13,+13 [5],92,XXYY,-7,+13 [2],46,XX [11]	Ala115LeufsX3	mut/mut	wt
13	AML M2	47,XY,+13 [12],46,XY [8]	Ser281SerfsX307	mut/wt	wt
14	AML M4	47,XY,+13 [2],46,XY [18]	Arg174Gln	mut/mut	*FLT3*-LM
15	AML M4	47,XX,+13 [1],47,XX,+13,der(16)t(11;16)(q11;p13) [3],46,XX [20]	Gly381ValfsX193	mut/wt	wt
16	AML M4	47,XX,+13 [16],46,XX [4]	Arg177X	mut/wt	wt
17	AML M4	47,XX,+13 [2],46,XX [25]	wt	wt/wt	*FLT3*-D835
18	AML M4	48,XX,del(12)(p12),+13,+21 [20]	[Ala297_Glu299delinsLeuGlnHis]+ [Asp299GlyfsX5]	mut/wt	wt
19	AML M5	47,XX,+13,der(18)t(11;18)(q23;q23) [20],46,XX [3]	Arg139X	mut/mut	wt
20	s-AML M2	47,XY,+13 [4],46,XY [9]	Ser114Leu	mut/wt	wt

*Nomenclature of the mutations is according to den Dunnen and Antonarakis.[33]

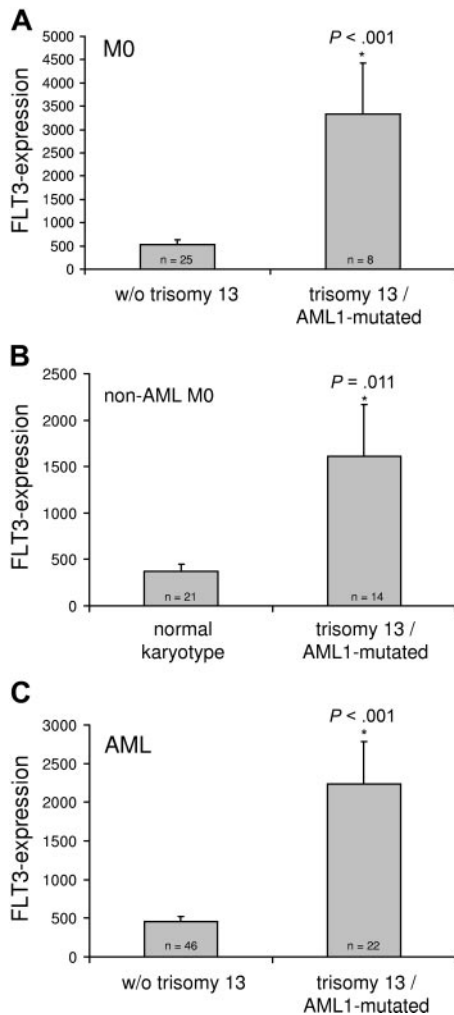AL indicates acute leukemia; mut, mutated; and ND, not determined.

Figure 2. FLT3 expression in AML cases with trisomy 13 and *RUNX1* mutation compared with cases without trisomy 13. *FLT3* transcripts were quantified by RQ-PCR on a LightCycler instrument. (A) Unselected AML M0 samples without trisomy 13 (w/o trisomy 13; n = 25) were compared with AML M0 with trisomy 13 plus a *RUNX1* mutation (trisomy 13/RUNX1-mutated; n = 8). (B) AML samples from FAB subgroups other than AML M0 with normal karyotype (n = 21) were compared with samples with trisomy 13 and RUNX1 mutation (n = 14). (C) Unselected AML M0 samples together with AML samples from other FAB subgroups without trisomy 13 (w/o trisomy 13; n = 46) were compared with AML with trisomy 13 plus a *RUNX1* mutation (trisomy 13/RUNX1-mutated; n = 22). *FLT3* expression is given as 100*FLT3/ *ABL* as previously described.[21] The bars represent mean values ± SEM. Each sample was analyzed in duplicate.

cases to a total number of 68 cases, consisting of 22 cases with trisomy 13 plus *RUNX1* mutation and 46 cases without trisomy 13. The comparison of both groups showed a highly significant ($P < .001$, t test), about 4-fold, increase of *FLT3* expression of the +13 samples (n = 22) versus the samples without trisomy 13 (n = 46) with mean ± SEM of 2237 ± 549 and 452 ± 69, respectively (Figure 2C).

Discussion

In this study we confirmed the high incidence of *RUNX1* mutations in AML M0 that has been reported previously. For the first time we detected a highly significant correlation of RUNX1 mutation to trisomy 13 as the sole primary aberration, which is independent of the FAB subtype. However, trisomy 13 in the context of a complex aberrant karyotype was not correlated to *RUNX1* mutation. *FLT3* is

located on chromosome 13 and quantification of *FLT3* transcript levels indicated an increased *FLT3* expression in samples with *RUNX1* mutation together with trisomy 13 compared with samples without trisomy 13, arguing for cooperation of both events in leukemogenesis.

The *RUNX1* gene is known to be involved in leukemogenesis since it was cloned as a fusion gene from the t(8;21) translocation.[35] Morphologically most of the t(8;21)-carrying AML cells are classified as AML M2 and less frequently as AML M1. Recently, somatic mutations have been identified in the *RUNX1* gene in myeloid leukemias.[11] These mutations occurred with the highest frequency in the RHD of the *RUNX1* gene in AML M0,[14] in cases with acquired trisomy 21,[14] and in certain MDS cases,[17,18,36] indicating that leukemogenesis is effected by different signaling pathways by *AML1-ETO* and by *RUNX1* somatic mutations. No mutations in *RUNX1* were detected outside the RHD (aa's 50-177) in AML M0 in 2 previous studies when the entire cDNA of *RUNX1β* was analyzed.[11,16] Therefore, most subsequent studies relied on screening of exons 3-5 in AML M0 containing the RHD up to R177.[14,19] In the present study we analyzed *RUNX1* mutations on the cDNA level (codons 1-227) in a large cohort of AML M0 samples at diagnosis (N = 90) to correlate the results to cytogenetic aberrations as well as to gene mutations in receptor-tyrosine kinase pathways. The percentage of AML M0 with *RUNX1* mutations in our study, 46%, is high compared with previous studies with values ranging between 16% and 27%.[14,16,19] Three possible explanations might account for this finding. First, our AML M0 patient population is characterized by a high incidence of trisomy 13 (20%; Table 3), a cytogenetic finding which is 100% correlated to *RUNX1* mutation in our study. Second, some of the previous studies screened only exons 3-5 of the *RUNX1* gene,[14,19] where the 3'-end of exon 5 represents the codon for R177, the extreme C-terminus of the RHD. In our study, we screened codons 1-227 of the *RUNX1β* cDNA of the AML M0, and 5 of the 41 *RUNX1*-mutated cases had mutations C-terminal to R177 (Table 3). Third, in contrast to previous reports, which applied single-strand conformation polymorphism (SSCP) analysis, our screening method for the detection of *RUNX1* mutations relied on the use of the more sensitive DHPLC technology.[37]

From structural analysis of the RHD of *RUNX1*,[34] many of the missense mutations detected in the AML M0 population in our study are supposed to disrupt DNA binding or have an impact on CBFB binding (Asn69, Arg80, Ser114, Asn171, Arg174, Arg177) while leaving the TA domain of *RUNX1* intact. On the other hand, the large majority of the residual mutations in our study displayed frame-shift or stop mutations in the RHD, which probably lead to a complete loss of RUNX1 protein function. Opposed to this are frame-shift mutations in 5 AML M0 samples that are C-terminal to the RHD, which are supposed to disrupt the TA domain while leaving the DNA-binding domain intact, indicating that different functional effects might result from these different classes of mutations. The next level of complexity is caused by the presence of these mutations on one or both alleles. In a previous study, almost all AML M0 patients with a *RUNX1* mutation carried the mutation on both alleles.[14] In the present study, homozygous and heterozygous mutations were about equally distributed in AML M0. However, homozygosity cannot be excluded because 20% to 30% of normal cells in the leukemic samples may mimic a pattern of heterozygosity.

Different from AML M0, a considerable portion of mutations were described to be located C-terminal to the RHD in therapy-related MDS as well as in MDS/AML.[17,18] Therefore, the entire

coding region of *RUNX1* was screened for mutations in the non-AML M0 cases with trisomy 13 in this study. The mutation pattern in the *RUNX1* gene in the non-AML M0 cases was similar to the AML M0 cases, with most of the mutation clustered inside the RHD (Table 5). However, we also detected 4 frame-shift mutations C-terminal to the RHD. These mutations would have escaped detection in our AML M0 screening, as they were located at codons 238, 281, 296, and 381 of the *RUNX1* transcript. The latter mutation is even located C-terminal to the TA domain (aa's 291-371) and the functional significance of this mutation remains to be determined.

RUNX1 mutations in AML M0 in our study were not restricted to trisomy 13, but all trisomy 13 samples (n = 18) were *RUNX1* mutated. In a second set of samples that were selected for trisomy 13 in different FAB subtypes other than AML M0, again an extremely high portion of cases was *RUNX1* mutated (n = 16/20, 80%).

Overall, trisomy 13 is a rare recurring clonal chromosomal aberration with an incidence of 2.4% in de novo AML,[38,39] and in agreement with our study these cases are mainly clustered in the AML M0 subtype.[40] The correlation of trisomy 13 and *RUNX1* mutations suggests that there might be a cooperating event between these two aberrations. Cooperation between hematopoietic transcription factors and members of the *RAS* or the RTK family has been proposed as a mechanism for leukemia development.[1] Mutations of *NRAS* and *KIT* have not been detected in the *RUNX1*-mutated samples in our study in the AML M0 cohort. Activating mutations of the RTK *FLT3*, which is located on chromosome 13, have been suggested as a cooperating event previously.[41] In de novo AML, these *FLT3* mutations have the highest incidence in normal karyotype (39%) as well as in t(15;17) with the *PML-RARA* fusion gene (36%).[29] Less frequent is the coincidence of *FLT3*-LMs with the *AML1-ETO* fusion gene from t(8;21) (9%).[29] *FLT3* mutations in our study did not correlate to *RUNX1* somatic mutations or to trisomy 13. Another mechanism of *FLT3* activation in addition to mutation is *FLT3* overexpression, which results in *FLT3* autophosphorylation and a decreased overall survival.[22] However, the mechanism of *FLT3* overexpression remains yet unknown.[22] In the present study, we hypothesized that trisomy 13 might be a mechanism to increase *FLT3* expression by means of a gene-dosage effect. This hypothesis is confirmed by quantitative real-time PCR, which detected a highly significant increase of *FLT3* transcript levels in AML with trisomy 13 and *RUNX1* mutation compared with samples without trisomy 13 (Figure 2). Trisomies as a gain-of-function mechanism by selection of a gain-of-function mutation or by gene dosage have been also implicated in other instances. Chromosome 4 harbors the *KIT* protooncogene, and AML patients with trisomy 4 as well as patients with t(8;21) both have the highest incidence of the activating *KIT*-D816 mutation,[6]

which lead to the amplification of the mutated allele by trisomy 4.[42] Similar to this, amplification of chromosome 21, the locus of *RUNX1*, by acquired trisomy 21 in myeloid malignancies is associated with *RUNX1* somatic mutations and duplication of the mutated allele.[14] Beyond hematologic malignancies, the same phenomenon is known to occur from solid tumors in hereditary renal carcinoma, where trisomy 7 amplifies activating mutations of the *MET* oncogene,[43,44] which belongs to the family of RTKs like *KIT*. In contrast to our study, trisomies in these studies effected the amplification of a mutated allele with an activating mutation. However, as shown by Ozeki et al,[22] overexpression of *FLT3* might already be sufficient for *FLT3* activation, and this activation by overexpression might be a general phenomenon in RTK signaling, as t(8;21)-induced overexpression of *KIT* was also associated with *KIT* autophosphorylation.[45] The gain of chromosomes (ie, trisomy 8, 11, and 13) as a mechanism for increased expression of genes located in the respective regions has been also demonstrated by gene-expression profiling in a previous study.[46]

Taken together, the data of the present study show a highly significant correlation of *RUNX1* mutation with trisomy 13 in various AML FAB subgroups but not with *FLT3*, *N-RAS,* or *KIT* mutations. Trisomy 13 correlates with an increase of *FLT3* transcript levels, which might cooperate with *RUNX1* mutation in leukemogenesis. As *FLT3* overexpression is associated with *FLT3* activation,[22] the subgroup of patients with *RUNX1* mutation plus trisomy 13 might benefit from treatment with *FLT3* inhibitors.[47,48]

Authorship

Contribution: F.D. was responsible for the molecular biology, was in part responsible for the study design, and wrote the paper; C.H. was responsible for cytogenetic analysis; W.K. was responsible for immunophenotyping; T.H. was responsible for cytomorphology; and S.S. was responsible for study design and contributed in writing the paper.

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