

## Detailed analysis of FLT3 expression levels in acute myeloid leukemia

Florian Kuchenbauer  
Wolfgang Kern  
Claudia Schoch  
Alexander Kohlmann  
Wolfgang Hiddemann  
Torsten Haferlach  
Susanne Schnittger

**Background and Objectives.** *FLT3* mutations are found in up to 30% of cases of acute myeloid leukemia (AML). Although new *FLT3* mutations are being investigated, the role of *FLT3* expression levels in wild type as well as in mutated *FLT3* has only been infrequently addressed.

**Design and Methods.** To further evaluate the role of *FLT3* in AML we investigated *FLT3* expression levels in 207 adult AML patients and 8 healthy donors by real-time polymerase chain reaction (PCR). The expression levels were correlated with clinical parameters, FAB types, cytogenetics, flow cytometry, microarray analysis, *FLT3* mutations, further molecular aberrations and prognosis.

**Results.** *FLT3* expression levels were different in certain FAB types with increasing levels in the following order: M3<M3v<M6<M2<M4eo<M4<M0<M1<M5a<M5b. These results correlate with the *FLT3* receptor surface expression (CD135) detected by flow cytometry ( $p<0.001$ ), showing the highest CD135 expression in FAB M5. Independent analysis of *FLT3* expression in cytogenetic AML subgroups showed the lowest levels in t(15;17) and the highest in the t(11q23) positive AML. In addition, *FLT3* expression levels correlated with high percentages of bone marrow blasts ( $p<0.001$ ) and high leukocyte counts ( $p<0.001$ ). On the molecular level, no differences in *FLT3* expression levels were detected between AML with and without any *FLT3* mutation as well as for FAB M5 with or without *MLL* abnormalities ( $p=0.495$ ). Furthermore, no significant difference could be found between the group of t(11q23) and *MLL-PTD* ( $p=0.180$ ) or between *MLL-PTD* positive and *MLL* negative normal karyotypes ( $p=0.859$ ). In patients with normal cytogenetics no impact on overall survival or event-free survival could be detected ( $p=0.128$  and  $p=0.305$ , respectively) regardless of *FLT3* mutation status, whereas investigating the group of patients with normal cytogenetics and wild-type *FLT3*, a clear tendency for a worse overall ( $p=0.059$ ) and event free ( $p=0.087$ ) survival was found.

**Interpretation and Conclusions.** *FLT3* expression levels are correlated with clinical data, genetic subgroups as well as prognosis. Furthermore, our data indicate that *FLT3* expression and signaling are closely associated with FAB M5.

Key words: multiple myeloma, high-dose therapy, autotransplant, remission, outcomes research.

Haematologica 2005; 90:1617-1625

From the Laboratory for Leukemia Diagnostics, Department of Internal Medicine III, Ludwig-Maximilians University of Munich, University Hospital Grosshadern, Munich, Germany (FK, WK, CS, AK, WH, TH, SS); Clinical Cooperative Group Leukemia (GSF), National Research Center for Environment and Health, Munich, Germany (FK,WH).

Correspondence:  
Susanne Schnittger Ph.D.,  
Laboratory for Leukemia,  
Diagnostics, Department of Internal  
Medicine III, University Hospital  
Grosshadern, Ludwig-Maximilians-  
University, Marchioninistraße 15,  
81377 München, Germany. E-mail:  
susanne.schnittger@med3.med.uni-  
muenchen.de

During the last few decades the classification of acute myeloid leukemia (AML) shifted from a morphologically based classification to a scheme including cytogenetics (WHO 1999).<sup>1</sup> In addition, molecular mutations are of increasing importance for stratification and risk assessment of AML. Investigations of genetic aberrations lead to a better understanding of molecular lesions in the pathogenesis of AML, thus enabling new AML subgroups and prognostic factors to be defined. *FLT3* length mutations (*FLT3-LM*) in the juxtamembrane domain-coding sequence of the *FLT3* gene, caused by internal tandem duplications, insertions, or both, have been found in approximately 20-25% of cases of adult AML.<sup>2-5</sup> Additionally, mutations in codons 835 and 836 in the tyrosine kinase domain (*TKD* mutations) can be detected in 7% of AML patients.<sup>3,6-8</sup> In con-

trast to *TKD* mutations, which do not have a clear prognostic impact, *FLT3-LM* were identified as a significant risk factor in AML patients.<sup>3,5,9-11</sup> Two large studies showed no difference in overall survival, but a shorter event-free survival because of a higher relapse rate in patients with *FLT3-LM*.<sup>2,3</sup> Smaller studies also indicated that these mutations affect complete remission rates and overall survival, especially in patients under the age of 60.<sup>2,3,6,11</sup> These different observations might be due to differences in the cohorts and treatment regimens of various intensities. Clinical trials showed that *FLT3-LM* are strongly associated with leukocytosis, high blast counts, normal cytogenetics and t(15;17).<sup>2,3</sup> The mutation is rare in AML cases with t(8;21), inv(16), 11q23 rearrangements and complex aberrant karyotypes. *FLT3-LM* are found in all FAB subtypes with the highest frequency in

**Table 1. Patients' characteristics.**

AML patients Bone marrow donor	n=207 n=8
Median age, years	61.4 (range: 17.4-83.6)
Gender	m: 107/f: 100
De novo AML	n=179
secondary AML following MDS therapy-related AML	n=6 n=22
Median WBC ( $\mu\text{L}$ ) (range: 80 - 514000)	16600
Median platelet count ( $\times 10^9/\text{L}$ ) (range: 11 - 846)	52500
Median hemoglobin (g/dL) (range: 4 - 15)	9.2

M3v.<sup>2,12,13</sup> A higher frequency of *FLT3-LM* has been reported in elderly patients, although without these mutations having a prognostic impact,<sup>14</sup> whereas in a previous study we found that the median age of the *FLT3-LM* positive patients was significantly lower than that of the *FLT3-LM* negative patients.<sup>2</sup> The frequency of *FLT3-LM* in childhood cases is 5-15%, thus being lower than in adults.<sup>15</sup>

Pathophysiologically, *FLT3-LM* were postulated to cause receptor dimerization resulting in constitutive auto-phosphorylation of tyrosine residues.<sup>15</sup> The activated receptor promotes ligand-independent proliferation, a block of myeloid differentiation and the induction of a myeloproliferative-like disease in a mouse model.<sup>6,14</sup> Considering the frequent expression of wild-type *FLT3* in leukemic and normal bone marrow cells and its functions as a regulator of differentiation and apoptosis and a modulator of the immune system, the role of the wild-type *FLT3* transcript level in adult AML is not fully understood.<sup>16-19</sup> Recent studies have started to characterize the clinical relevance of the *FLT3* transcript level and differences in *FLT3* expression in specific AML subgroups.<sup>13,20-22</sup> To further clarify the significance of the *FLT3* expression level in AML, we studied *FLT3* transcript ratios by quantitative real time PCR in 207 AML patients and correlated these cytogenetic data, cytomorphology, *FLT3* mutation status, CD135 and CD34 protein expression as well as with clinical parameters and prognosis.

## Design and Methods

### Patients' samples

Of the 215 samples analyzed, 179 were from patients with *de novo* AML, 6 from patients with sec-

ondary AML after myelodysplastic syndrome (s-AML), 22 from patients with AML after treatment of a previous malignant disease (t-AML) and 8 were from healthy bone marrow donors (Table 1). All samples were referred to the Laboratory for Leukemia Diagnostics between 1999-2004. The samples were chosen according to standard FAB criteria, cytogenetics and *FLT3* mutation and underwent a standardized processing including central sample registration, preparation, and evaluation by cytomorphology, cytochemistry, multiparameter immunophenotyping, cytogenetics, fluorescence *in situ* hybridization, and molecular genetics. Prior to therapy, all patients gave their informed consent to participation in the AMLCG study.<sup>23</sup> The study design adhered to the declaration of Helsinki and was approved by the ethics committees of the participating institutions prior to its initiation.<sup>23</sup> Initially, patients' samples were randomly chosen from our database according to FAB type, cytogenetics and molecular aberrations. In the course of the project we expanded certain groups to confirm some results.

### Treatment protocol of the German AMLCG Study

Treatment in the 1999 trial was identical to that in the previously described 1992 trial with the following exceptions:<sup>24</sup> patients were randomized (i) to receive TAD/HAM or HAM/HAM as double induction therapy and (ii) to receive autologous stem cell transplantation or three years of maintenance after consolidation therapy (patients under the age of 60 years only; all patients  $\geq 60$  years old received maintenance treatment). All patients were randomized to receive or not priming with granulocyte colony-stimulating factor (G-CSF) two days before and during chemotherapy.

### Cytogenetics

Cytogenetic G-banding analysis was performed according to standard methods.<sup>25</sup> The definition of a cytogenetic clone and descriptions of karyotypes followed the International System for Human Cytogenetic Nomenclature (ISCN).<sup>26</sup>

### Sample preparation

Mononucleated bone marrow cells were obtained by Ficoll Hypaque density gradient centrifugation. Samples were analyzed by FACS for percentage of blasts.<sup>27</sup> The average range of blasts in all samples after Ficoll Hypaque separation was between 90 and 100 percent. mRNA extraction and cDNA synthesis was performed as already described.<sup>2</sup>

### Quantification of the *FLT3* and *ABL* transcripts by real-time PCR

Real-time PCR using the LightCycler technology (Roche Applied Science, Mannheim, Germany) was performed to quantify the *FLT3* and *ABL* transcripts in

individual samples. Primers used for *FLT3* amplification were *FLT3-F*: CCGCCAGGA-ACGTGCTTG and *FLT3-R*: ATGCCAGGGTAAGGATTCACACC and *ABL* primers as published previously.<sup>28</sup> Each quantitative reverse transcription PCR reaction was carried out in a 20  $\mu$ L reaction volume with 0.5  $\mu$ M each of forward and reverse primer, 4 mM MgCl<sub>2</sub>, 2  $\mu$ L LightCycler-Fast Start DNA Master SYBR Green I (Roche Applied Science) and 2  $\mu$ L cDNA. Amplification was done with 45 rounds of denaturation for 1 sec at 95°C, annealing for 10 sec at 60°C and elongation for 10 sec at 72°C. LightCycler data were analyzed using the LightCycler 3.0 software and the second derivative maximum method. LightCycler relative quantification software 1.0 (Roche Applied Science) was used for the quantification. A standard curve was produced for *FLT3* and *ABL* by 10-fold dilution series of six different plasmid concentrations. The standard curve was saved in a standard curve file. In all following runs a reference dilution was analyzed and the standard curve loaded over this reference sample. *ABL* expression was assessed to normalize for the quality and quantity of the isolated RNA as well as the efficiency of cDNA synthesis. *ABL* was chosen as a housekeeping gene as it was previously shown to be the most adequate for quantitative analysis in AML.<sup>29</sup> A relative quantification was performed by calculating the ratios of the target fusion gene using a plasmid dilution containing the respective fusion target as a standard curve in relation to the housekeeping gene. For simplicity the ratios were multiplied by a factor of 100. Thus, all expression ratios are given as:  $100 \times FLT3/ABL$ . Any samples that had *ABL* values  $< 1$  fg/2 $\mu$ L cDNA ( $< 1.3 \times 10^4$  *ABL* copies), isolated from  $2 \times 10^5$  cells, were excluded from subsequent analysis.

#### Detection of *MLL-PTD* and *FLT3*-mutations

*MLL-PTD* and *FLT3* mutations were analyzed as previously described.<sup>2,30,31</sup>

#### Reproducibility and accuracy of assays

Positive and negative controls were included in all assays. In particular, RNA from any diagnostic sample was used as a positive control. Negative controls included reactions with no cDNA. All assessments were conducted at least in duplicate to confirm the results. Precautions taken to avoid contamination included the use of an UV cabinet (Appligene Oncor, Heidelberg, Germany) and PCR-designated pipettes with filtered tips.

#### Quantitative analysis of the *FLT3* mutations

Analyses were performed as previously described.<sup>2</sup> *FLT3-LM* were divided into five categories according to densitometric estimations by Genescan analysis:<sup>3</sup> (i) mutant fragment less intense than the wild-type band

(ratio  $< 0.6$ ), (ii) mutant fragment equally intense as the wild-type band (ratio  $\geq 0.6-1$ ), (iii) mutant fragment more intense than the wild-type band (ratio  $> 1$ ), (iv) only mutant fragment with loss of the wild-type band and (v) more than one mutated fragment.

#### Flow cytometry

Phycoerythrin (PE)-conjugated anti-CD135 monoclonal antibody and PE-conjugated anti-CD34 (both from Immunotech, Marseilles, France) were used. Flow cytometry analysis was performed gating on all cells acquired after Ficoll separation. Thus, the cell populations analyzed were identical for flow cytometry and mRNA analysis. The percentages of positive cells were determined using isotype controls as described previously.<sup>32</sup> The antibody was added to  $1 \times 10^6$  mononuclear cells (volume: 100  $\mu$ L) and incubated for 10 minutes. After addition of 2mL lysing solution the samples were incubated for another 10 minutes and were washed twice in phosphate-buffered saline (PBS) and resuspended in 0.5 mL PBS. Multiparameter flow cytometry analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CS, USA).

#### Microarray analysis

Samples, prepared as described elsewhere,<sup>33</sup> were profiled using U133A GeneChip<sup>®</sup> expression microarrays (Affymetrix, Santa Clara, USA). The Affymetrix software package (Microarray Suite 5.0.1) was used to detect the cells (U133A mask file; TGT value: 5000).<sup>34</sup> The minimal quality control parameters for inclusion of an expression profile in our study were more than 30% present calls and a 3'/5' ratio of 3 or less of represented glyceraldehyde-3'-phosphate dehydrogenase gene (*GAPDH*) probe sets. All probe sets were functionally annotated by the current NetAffx database release.<sup>35</sup> For this study, the probe set 206674\_at, representing *FLT3*, was of interest. In order to compare gene expression profiling intensities with quantitative reverse transcription PCR results, present and marginal calls were rated positive and absent calls were rated negative (signal intensity values were reset to 1).

#### Statistical analysis

Overall survival was calculated from the first day of therapy to death and event-free survival to non-response, relapse or death using Kaplan-Meier estimates. Survival curves were compared using a two-sided log-rank-test. Results were considered statistically significant at a level of  $p < 0.05$ . Rank sum analysis was used to test differences in distribution of dichotomous variables and in the median values of continuous distributions. The normalized expression ratios at diagnosis were correlated to event-free and overall survival using Cox regression analysis with the initial expres-

sion ratio as a co-variate and by Spearman's rank correlation. Analyses were performed using the SPSS 11.0.1 software package (SPSS, Chicago, IL, USA).

## Results

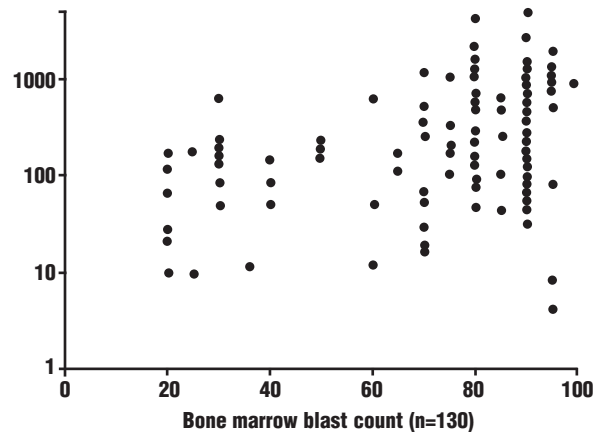
We measured the expression of *FLT3* transcripts in a total of 207 patients with AML and in 8 healthy bone marrow donors using a real-time fluorescence detection method (Table 1). The mean expression level in the AML samples was 3609 and the median 390 (range: 0.7-5880.5). The mean expression in the healthy bone marrow samples was 62.5 and the median 69 (range: 16.3-127.7). Thus the expression of *FLT3* was significantly higher in AML patients than in healthy bone marrow donors ( $p < 0.001$ ).

### *FLT3* expression level and its relation to clinical parameters

Clinical information, such as age, platelet count, bone marrow blast count (Figure 1) and peripheral leukocyte count, was completely available for 186 patients (Table 1). Among the whole group Spearman's rank correlation showed that blast and leukocyte counts were significantly related to *FLT3* expression ( $p < 0.001$  and  $r = 0.422$ , and  $p < 0.001$  and  $r = 0.341$ , respectively). No significant correlation was found with respect to age and platelet count. No difference was observed between secondary AML, therapy-related AML and *de novo* AML ( $p = 0.868$ ,  $p = 0.562$ , and  $p = 0.570$ , respectively).

### Relation to cytomorphology

Morphologic information was available for 184 AML patients and for the 8 bone marrow donors (Table 2). *FLT3* expression levels were not equally distributed among the FAB subtypes (Table 2) (Figure 2A). The lowest median level was found in FAB M3 and the highest in FAB M5a, with the following ascending order  $M3 < M3v < M6 < M2 < M4eo < M4 < M0 < M1 < M5b < M5a$ . No significant difference was detected between the groups of FAB M3 and M3v, M5a and M5b, as well as M4 and M4eo. No significant difference ( $p = 0.495$ ) was found by rank sum analysis in the group of M5 with ( $n = 13$ ) or without *MLL* abnormalities ( $n = 13$ ). Thus, high *FLT3* expression is not independently related to *MLL* rearrangement but to the M5 phenotype. In addition, when *FLT3* expression levels in the various FAB groups were compared to those in healthy bone marrow significant differences were found for M0 ( $p < 0.001$ ), M1 ( $p < 0.001$ ), M2 ( $p = 0.009$ ), M4 ( $p = 0.001$ ) and M5a/b ( $p < 0.001$  each).



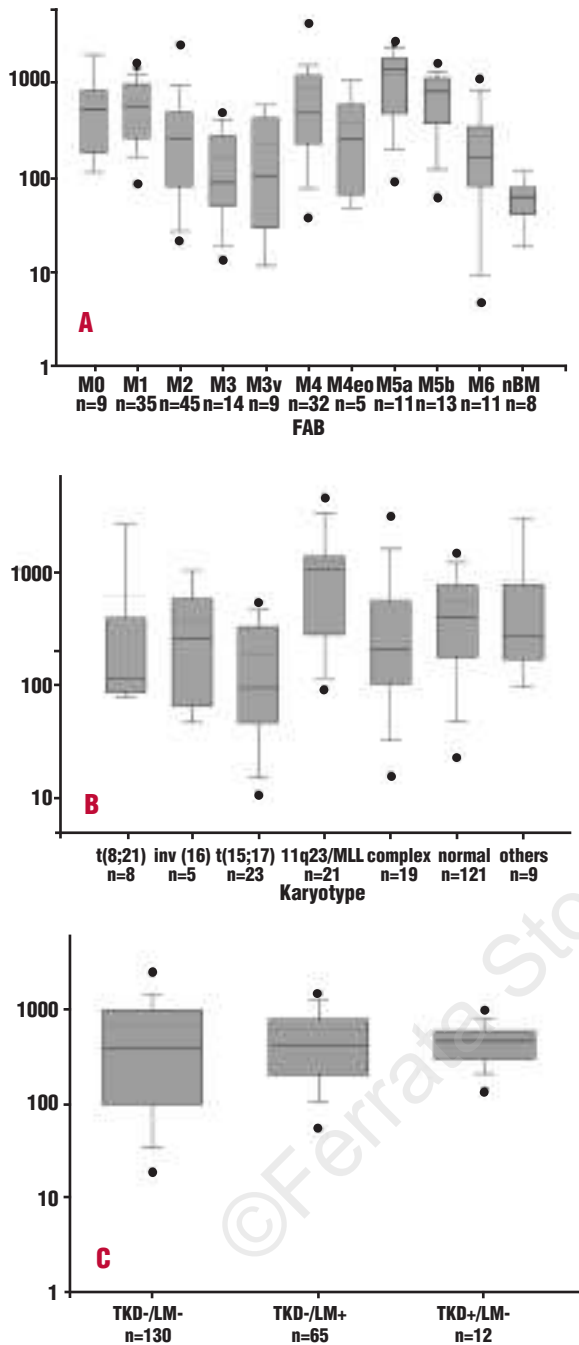
**Figure 1.** Expression level of the *FLT3* transcript according to the percentage of bone marrow blasts. The plot indicates the linear regression. *FLT3* expression levels increase with the number of bone marrow blasts ( $r = 0.422$ ;  $p < 0.001$ ).

**Table 2.** Mean and median expression levels of the *FLT3* transcript according to the FAB classification.

FAB	Number	Range (min-max)	Mean	Median	Standard deviation
M0	9	92.1-2366.8	685.6	523.6	735.4
M1	35	32.3-2284.95	642.4	537.2	474.14
M2	45	9.8-4101.6	478.1	243.7	823.7
M3	14	12.0-487.0	156.4	85.862	153.0
M3v	9	8.5-637.8	221.3	102.1	232.4
M4	32	9.7-4747.7	839.46	468.5	1093.9
M4eo	5	46.1-1028.6	365.9	254.9	401.6
M5a	11	81.1-2562.0	1189.9	1354.8	816.1
M5b	13	49.9-1620.3	777.46	804.5	458.5
M6	11	4.2-1128.9	271.34	160.3	334.4
NBM	8	16.3-127.7	62.5	69.0	34.8

### Cytogenetic groups and *FLT3* expression

Karyotype information was available for 206 patients. The karyotypes were grouped into six categories:  $t(8;21)$  ( $n = 8$ ),  $inv(16)$  ( $n = 5$ ),  $t(15;17)$  ( $n = 23$ ),  $t(11q23)$  ( $n = 21$ ), complex karyotype ( $n = 19$ ), normal karyotype ( $n = 121$ ), and all others ( $n = 9$ ). The median *FLT3* expression was not equally distributed among the genetic subtypes. The lowest expression level was found in  $t(15;17)$  and the highest in  $t(11q23)$  with the following ascending order:  $t(15;17) < t(8;21) < \text{complex} < inv(16) < \text{others} < \text{normal} < t(11q23)$  (Table 3) (Figure 2B). Rank sum analysis showed a significant difference in *FLT3* expression when comparing normal karyotype to  $t(15;17)$  ( $p < 0.001$ ) and  $t(11q23)$  ( $p < 0.011$ ) as well as between  $t(11q23)$  and all other karyotypes except the group defined as *other* karyotypes. *MLL* tandem duplications were found in eight patients with normal karyotypes with a median expression level of 504



**Figure 2.** The box plots indicate the median (solid), mean (dotted), 10<sup>th</sup>, 25<sup>th</sup>, 75<sup>th</sup> and 90<sup>th</sup> percentiles. Outliers represent the 5<sup>th</sup> and 95<sup>th</sup> percentiles. **A.** FLT3 expression levels and FAB classification. The lowest median expression levels were detected in M3 and M3v and the highest in M5a and M5b. Rank sum analysis revealed a significantly lower FLT3 expression in the healthy bone marrow (nBM) compared to AML subtypes M0, M1, M2, M4 and M5. **B.** FLT3 expression levels and karyotype. Cytogenetic grouping into six categories (t(8;21), inv(16), t(15;17), t(11q23), complex, normal, others). The median FLT3 expression levels were not equally distributed among the genetic subtypes. The lowest level was found in t(15;17) and the highest in t(11q23). Rank sum analysis showed a significant difference when comparing normal karyotype to t(15;17) ( $p < 0.001$ ) and t(11q23) ( $p < 0.011$ ) as well as between t(11q23) and all other karyotypes except for others. **C.** FLT3 expression levels and FLT3 mutations. Of the 204 patients analyzed 130 had no FLT3 mutation (64%), 62 patients had a FLT3-LM (30%) and in 12 patients a FLT3-TKD mutation could be demonstrated (6%). No statistically significant difference could be demonstrated in FLT3 expression between the three groups.

**Table 3.** Mean and median expression levels of the FLT3 transcript according to the karyotype.

Karyotype	N	Range (min-max)	Mean	Median	Standard deviation
t(8;21)	8	77.6-461.0	179.3	101.7	179.8
inv(16)	5	46.4-1028.6	365.9	254.9	401.6
t(15;17)	23	8.5-637.8	181.7	94.0	181.4
t(11q23)/MLL	21	81.1-4747.6	1234.6	1052.4	1313.9
complex	19	4.2-4101.6	582.5	206.0	971.5
normal	121	9.7-2366.8	547.5	403.1	485.7
all others	9	69.9-4309.0	834.6	269.6	1343.1

**Table 4.** Mean and median expression levels of the FLT3 transcript according to the gene mutations.

Mutation	N	Range (min-max)	Mean	Median	Standard deviation
TKD-/LM-	130	4.2-4747.6	665.0	368.5	667.4
TKD-/LM+	62	15.7-5880.5	456.9	396.0	841.3
TKD+/LM-	12	131.7-993.2	459.2	420.8	232.5

Statistical analysis showed no significant difference between the various groups.

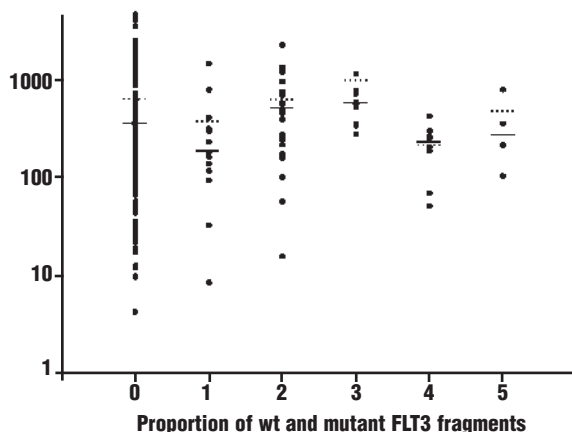
(range: 103.9-1238.1). No significant difference could be found between the group of t(11q23) and MLL-PTD ( $p=0.180$ ) or between MLL-PTD positive and MLL-PTD negative normal karyotypes ( $p=0.859$ ).

**Influence of FLT3-LM and FLT3-TKD on FLT3 expression levels**

FLT3 mutations were analyzed in 204 out of 207 patients. In 130 samples (63%) neither a FLT3-LM nor a FLT3-TKD was detected. In 62 patients a FLT3-LM was found (30%) and in 12 patients a FLT3-TKD mutation could be demonstrated (6%). Thus the cohort has a normal distribution of these two mutations. Except for three samples, all of those with a FLT3-LM had either a normal karyotype or a t(15;17). No statistically significant differences were found among the three groups ( $p$ -range: 0.3628 - 0.9943) (Table 4) (Figure 2C).

**Relation of FLT3 expression levels to FLT3-LM status**

The quantification of FLT3 expression levels detected both wild-type (WT) - and mutant-FLT3 transcripts. As there was no significant difference between the different mutated FLT3 groups we performed a quantitative analysis of the amplified fragments representing the mutation in relation to the intensity of the wild-type allele in 60 patients of the FLT3-LM group to look for further genetic subgroups. On the basis of the FLT3-LM /WT ratios five different status groups were defined. Twenty-one percent of the patients had a low muta-



**Figure 3.** *FLT3* expression levels and classification of *FLT3-LM* according to mutation status. The point plots indicate the median (solid) and mean (dotted) values. (0) no mutation, (1) mutant fragment less intense than the wild-type band (ratio <0.6), (2) mutant fragment equally intense as the wild-type band (ratio ≥0.6-1), (3) mutant fragment more intense than the wild-type band (ratio >1), (4) only mutant fragment with loss of the wild-type band and (5) more than one mutated fragment. Rank sum analysis showed a significant difference between groups 1 and 3 ( $p=0.005$ ) and between groups 3 and 4 ( $p<0.001$ ).

**Table 5.** Mean and median expression levels of the *FLT3* transcript levels are indicated according to the gene mutations.

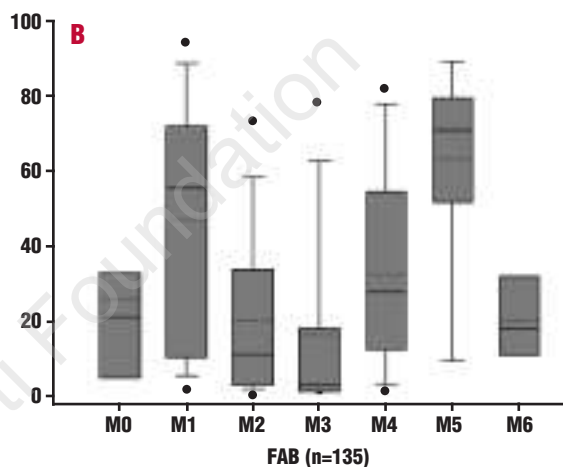
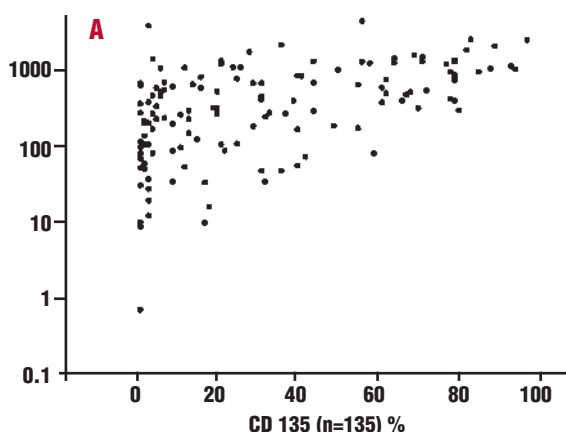
Status of mutation	N	Range (min-max)	Mean	Median	Standard deviation
0	128	4.2-4747.6	665.0	368.5	667.4
1	13	8.5-1481.5	330.5	185.7	402.3
2	24	15.7-2284.1	624.9	487.5	536.9
3	12	280.6-5880.5	1045.3	576.5	1541.5
4	8	51.7-429.0	221.5	232.5	122.8
5	5	103.9-804.5	371.2	287.5	307.0

Status (1) mutant fragment less intense than the wild-type band (ratio <0.6); (2) mutant fragment equally intense as the wild-type band; (3) mutant fragment more intense than the wild-type band; (4) only mutant fragment with loss of the wild-type band and (5) more than one mutated fragment.

tional status indicating that the mutation was only present in a part of the leukemic cells (type 1). In 38% of the patients studied, the ratio between the mutated allele and the wild-type allele was balanced (type 2). Eighteen percent had a high mutation to wild-type ratio (type 3) and 13% revealed only the mutated allele and loss of the wild-type allele (type 4). Finally, 7% showed more than one mutated fragment (type 5). The highest median mRNA expression was found in type 3 and the lowest in types 1 (Table 5) (Figure 3). Rank sum analysis revealed significant differences between type 1 and 3 as well as between types 3 and 4 ( $p=0.005$  and  $p<0.001$ , respectively).

**Correlation of *FLT3* mRNA expression levels to CD135 and CD34 surface protein expression**

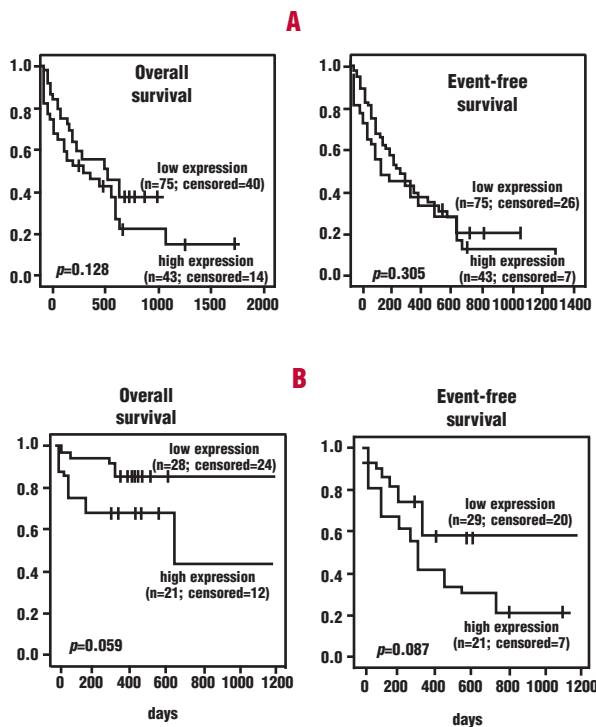
To investigate the correlation of surface expression



**Figure 4.** A. Correlation of *FLT3* expression with CD135 flow cytometry. The percentages of CD135 positive cells were used for the correlation analysis. Spearman’s rank correlation shows a significant correlation between receptor expression and mRNA expression ( $r=0.561$ ,  $p<0.001$ ). B. Correlation of CD135 flow cytometry with FAB status. The percentages of CD135 positive cells were used for the correlation analysis. The highest CD135 expression was found in FAB M5.

of *FLT3* protein and *FLT3* mRNA expression levels we performed CD135 flow cytometry in 135 patients and CD34 flow cytometry in 140 patients. The percentages of CD135 and CD34 positive cells were used for the correlation. Spearman’s rank correlation showed that CD135 receptor expression was correlated with mRNA expression ( $r=0.561$ ,  $p<0.001$ ) (Figure 4A). Examining CD135 expression with respect to FAB group, the highest expression was found in FAB M5 (Figure 4B).

No significant discrimination between FAB M5a and M5b could be made ( $p=0.164$ ). No significant differences were found dividing each FAB group according to wt-*FLT3* or mutant *FLT3* (results not shown). Furthermore, Spearman’s rank correlation showed no correlation between CD34 surface expression and *FLT3* mRNA expression ( $r=0.0502$ ,  $p=0.557$ ).



**Figure 5. A.** Prognostic relevance of *FLT3* expression within the cytogenetically normal karyotype group. Kaplan Meier analyses of two groups with *FLT3* expression levels above or below the median expression value. Overall survival and event-free survival were assessed in 118 patients. No significant difference was demonstrated between the two groups ( $p=0.128$  and  $p=0.305$ , respectively). **B.** Prognostic relevance of *FLT3* expression within the group with wt-*FLT3*. Kaplan Meier analyses of *FLT3* expression levels in the group with wt-*FLT3* above or below the mean expression value. Overall survival and event-free survival were assessed in 118 patients. A tendency towards a lower overall survival and event-free survival was demonstrated in the group with high *FLT3* expression ( $p=0.058$  and  $p=0.087$ , respectively).

**Relation of *FLT3* expression assessed by real time PCR or microarray analysis**

To confirm the real time PCR results with a second independent method, we compared *FLT3* expression levels as assessed by real time PCR with those measured by microarray analysis in 89 AML patients. Spearman’s rank correlation showed that the two methods, real time PCR and microarray analysis, gave comparable results ( $r=0.412$ ,  $p<0.001$ ).

**Analysis of the prognostic relevance of *FLT3* expression levels**

Since the expression of *FLT3* was lowest in M3, the prognostically most favorable group, and highest in the poor t(11q23) group, expression level may have implications for prognosis. We performed a Cox regression analysis of all cases except for AML M3 was found no difference for overall survival ( $p=0.5663$ ) and event-free survival ( $p=0.9049$ ). Thus, to analyze whether high *FLT3* expression is a prognostic parameter independent of karyotype, 118 AML cases with normal karyotype were divided into two groups. Group 1 (n=75) was

defined to have less and group 2 (n=43) more than the median of the *FLT3* expression level found in the total group. Using Cox regression analysis no impact on either overall or event-free survival could be shown (608 vs. 311 days,  $p=0.128$ , and 398 vs. 208 days,  $p=0.305$ ) (Figure 5A). No other threshold such as the mean, the 25<sup>th</sup> or the 75<sup>th</sup> percentile had a prognostic significance, nor was *FLT3* expression as a continuous variable was significantly associated with prognosis. The comparison of wt-*FLT3* expression including FAB M3/M3v cases did not show a significantly worse overall survival ( $p=0.48$ ) or event-free survival ( $p=0.6732$ ) using the median as the threshold for separating high and low expression levels. Omitting FAB M3 and M3v, which are characterized by low *FLT3* expression levels and very good overall and event-free survivals, log rank analysis showed a clear tendency towards a worse overall survival ( $p=0.059$ ) and event-free survival ( $p=0.0870$ ) applying the mean *FLT3* expression level as the threshold for separating between high and low expression (Figure 5B). The 75<sup>th</sup> percentile showed no significant difference ( $p=0.230$ ), probably because of the small number of patients.

**Discussion**

During the last years the *FLT3* receptor and its gene mutations have been extensively investigated in AML. Beside the prognostic relevance of *FLT3* mutations, only a very few studies have reported on the role of *FLT3* mRNA expression levels in AML.<sup>13,20</sup> In this study we analyzed *FLT3* expression levels by quantitative real time PCR in 207 AML patients and 8 healthy bone marrow donors. We investigated correlations with cytomorphology, cytogenetics, flow cytometry, microarray analysis, *FLT3* mutation status and other clinical parameters. Furthermore, we examined whether *FLT3* expression levels are prognostic of outcome in AML patients. We found that *FLT3* expression differed according to cytomorphological type of AML with the lowest expression occurring in M3 and the highest in M5. Although different PCR quantification systems were used similar results have been shown previously by two other groups.<sup>13,20</sup> As already demonstrated, in FAB M3 especially in the M3v group both with t(15;17) a high percentage of patients had *FLT3-LM*.<sup>2</sup> The low level of *FLT3* expression in AML M3/M3v remains unexplained. It is known that *FLT3* is expressed in the myeloid progenitor compartment, thus a possible explanation could be that AML M3/M3v are characterized by a more differentiated blast population.<sup>14</sup> As FAB M3 leukemias rarely express CD34, the lack of correlation between CD34 and *FLT3* expression levels does not contradict this.<sup>36</sup> This finding is also in line with the results of Munoz *et al.*<sup>37</sup> Although this work was restricted to

*FLT3-LM* positive cells, only 42% were CD34 positive. The lack of correlation with CD34, which still remains speculative, might be due to the fact that the *FLT3* mRNA level does not reflect the stage of blast maturation, but has an as yet unknown biological cause. Previously, a correlation between *FLT3-LM* and high leukocyte count was described.<sup>13</sup> In our study this correlation could be extended to a high level of *FLT3* expression, independently of the mutation status. The highest expression was found in FAB M5a and M5b, which are frequently associated with chromosome 11q23 (*MLL*) translocations and a high leukocyte count.<sup>20</sup> This is in line with CD135 expression, which was highest in FAB M5 and consistent with the findings of McKenna *et al.*, who reported that M5 AML samples proliferated in response to *FLT3* ligand.<sup>38,39</sup> Consequently, it might be speculated that *FLT3* mutations as well as high expression trigger the leukocyte count. As already mentioned, previous studies have shown that t(11q23) is correlated with high *FLT3* expression.<sup>22,40,41</sup> Comparing the group of M5 with and without *MLL* translocation, no significant difference between their high *FLT3* expression levels could be demonstrated. Thus, the expression level is not independently correlated with t(11q23) but more significantly with FAB M5. In addition, it should be emphasized that the high expressing t(11q23) positive AML very rarely have *FLT3* mutations whereas the low expression found in t(15;17) is associated with a high mutation rate.<sup>2</sup> It may be speculated that high *FLT3* mRNA expression in AML is an alternative to mutational activation of the *FLT3* receptor.

*MLL* tandem duplications were found in only 3.8% of the total AML cohort analyzed in the present study and we observed only cases with normal karyotype. Contrasting with the findings in t(11q23), no elevated *FLT3* expression was shown in this group.

It was previously reported that *MLL* rearrangements are associated with a constitutive activation of wild type *FLT3* kinase independently of *FLT3* mutations.<sup>13</sup> This seems not to be the case in *MLL-PTD* positive cases, suggesting that this *MLL* aberration acts through different downstream pathways than the t(11q23). It was recently reported that *FLT3-TKD* and *FLT3-LM* are associated with high *FLT3* expression levels in AML.<sup>13</sup> The analysis of an even larger cohort in the present study did not confirm a significant difference in expression levels in AML with or without *FLT3* mutations. However, within the group of *FLT3-LM* we did detect a difference between mutation status 3 (with high *FLT3* gene expression) and mutation status 4 and 1 (showing decreased *FLT3* mRNA expression).<sup>13</sup> Thus *FLT3* upregulation is associated with a partial loss of the wild-type allele, whereas a total loss of the wild-type fragment leads to low *FLT3* expression. These results demonstrate that *FLT3* expression levels within the group of AML with *FLT3-LM* depend on the co-expression of

the wild-type and the mutant alleles. It remains unclear whether regulation takes place at the level of gene expression or protein synthesis. These results could also indicate a negative feedback between highly activated *FLT3-LM* signaling and the regulation of *FLT3* expression. That said, it is also possible that our observations may be due to the small numbers of patients in the single subgroups. Overall the mutation status did not seem to have any impact on the expression level. Consequently the expression level is not associated with the activating status with the kinase.

Although the loss of the wild type-allele is associated with a worse clinical course, no clear association with *FLT3* expression level could be found until now.<sup>2,3</sup> The correlation of FAB status with CD135 flow cytometry and *FLT3* mRNA levels shows that the mRNA expression reflects the cellular protein levels i.e. the level of receptor tyrosine kinase expression. This is in line with the findings of Graf *et al.*, who reported that FAB M5 is also associated with a high CD135 expression.<sup>39</sup> This corroborates the conclusion that *FLT3* signaling is likely associated with FAB M5 and raises the questions of whether monocytic differentiation induces *FLT3* expression or *FLT3* signaling leads to monocytic differentiation. What argues against these speculations is the fact that in the mouse model wild-type *FLT* and *FLT-LM* overexpression did not cause a monocytic phenotype.<sup>42</sup>

*FLT3* mutations within certain subgroups of AML are associated with a worse prognosis.<sup>2,3</sup> It has been postulated that high *FLT3* expression levels are associated with an unfavorable prognosis.<sup>13,21,21</sup> Although in the present study high expression of *FLT3* was also correlated with t(11q23) and low expression was found in t(15;17), two groups with very different prognoses, in the prognostically intermediate karyotype group no independent statistical differences were found for overall survival, event-free survival, and relapse-free survival. However, separating the wild-type-*FLT3* group (excluding FAB M3/M3v) according to the mean *FLT3* expression level revealed a clear tendency towards worse overall and event-free survival, which is in line with the findings of Ozeki *et al.*, indicating that *FLT3* expression level is a possible risk factor in patients with wild-type *FLT3*.

In conclusion, we found significant correlations between *FLT3* expression levels and leukocyte and bone marrow blast counts, cytomorphology, cytogenetics, flow cytometry and a tendency towards a worse survival.

FK: realtime PCR, manuscript, statistics, interpretation of data, figures; CS: cytogenetics, manuscript; WK: immunophenotyping, manuscript, statistics, interpretation of data; AK: array results, manuscript; TH: microscopy, manuscript; WH: manuscript and contents; SS: main investigator, morphology, manuscript, statistics, interpretation of data. Manuscript received April 29, 2005. Accepted September 19, 2005.



## References

- Harris NL, Jaffe ES, Diebold J, Flandrin G, Muller-Hermelink HK, Vardiman J, et al. World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues: report of the Clinical Advisory Committee meeting-Airlie House, Virginia, November 1997. *J Clin Oncol* 1999;17: 3835-49.
- Schnittger S, Schoch C, Dugas M, Kern W, Staib P, Wuchter C, et al. Analysis of FLT3 length mutations in 1003 patients with acute myeloid leukemia: correlation to cytogenetics, FAB subtype, and prognosis in the AMLCG study and usefulness as a marker for the detection of minimal residual disease. *Blood* 2002; 100: 59-66.
- Thiede C, Steudel C, Mohr B, Schaich M, Schakel U, Platzbecker U, et al. Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: association with FAB subtypes and identification of subgroups with poor prognosis. *Blood* 2002;99:4326-35.
- Nakao M, Janssen JW, Erz D, Seriu T, Bartram CR. Tandem duplication of the FLT3 gene in acute lymphoblastic leukemia: a marker for the monitoring of minimal residual disease. *Leukemia* 2000; 14:522-4.
- Nakao M, Yokota S, Iwai T, Kaneko H, Horiike S, Kashima K, et al. Internal tandem duplication of the flt3 gene found in acute myeloid leukemia. *Leukemia* 1996; 10:1911-8.
- Gilliland DG, Griffin JD. The roles of FLT3 in hematopoiesis and leukemia. *Blood* 2002; 100:1532-42.
- Abu-Duhier FM, Goodeve AC, Wilson GA, Gari MA, Peake IR, Rees DC, et al. FLT3 internal tandem duplication mutations in adult acute myeloid leukaemia define a high-risk group. *Br J Haematol* 2000;111:190-5.
- Yamamoto Y, Kiyoi H, Nakano Y, Suzuki R, Koderia Y, Miyawaki S, et al. Activating mutation of D835 within the activation loop of FLT3 in human hematologic malignancies. *Blood* 2001; 97:2434-9.
- Yanada M, Matsuo K, Suzuki T, Kiyoi H, Naoe T. Prognostic significance of FLT3 internal tandem duplication and tyrosine kinase domain mutations for acute myeloid leukemia: a meta-analysis. *Leukemia* 2005;19:1345-9.
- Gilliland DG, Griffin JD. Role of FLT3 in leukemia. *Curr Opin Hematol* 2002; 9: 274-81.
- Kottaridis PD, Gale RE, Linch DC. Prognostic implications of the presence of FLT3 mutations in patients with acute myeloid leukemia. *Leuk Lymphoma* 2003;44:905-13.
- Kuchenbauer F, Schoch C, Kern W, Hiddemann W, Haferlach T, Schnittger S. Impact of FLT3 mutations and promyelocytic leukaemia-breakpoint on clinical characteristics and prognosis in acute promyelocytic leukaemia. *Br J Haematol* 2005;130:196-202.
- Ozeki K, Kiyoi H, Hirose Y, Iwai M, Ninomiya M, Koderia Y, et al. Biologic and clinical significance of the FLT3 transcript level in acute myeloid leukemia. *Blood* 2004;103:1901-8.
- Stirewalt DL, Radich JP. The role of FLT3 in hematopoietic malignancies. *Nat Rev Cancer* 2003;3:650-65.
- Zwaan CM, Meshinchi S, Radich JP, Veerman AJ, Huismans DR, Munske L, et al. FLT3 internal tandem duplication in 234 children with acute myeloid leukemia: prognostic significance and relation to cellular drug resistance. *Blood* 2003;102:2387-94.
- Birg F, Rosnet O, Carbuca N, Birnbaum D. The expression of FMS, KIT and FLT3 in hematopoietic malignancies. *Leuk Lymphoma* 1994; 13:223-7.
- Carow CE, Levenstein M, Kaufmann SH, Chen J, Amin S, Rockwell P, et al. Expression of the hematopoietic growth factor receptor FLT3 (STK-1/Flk2) in human leukemias. *Blood* 1996;87:1089-96.
- Rosnet O, Buhring HJ, deLapeyriere O, Beslu N, Lavagna C, Marchetto S, et al. Expression and signal transduction of the FLT3 tyrosine kinase receptor. *Acta Haematol* 1996; 95:218-23.
- Meierhoff G, Dehmel U, Gruss HJ, Rosnet O, Birnbaum D, Quentmeier H, et al. Expression of FLT3 receptor and FLT3-ligand in human leukemia-lymphoma cell lines. *Leukemia* 1995; 9:1368-72.
- Libura M, Asnafi V, Tu A, Delabesse E, Tigaud I, Cymbalista F, et al. FLT3 and MLL intragenic abnormalities in AML reflect a common category of genotoxic stress. *Blood* 2003;102:2198-204.
- Bullinger L, Dohner K, Bair E, Frohling S, Schlenk RF, Tibshirani R, et al. Use of gene-expression profiling to identify prognostic subclasses in adult acute myeloid leukemia. *N Engl J Med* 2004; 350:1605-16.
- Armstrong SA, Kung AL, Mabon ME, Silverman LB, Stam RW, Den Boer ML, et al. Inhibition of FLT3 in MLL. Validation of a therapeutic target identified by gene expression based classification. *Cancer Cell* 2003;3:173-83.
- Haferlach T, Schoch C, Loffler H, Gassmann W, Kern W, Schnittger S, et al. Morphologic dysplasia in de novo acute myeloid leukemia (AML) is related to unfavorable cytogenetics but has no independent prognostic relevance under the conditions of intensive induction therapy: results of a multiparameter analysis from the German AML Cooperative Group Studies. *J Clin Oncol* 2003;21:256-65.
- Haferlach T, Kern W, Schoch C, Schnittger S, Sauerland MC, Heinecke A, et al. A new prognostic score for patients with acute myeloid leukemia based on cytogenetics and early blast clearance in trials of the German AML Cooperative Group. *Haematologica* 2004;89:408-18.
- Schoch C, Schnittger S, Bursch S, Gerstner D, Hochhaus A, Berger U, et al. Comparison of chromosome banding analysis, interphase- and hypermetaphase-FISH, qualitative and quantitative PCR for diagnosis and for follow-up in chronic myeloid leukemia: a study on 350 cases. *Leukemia* 2002; 16:53-9.
- ISCN. Guidelines for Cancer Cytogenetics, Supplement to: An International System for Humann Cytogenetic Nomenclature. F. Mitelman, ed. S. Karger. 1995.
- Haferlach T, Schoch C, Loffler H, Gassmann W, Kern W, Schnittger S, et al. Morphologic dysplasia in de novo acute myeloid leukemia (AML) is related to unfavorable cytogenetics but has no independent prognostic relevance under the conditions of intensive induction therapy: results of a multiparameter analysis from the German AML Cooperative Group studies. *J Clin Oncol* 2003;21:256-65.
- Schnittger S, Weissner M, Schoch C, Hiddemann W, Haferlach T, Kern W. New score predicting for prognosis in PML-RARA+, AML1-ETO+, or CBFB-MYH11+ acute myeloid leukemia based on quantification of fusion transcripts. *Blood* 2003;102:2746-55.
- Weisser M, Haferlach T, Schoch C, Hiddemann W, Schnittger S. The use of housekeeping genes for real-time PCR-based quantification of fusion gene transcripts in acute myeloid leukemia. *Leukemia* 2004;18:1551-3.
- Schnittger S, Kinkel U, Schoch C, Heinecke A, Haase D, Haferlach T, et al. Screening for MLL tandem duplication in 387 unselected patients with AML identify a prognostically unfavorable subset of AML. *Leukemia* 2000;14:796-804.
- Bagrintseva K, Schwab R, Kohl TM, Schnittger S, Eichenlaub S, Ellwart JW, et al. Mutations in the tyrosine kinase domain of FLT3 define a new molecular mechanism of acquired drug resistance to PTK inhibitors in FLT3-ITD-transformed hematopoietic cells. *Blood* 2004; 103:2266-75.
- Kern W, Danhauser-Riedl S, Ratei R, Schnittger S, Schoch C, Kolb HJ, et al. Detection of minimal residual disease in unselected patients with acute myeloid leukemia using multiparameter flow cytometry for definition of leukemia-associated immunophenotypes and determination of their frequencies in normal bone marrow. *Haematologica* 2003; 88:646-53.
- Kohlmann A, Schoch C, Schnittger S, Dugas M, Hiddemann W, Kern W, et al. Molecular characterization of acute leukemias by use of microarray technology. *Genes Chromosomes Cancer* 2003; 37:396-405.
- Liu WM, Mei R, Di X, Ryder TB, Hubbell E, Dee S, et al. Analysis of high density expression microarrays with signed-rank call algorithms. *Bioinformatics* 2002;18: 1593-9.
- Liu G, Loraine AE, Shigeta R, Cline M, Cheng J, Valmееkam V, et al. NetAffx: Affymetrix probesets and annotations. *Nucleic Acids Res* 2003; 31:82-6.
- Dunphy CH. Applications of flow cytometry and immunohistochemistry to diagnostic hematopathology. *Arch Pathol Lab Med* 2004;128:1004-22.
- Munoz L, Aventin A, Villamor N, Junca J, Acebedo G, Domingo A, et al. Immunophenotypic findings in acute myeloid leukemia with FLT3 internal tandem duplication. *Haematologica* 2003;88:637-45.
- McKenna HJ, Smith FO, Brasel K, Hirschstein D, Bernstein ID, Williams DE, et al. Effects of flt3 ligand on acute myeloid and lymphocytic leukemic blast cells from children. *Exp Hematol* 1996;24:378-85.
- Graf M, Hecht K, Reif S, Pelka-Fleischer R, Pfister K, Schmetzer H. Expression and prognostic value of hemopoietic cytokine receptors in acute myeloid leukemia (AML): implications for future therapeutic strategies. *Eur J Haematol* 2004;72:89-106.
- Armstrong SA, Mabon ME, Silverman LB, Li A, Gribben JG, Fox EA, et al. FLT3 mutations in childhood acute lymphoblastic leukemia. *Blood* 2004; 103: 3544-6.
- Armstrong SA, Golub TR, Korsmeyer SJ. MLL-rearranged leukemias: insights from gene expression profiling. *Semin Hematol* 2003;40:268-73.
- Kelly LM, Liu Q, Kutok JL, Williams IR, Boulton CL, Gilliland DG. FLT3 internal tandem duplication mutations associated with human acute myeloid leukemias induce myeloproliferative disease in a murine bone marrow transplant model. *Blood* 2002;99:310-8.