Detailed analysis of FLT3 expression levels in acute myeloid leukemia

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Background and Objectives. FLT3 mutations are found in up to 30% of cases of acute myeloid leukemia (AML). Although new FLT3 mutations are being increasingly 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<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 or 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.

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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). 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.1-4 Additionally, mutations in codons 835 and 836 in the tyrosine kinase domain (TKD mutations) can be detected in 7% of AML patients.4,8 In contrast to TKD mutations, which do not have a clear prognostic impact, FLT3-LM were identified as a significant risk factor in AML patients.5,9-11 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.2,12 Smaller studies also indicated that these mutations affect complete remission rates and overall survival, especially in patients under the age of 60.2,3,6,11 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).2,3 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...
A higher frequency of FLT3-LM has been reported in elderly patients, although without these mutations having a prognostic impact, 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. The frequency of FLT3-LM in childhood cases is 5-15%, thus being lower than in adults.

Pathophysiologically, FLT3-LM were postulated to cause receptor dimerization resulting in constitutive auto-phosphorylation of tyrosine residues. The activated receptor promotes ligand-independent proliferation, a block of myeloid differentiation and the induction of a myeloproliferative-like disease in a mouse model. 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. Recent studies have started to characterize the clinical relevance of the FLT3 transcript level and differences in FLT3 expression in specific AML subgroups. 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 secondary 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. The study design adhered to the declaration of Helsinki and was approved by the ethics committees of the participating institutions prior to its initiation. 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: 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 ≥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. The definition of a cytogenetic clone and descriptions of karyotypes followed the International System for Human Cytogenetic Nomenclature (ISCN).

#### Sample preparation

Mononucleated bone marrow cells were obtained by Ficoll Hypaque density gradient centrifugation. Samples were analyzed by FACS for percentage of blasts. 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.

#### 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

### Table 1. Patients’ characteristics.

<table>
<thead>
<tr>
<th>AML patients</th>
<th>n=207</th>
<th>Bone marrow donor</th>
<th>n=8</th>
</tr>
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<tr>
<td>Median age, years</td>
<td>61.4 (range: 17.4-83.6)</td>
<td></td>
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<tr>
<td>Gender</td>
<td>m: 107/f: 100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>De novo AML</td>
<td>n=19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>secondary AML following MDS</td>
<td>n=6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>therapy-related AML</td>
<td>n=22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median WBC (x10^9/L)</td>
<td>16600 (range: 60 - 514000)</td>
<td></td>
<td></td>
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<tr>
<td>Median platelet count (x10^9/L)</td>
<td>52500 (range: 11 - 846)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median hemoglobin (g/dL)</td>
<td>9.2 (range: 4 - 15)</td>
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</table>
individual samples. Primers used for FLT3 amplification were FLT3-F: CCGCCCAGGGA-ACGTGCTTG and FLT3-R: ATGCCAGGGTAAAGGATTCAACC and ABL primers as published previously. Each quantitative reverse transcription PCR reaction was carried out in a 20 µL reaction volume with 0.5 µM each of forward and reverse primer, 4 mM MgCl₂, 2 µL LightCycler-Fast Start DNA Master SYBR Green I (Roche Applied Science) and 2 µL 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. 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×FLT3/ABL. Any samples that had ABL values <1 fg/2µL cDNA (<1.5×10⁶ ABL copies), isolated from 2×10⁶ cells, were excluded from subsequent analysis.

Detection of MLL-PTD and FLT3-mutations

MLL-PTD and FLT3 mutations were analyzed as previously described. 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. FLT3-LM were divided into five categories according to densitometric estimations by Genescan analysis: (i) mutant fragment less intense than the wild-type band (ratio <0.6), (ii) mutant fragment equally intense as the wild-type band (ratio ≥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. The antibody was added to 1×10⁶ mononuclear cells (volume: 100 µL) and incubated for 10 minutes. After addition of 2 mL 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, CA, USA).

Microarray analysis

Samples, prepared as described elsewhere, were profiled using U133A GeneChip 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). The minimal quality control parameters for inclusion of an expression profile in our study were more than 50% present calls and a 3'/5' ratio of 0.3 or less of represented glyceraldehyde-3'-phosphate dehydrogenase gene (GAPDH) probe sets. All probe sets were functionally annotated by the current NetAffx database release. 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 M5v, 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).

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) <complex <inv(16) <others <normal <t(11q23) (Table 3) (Figure 2B). Rank sum analysis showed a significant difference in FLT 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.
FLT3 expression in AML

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 (64\%) 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 \( (p\text{-range: 0.3628 - 0.9943}) \) (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-

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<th>Standard deviation</th>
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<td>inv(16)</td>
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<td>46.4–1028.6</td>
<td>365.9</td>
<td>254.9</td>
<td>401.6</td>
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<tr>
<td>t(15;17)</td>
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<td>181.7</td>
<td>94.0</td>
<td>181.4</td>
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<td>t(11q23)/MLL</td>
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<td>1052.4</td>
<td>1313.9</td>
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<td>582.5</td>
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<td>971.5</td>
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<td>9.7–2366.8</td>
<td>547.5</td>
<td>403.1</td>
<td>485.7</td>
</tr>
<tr>
<td>all others</td>
<td>9</td>
<td>69.9–4309.0</td>
<td>834.6</td>
<td>269.6</td>
<td>1943.1</td>
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<table>
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<th>Standard deviation</th>
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<tr>
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<td>665.0</td>
<td>368.5</td>
<td>667.4</td>
</tr>
<tr>
<td>TKD-/LM+</td>
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<td>15.7–5880.5</td>
<td>456.9</td>
<td>396.0</td>
<td>841.3</td>
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<tr>
<td>TKD+/LM-</td>
<td>12</td>
<td>131.7–993.2</td>
<td>459.2</td>
<td>420.8</td>
<td>232.5</td>
</tr>
</tbody>
</table>

Statistical analysis showed no significant difference between the various groups.

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

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

![Figure 2. The box plots indicate the median (solid), mean (dotted), 10\%, 25\%, 75\% and 90\% percentiles. Outliers represent the 5\% and 95\% percentiles.](image-url)
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 type 1 (Table 5) (Figure 3). 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.

<table>
<thead>
<tr>
<th>Status of mutation</th>
<th>N</th>
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<th>Mean</th>
<th>Median</th>
<th>Standard deviation</th>
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<td>0</td>
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<td>665.0</td>
<td>368.5</td>
<td>667.4</td>
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<td>1</td>
<td>13</td>
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<td>330.5</td>
<td>185.7</td>
<td>402.3</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>15.7-2284.1</td>
<td>624.9</td>
<td>487.5</td>
<td>536.9</td>
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<tr>
<td>3</td>
<td>12</td>
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<td>1045.3</td>
<td>576.5</td>
<td>1541.5</td>
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<tr>
<td>4</td>
<td>8</td>
<td>51.7-429.0</td>
<td>221.5</td>
<td>232.5</td>
<td>122.8</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>103.9-804.5</td>
<td>371.2</td>
<td>287.5</td>
<td>307.0</td>
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</table>

Status (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.

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

To investigate the correlation of surface expression 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 positive cells were used for the correlation analysis. Spearman’s rank correlation showed 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.
In this study we observed that FLT3 expression differed according to cytogenetic status. The expression level was lowest in M3, the cytogenetically normal karyotype group. Kaplan Meier analyses of two groups with FLT3 expression levels above or below the median expression value showed no significant difference between the two groups (p=0.128 and p=0.305, respectively). Overall survival and event-free survival were assessed in 118 patients. No significant difference was demonstrated between the two groups (p=0.6732) using the median as the threshold for separating high and low expression levels. Omitting M3 and M5v, which are characterized by low FLT3 expression levels and very good overall and event-free survivals, the low level of FLT3 expression occurring in M3 and the highest in M5v, is expressed in the myeloid progenitor compartment, thus a possible explanation could be that AML M3/M5v are characterized by a more differentiated blast population.14 As FAB M3 leukemias rarely express CD34, the lack of correlation between CD34 and FLT3 expression levels does not contradict this.15 This finding is also in line with the results of Munoz et al.27 Although this work was restricted to

**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 25th or the 75th 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 M5v, 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 75th 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.129 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 cytology, 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 cytological 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.131 As already demonstrated, in FAB M3 especially in the M3v group both with t(15;17) a high percentage of patients had FLT3-LM.13 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.14 As FAB M3 leukemias rarely express CD34, the lack of correlation between CD34 and FLT3 expression levels does not contradict this.15 This finding is also in line with the results of Munoz et al.27 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 maturato, but has an as yet unknown biological cause. Previously, a correlation between FLT3-LM and high leukocyte count was described. 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. 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. 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. 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. 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. 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. 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 5 (with high FLT3 gene expression) and mutation status 4 and 1 (showing decreased FLT3 mRNA expression). 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. 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. 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. FLT3 mutations within certain subgroups of AML are associated with a worse prognosis. It has been postulated that high FLT3 expression levels are associated with an unfavorable prognosis. 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 M5/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.

F. Kuchenbauer et al.
References


