ORIGINAL ARTICLE

Expression of pSTAT5 predicts *FLT3* internal tandem duplications in acute myeloid leukemia

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Abstract Mutations of the *Fms-like tyrosine kinase 3 (FLT3)* can be detected in a significant number of acute myeloid leukemias (AML). Seventy-five cases of acute myeloid leukemia were evaluated for *FLT3*-internal tandem duplications (ITD) by polymerase chain reaction. Paraffin-embedded formalin-fixed trephine biopsies of these cases were evaluated for expression of phosphorylated signal transducer and activator of transcription 1 (pSTAT1), pSTAT3, and pSTAT5. Specific expression of pSTAT5 was proven in leukemic blasts in situ by double staining with a blast-specific marker. Expression of pSTAT5 in ≥1% of blasts was highly predictive of *FLT3*-ITD. Neither expression of pSTAT1 nor pSTAT3 were associated with *FLT3* mutations. Altogether we conclude that pSTAT5 expression can precisely be assessed by immunohistochemistry in routinely processed bone marrow trephines, STAT5 is highly likely the preferred second messenger of FLT3-mediated signaling in AML, and expression of pSTAT5 is predictive of *FLT3*-ITD.

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Introduction

The gene for *Fms-like tyrosine kinase 3 (FLT3)* encodes a receptor tyrosine kinase that plays an important role in hematopoiesis [1]. *FLT3* is recurrently targeted by genetic aberrations in leukemias. *FLT3* mutations may be internal tandem duplications (ITD) of the exons encoding for the juxtamembrane domain or point mutations in the tyrosine kinase domain [2]. *FLT3* may possibly be activated by translocations as well [3, 4]. *FLT3*-ITD can be found in 20% to 30% of patients with de novo acute myelogenous leukemia (AML) [5–8]. It leads to the disruption of a repressor sequence in the juxtamembrane domain of the *FLT3* gene, which causes a constitutive autophosphorylation of the receptor [9]. This in turn promotes cell proliferation and inhibits apoptosis in leukemic blasts [9, 10]. Alterations of *FLT3* seem to be of significant consequence in the clinical behavior of AML [5, 6, 8, 11]. Though the most recent edition of the World Health Organization (WHO)-Classification of Tumours of Haematopoietic and Lymphoid Tissues introduced the incorporation of genetic abnormalities into diagnostic algorithms for the diagnosis of AML, *FLT3* mutations were not included as a defining criterion for any distinct entity since they frequently accompany other well-recognized genetic lesions. However, in cases of cytogenetically normal AML and in AML with myelodysplasia-related changes, assessment of *FLT3* mutations is recommended, and, if present, the abnormality should be noted along with the diagnosis [11].

The *signal transducers and activators of transcription (STAT)* gene family consist of seven proteins [12]. STAT-mediated signal transduction pathways influence neoplastic processes on a broad basis by perturbing control over survival, differentiation, proliferation, and apoptosis [13]. Aberrant activation of STAT3 and STAT5 (less often STAT1) has been demonstrated in diverse groups of neoplasms, including AML [14–16]. Activation of STAT is caused by tyrosine phosphorylation followed by the consecutive translocation of pSTAT into the nucleus [17]. In normal hematopoiesis, STAT5 is activated by receptor ligation of FLT3 to its ligand as well as by several other cytokines [18, 19], thus STAT5 being the preferred second messenger of FLT3-mediated signaling.

The aim of this study was to assess protein expression of pSTAT1, pSTAT3, and pSTAT5 in a well-defined set of AML with known status of the *FLT3* gene, in order to address whether STAT5 is the preferred signal transducer of FLT3 in AML and if presence of *FLT3*-ITD can be predicted by quantification of pSTAT5 in leukemic blasts.

Materials and methods

Patients

A retrospective cohort of 75 patients with AML on first diagnosis was included in this study. All newly diagnosed patients with AML between 2005 and 2007 at the University Hospital Basel, who had been fully evaluated for cytogenetic alterations and molecular markers including presence of *FLT3*-ITD, were considered if paraffin blocks of bone marrow trephine biopsies were available and adequately preserved for immunohistochemical analyses. All cases were reclassified according to the most recent WHO criteria [11]. Clinical and follow-up data were obtained by reviewing the charts. Retrieval of tissue and clinical data were performed according to data safety laws. Of the 75 patients included in this study, 37 were male (mean age 59; median age 57; range 26–95), and 38 were female (mean age 56; median 59; range 20–89). The average follow-up time was 16.5 months (median 10; range 0–62 range). Within this time, 37 patients passed away. Most of the patients ($n=56$) were treated with curative intention (intensive chemotherapy alone, $n=23$; intensive chemotherapy followed by autologous hematopoietic stem cell transplantation, $n=5$; intensive chemotherapy followed by allogeneic hematopoietic stem cell transplantation, $n=28$). Nineteen patients, who did not qualify for intensive treatment, received best supportive care. Five of them were given hydroxyurea. The distribution of cases according to the WHO classification is presented in Table 1.

Immunohistochemistry

Assessment of pSTAT expression profiles

Four-micrometer sections were cut to adhesive-coated slides and stained using standard procedures. The precise percentage of infiltrating blasts was identified by case-specific markers using standard staining procedures. The following antibodies were used: CD34 (M7165, DAKO, final dilution 1:10), CD117 (A4502, DAKO, final dilution 1:80), CD68 (M7165, DAKO, final dilution 1:10), CD14 (MS-1080, Neomarkers, final dilution 1:10), CD163 (MS-1103, Neomarkers, final dilution 1:5,000), and myeloperoxidase (A0398, DAKO, final dilution 1:10). Staining of pSTAT1, pSTAT3, and pSTAT5 was performed as described previously [4, 16] using commercially available antibodies (pSTAT1: no. 9167; pSTAT3: no. 9145; pSTAT5: no. 9359; all by Cell Signaling). The specificity of the pSTAT3 and pSTAT5 stainings was corroborated by preabsorption experiments with corresponding blocking peptides (no. 1195, Cell Signaling, and no. 06-785-213045, GenWay, respectively). One high-power field containing at least 350 cells was assessed in each case to identify the percentage of positive cells (i.e., cells displaying a distinct nuclear staining). The slides were evaluated without knowledge of clinical data or mutational status. Of the cases, 20% were reassessed by a second observer.

Assessment of coexpression of pSTAT5 and blast-specific markers

Six cases were double stained with the anti-pSTAT5 antibody and a blast-specific marker (either CD34 or CD117) in order to prove that pSTAT5 is expressed in the leukemic population.

For double stains of CD34 and pSTAT5, the antibody for pSTAT5 was applied first and incubated at room temperature overnight. The alkaline-phosphatase (AK-5000, Vector Laboratories) technique was used for developing with Substrat Kit III (SK-5300, Vector Laboratories) as chromogen. The antibody against CD34 was consecutively applied for an incubation time of 120 min at 37°C; utilizing the avidin–biotin complex (AK-5100, Vector Laboratories) either Nova-Red (SK-4800, Vector Laboratories) or VIP (SK-4600, Vector Laboratories) served as chromogens. For double stains of CD117 and pSTAT5, antibodies against CD117 were applied first for an incubation time of 120 min at 37°C; the reaction was visualized using the alkaline-phosphatase method with the chromogen Substrat Kit III. Antibodies against pSTAT5 were afterward incubated overnight at room temperature; the labeling was visualized with the aid of the avidin–biotin complex and Nova-Red as chromogen.

Table 1 Patient characteristics and distribution of *FLT3*-ITD

AML, classified according to WHO [11]	Number of cases	Cases with *FLT3*-ITD	Cases[a] with *NPM1* mutation
AML with recurrent genetic abnormalities			
AML with t(8;21); *RUNX1-RUNX1T1*	4	0	
AML with inv(16); *CBFB-MYH11*	4	0	
Acute promyelocytic leukemia with t(15;17); *PML-RARA*	1	1	
AML with myelodysplasia-related changes	21	1	3/4
Therapy-related AML[b]	10	0	0/2
AML, not otherwise specified (NOS)			
AML, NOS with normal karyotype	19	11	14/17
AML, NOS with single genetic aberrations	7	2	
AML, NOS with complex aberrations	5	0	
Others			
Transformation of myeloproliferative neoplasm[c]	4	1	0/1
Total	75	16	

[a] All 26 AML cases with normal karyotypes were tested for *NPM1* mutations, irrespective of the disease subtype according to the WHO classification; analysis succeeded in 24 cases; the results represent mutated cases out of all analyzable cases with normal karyotypes within the respective entity

[b] There were three additional acute promyelocytic leukemia with t(15;17) cases in this group, but considering the clinical history, these patients had to be properly classified as therapy-related AML

[c] All four cases were *JAK2* wild type

Cytogenetic analysis

Cytogenetic analyses were carried out at the Centre Hospitalier Universitaire Vaudois in Lausanne (Unité cytogénétique du cancer). Cell culture and chromosome preparation were performed as reported previously [20]. Chromosomes were stained in G-bands. Karyotypes were described according to the International System for Human Cytogenetic Nomenclature [21].

FLT3-ITD and *nucleophosmin (NPM1)* mutations detection

All samples were analyzed for *FLT3*-ITD in exons 14 and 15 by reverse transcription-polymerase chain reaction (RT-PCR) and agarose gel electrophoresis as previously described [22]. RNA and cDNA were prepared from bone marrow or from peripheral blood in patients with a dry tap. cDNA (1 µg) was added to a total of 30-µl reaction mix containing nuclease-free water (Catalyse AG), 15 mM magnesium chloride (10× Puffer Qiagen), 10 mM dNTP (Invitrogen), 10 µM forward primer, 10 µM reverse primer, and Taq polymerase (Qiagen). The following primers were used: 5′-AGC AAT TTA GGT ATG AAA GCC AG-3′ as forward primer and 5′-CCT TCC CAA ACT CTA AAT TTT CTC T-3′ as reverse primer (Microsynth). Preheating of the samples at 95°C for 5′ was followed by amplification for 35 cycles consisting of 30-s denaturation at 95°C, 30-s annealing at 60°C, and 45-s elongation at 72°C. After completion of these cycles, a final elongation of 5 min at 72°C was performed. Amplified PCR products were supplemented with 6 µl loading buffer (Catalyse AG). Of the final product, 15 µl were loaded on a 3% agarose gel and analyzed by electrophoresis and ethidium bromide staining. As a control, the cell line MV4-11 (ATCC) known to contain the *FLT3*-ITD at 171 bp was included in every analysis. The sensitivity of the assay was 10^{-2}. Samples of all AML patients with normal karyotypes, irrespective of the disease subtype according to the WHO classification, were analyzed for *NPM1* mutations as described [23]. The following primers were used: 5′-GCG CCA GTG AAG AAA TCT ATA C-3′ as forward primer and 5′GGA CAA CAT TTA TCA AAC ACG G-3′ as reverse primer (Microsynth). PCR products were analyzed on the ABI3130 Genetic Analyzer (Applied Biosystems). *NPM1*-positive samples were sequenced to determine the type of mutation.

Statistical analysis

Statistical analyses were performed using SPSS version 15.0 (SPSS, Chicago, IL). Incomplete data were not excluded from the tests. The degree of agreement between the quantitative immunohistochemical values by the different observers was evaluated by intra/interclass correlation coefficients, using reliability Cronbach's Alpha analysis. The Fisher's exact test was used to analyze differences in case numbers distribution between groups. Mann–Whitney or Kruskal–Wallis tests were applied as appropriate to assess mean differences between groups. The prognostic performance of the variables and determination of optimal cutoff values was established by receiver

operating characteristic (ROC)-curves plotting sensitivity vs 1-specificity with special considerations of the respective area under the ROC (AUROC). The optimal cutoff point was calculated using Youden's index (Y), denoting Y=sensitivity+specificity−1, since this method can be applied to find the optimal cutoff value with the highest sensitivity and specificity when there is no particular requirement on sensitivity and/or specificity [24]. Disease-specific survival was analyzed by the Kaplan–Meier method and compared by the logrank test in univariable modus and by the Cox regression analysis in multivariable modus. Solely factors, which turned out to be of prognostic significance in the univariable modus, were entered into the multivariable analysis. Statistical significance was defined as $p<0.05$ and corrected for multiple testing, when necessary. Two-sided tests were used throughout.

Results

### Distribution of *FLT3*-ITD and *NPM1* mutations

Altogether, 16 out of 75 cases (21%) showed *FLT3*-ITD (males 11/38; females 5/37). The distribution of *FLT3*-ITD among the different types of AML is displayed in Table 1.

NPM1 mutations were detected in 17/24 evaluable AML patients with normal karyotypes (Table 1); 16 of these were type A mutations, and 8 were observed in *FLT3*-ITD cases.

Immunohistochemistry

Immunohistochemical stainings were evaluable in 65 cases for pSTAT1, 65 cases for pSTAT3, and 60 cases for pSTAT5 (Fig. 1a–f). pSTAT3 and pSTAT5 stainings could be fully oppressed by preabsorption with the respective blocking peptides. Blasts were considered to express the respective marker if their nuclear staining was clearly detectable against the background. Positivity in nuclei of lymphocytes, neutrophils, erythroid precursors, and vascular walls served as an internal control (Fig. 1a–f). The interclass correlation for interobserver variability was excellent for pSTAT5 (p=0.002, alpha=0.740), good for pSTAT1 (p=0.006, alpha=0.651), and acceptable for pSTAT3 (p=0.05, alpha=0.517).

pSTAT5 was expressed in a mean 11±13% blasts (median 2.5%, range 0.8–39%) in AML with *FLT3*-ITD as opposed to 0.7±0.9% (median 0.4%, 0–3.7%) in cases without *FLT3* abnormalities ($p^{\text{Mann–Whitney-}U}<0.0001$), but there were no significant differences for pSTAT1 or pSTAT3, respectively (Fig. 2, Table 2). Importantly, pSTAT5 was expressed in only 0.2±0.3% blasts in the three analyzable AML transformations of myeloprolifera-
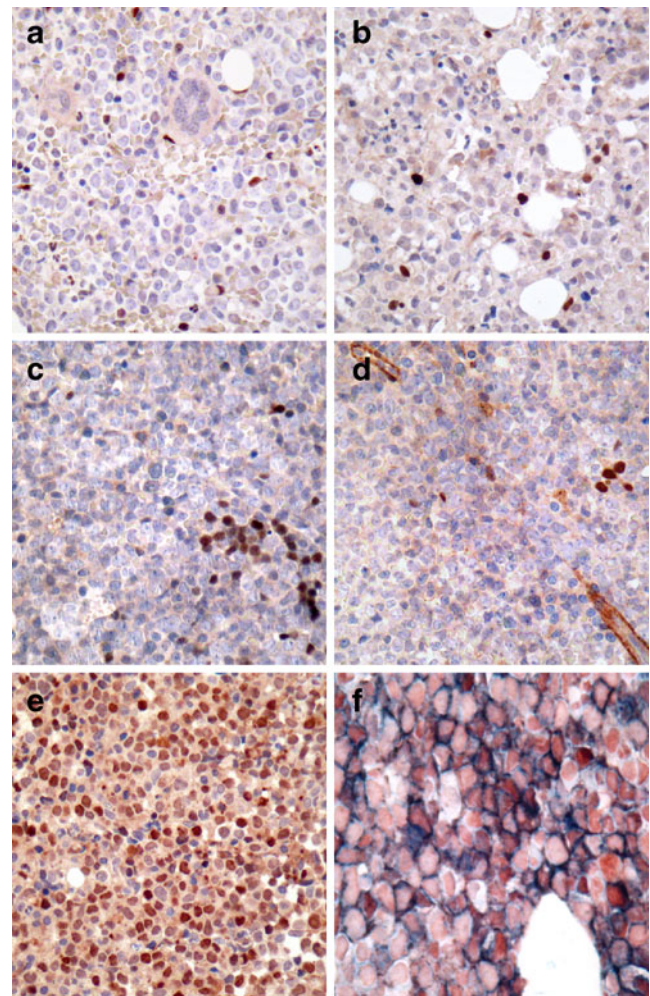
Fig. 1 a pSTAT1 expression in AML; note scattered positive neutrophils and lymphocytes as well as negative megakaryocytes; immunoperoxidase stain, 200×. **b** pSTAT3 expression in AML; note scattered positive blasts; immunoperoxidase stain, 200×. **c, d** pSTAT5 expression in erythroid precursor islet and in microvascular walls of *FLT3* wild-type AML; immunoperoxidase stain, 200×. **e** Abundant pSTAT5 expression in *FLT3*-ITD AML; immunoperoxidase stain, 200×. **f** CD117 (*blue*)/pSTAT5 (*red*) double stain showing colocalization of signals; immunoperoxidase stain, 640×

tive neoplasm cases; one AML transformation of myeloproliferative neoplasm case with *FLT3*-ITD was not analyzable for pSTAT5. Double staining of pSTAT5 and the blast-specific markers CD34 or CD117 showed coexpression of the two markers, proving the correct labeling of blasts by the antibody against pSTAT5 (Fig. 1f).

The AUROC of the predictive value of pSTAT5 expression for *FLT3*-ITD was 0.901 (95% confidence interval 0.822–0.980) with a cutoff with both maximum specificity (73%) and sensitivity (100%) suggested by the Youden's index of >0.8% (Fig. 3). The positive predictive value (PPV) for the presence of *FLT3*-ITD in AML cases with expression of pSTAT5 in >0.8% of blasts was 57%; the negative predictive value (NPV) was 100%. Applying
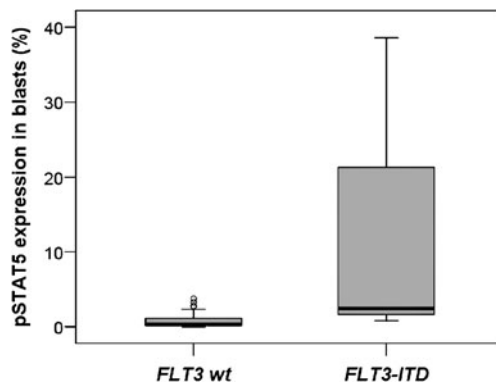
Fig. 2 Boxplot of pSTAT5 expression in *FLT3* wild type (*wt*) and mutated (*ITD*) acute myelogenous leukemia cases; $p^{\text{Mann–Whitney-}U} < 0.0001$

Fig. 3 ROC curve indicating a significant discriminatory potential of the amount of pSTAT5 expressing blasts for predicting *FLT3*-ITD. Note that the *dark ROC curve* never crosses the *dotted reference line* (corresponding to random guess or variable without diagnostic capability) and that the AUROC is 0.901, indicating that consideration of pSTAT5 is as 40% more accurate to predict presence/absence of *FLT3*-ITD as a random guess (for more details, see [24]). The curve point at a sensitivity of 1 (100%) and specificity of 0.73 (73%; 1-specificity=0.27) is at greatest distance away from the reference line. Considering increased pSTAT5 expressing blasts, these coordinates correspond to an optimal cutoff for the prediction of *FLT3*-ITD of >0.8%

this cutoff, all 12 *FLT3*-ITD cases were identifiable, while seven out of 48 cases without *FLT3*-ITD also expressed pSTAT5 in >0.8% of blasts ($p^{\text{Fisher's}} < 0.001$). The cutoff with highest specificity was 4.7% with a PPV of 100% (since not a single case without *FLT3*-ITD expressed pSTAT5 in >4.7% of blasts) and a NPV of 86%.
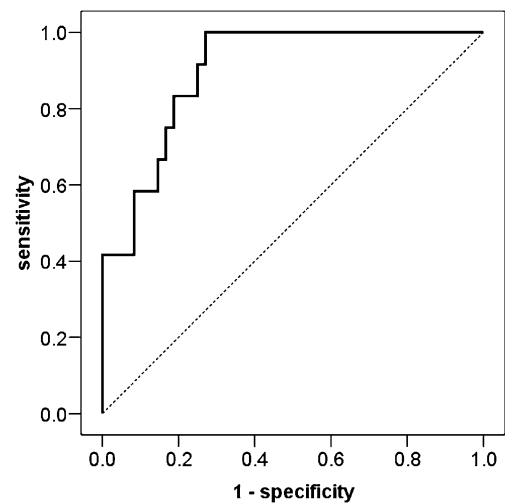
Clinical outcome

Survival data were available for all patients. Thirty-seven patients died within 16 months with a mean survival of 6.1 months. The univariable analysis of clinical parameters (age below vs equal/above 60 years, sex), disease subtype according to the WHO classification, *FLT3* status, *NPM1* status, expression of pSTAT1, 3, and 5, and type of therapy, for their prognostic significance showed that the disease subtype according to the WHO classification ($p < 0.001$), age at diagnosis ($p < 0.001$), and therapy type ($p < 0.001$) predicted survival, while the other parameters showed no influence. Only in AML not otherwise specified (NOS) with normal karyotype ($n = 19$, all but two treated in curative intention) presence of *FLT3*-ITD but not of *NPM1* mutations turned out to be of prognostic significance ($p = 0.033$; Fig. 4). Consideration of the *NPM1*

status in our series of AML, NOS with normal karyotype was of no additional prognostic value neither in all cases nor in cases split according to their *FLT3* status. This was also the case for the prognostic significance *NPM1*

Table 2 Expression of pSTAT1, 3, and 5 with respect to *FLT3*-ITD status

	Mean number of positive blasts per HPF[a]		
	pSTAT1	pSTAT3	pSTAT5
FLT3 wild type	9±21 (50)	38±39 (45)	21±24 (48)
FLT3-ITD	6±8 (15)	46±42 (10)	167±262 (12)
p value	ns	ns	0.004

[a] *HPF* high-power field, i.e., 0.177 mm^2, *ns* not significant

Number of evaluable cases is shown in brackets
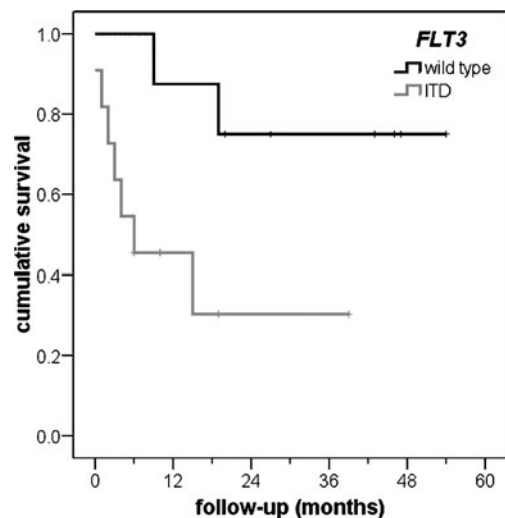
Fig. 4 Kaplan–Meier survival curves of AML, not otherwise specified cases with normal karyotype with respect to the *FLT3* status; the *p* value has been calculated by applying the logrank test. Two out of eight AML patients without *FLT3*-ITD died (mean survival 44 months, median not reached), while seven out of 11 AML patients with *FLT3*-ITD died (mean survival 16 months, median 6 months; *p*=0.033)

mutations in AML with myelodysplasia-related changes with normal karyotypes.

When the prognostic parameters were assessed in a multivariable manner, only type of therapy ($p=0.03$) and age ($p<0.001$) retained their statistical significance for all AML. In a multivariable model in AML, NOS with normal karyotype FLT3-ITD turned to be an age- and therapy type-independent negative prognostic factor, even after correction for multiple testing ($p=0.026$, relative risk 7.62, 95% confidence interval 1.28–45.34).

Discussion

Here, we were able to show that assessment of pSTAT1-, 3-, and, particularly, pSTAT5-positive blasts is reproducibly possible in formalin-fixed paraffin-embedded trephine bone marrow biopsies by immunohistochemistry. Of the markers analyzed, assessment of pSTAT3 showed only acceptable interobserver reproducibility, primarily due to the presence of weakly stained blasts, which were variably taken into consideration by the observers. This finding should be considered in future in situ bone marrow studies addressing pSTAT3 expression.

In addition, we demonstrated that pSTAT5 expression is significantly correlated to FLT3-ITD and proved by double labeling that pSTAT5 is specifically expressed in myeloid blasts (Fig. 1f), indicating that, similarly to normal hematopoiesis, STAT5 is highly likely the preferred second messenger of FLT3-mediated cell signaling. The distribution of FLT3-ITD AML cases (21% of the studied samples) and the observed strong prognostic impact of FLT3-ITD in AML with normal karyotypes are in line with the literature, reporting such mutations in approximately 25% of AML and association with an adverse clinical outcome with poor overall survival and a higher risk of relapse, particularly in AML with normal karyotypes [5, 6]. These basic characteristics of our cohort additionally point toward its representativeness.

FLT3 and its downstream STAT-related pathways play a fundamental role in AML [14, 15]. Therefore, assessment of FLT3 mutations as well as addressing their functional significance, e.g., by measurement of pSTAT5 might be important. Analysis of pSTAT has been attempted by different methodologies such as flow cytometry [25], electrophoretic mobility shift assays [15, 26], and western blot analysis [27, 28]. Interestingly, the percentage of cases with pSTAT expression varied between 7% [26] and 95% [28]. Some discrepancies can probably be attributed to the applied methodologies as well as the sensitivity and specificity of the antibodies. However, the specificity of the anti-pSTAT5 antibody applied in this study has been proven previously [4, 16] and additionally in the present

study by preabsorption with a corresponding blocking peptide. Addressing the strengths and drawbacks of western blot analysis, samples may contain other cells, such as vascular structures or erythroid precursors, which can also express pSTAT5, as shown in Fig. 1c, d. Association of pSTAT5 expression and FLT3-ITD in AML is obviously only relevant for pSTAT5 expression in blasts. Identification of in situ pSTAT5 expression in blasts seems to be possible only when the microtopography of the evaluated tissue sample is preserved, which is the case in our technique, but not in western blot or electrophoretic mobility shift assays. Assessment of STAT5 gene expression by quantitative PCR would not be helpful as well since the phosphorylated (active) protein amount and its microtopographic distribution is of relevance. Importantly, on the functional level, it has been clearly shown that mutations of FLT3 result in the strong activation of STAT5 [2, 29], and vice versa, constitutive phosphorylation of STAT5 can efficiently be downregulated by a FLT3 tyrosine kinase inhibitor [30].

Our findings support the hypothesis that FLT3 mutations lead to an activation of STAT5, which in turn can be assessed in order to predict the FLT3 status. Furthermore, both FLT3 and STAT5 have been identified as potential new therapeutic targets for treatment of patients with AML [31]. In particular, combining FLT3 inhibitors and agents, such as antisense nucleotides, small molecular inhibitors, or shRNA, targeting STAT and STAT-related pathways has been recommended by some authors [32]. Therefore, simultaneous assessment of both FLT3 mutation status and STAT5 expression and phosphorylation status might be required in the future for selecting AML patients for targeted therapies.

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