Correlation of minimal residual disease cell frequency with molecular genotype in patients with acute myeloid leukemia

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ABSTRACT

Background

About 70-80 percent of patients with acute myeloid leukemia enter complete remission, but at least half of these patients who achieve remission go on to relapse. Improved treatment is likely to come from increasing the time to relapse, especially for younger patients. With the vastly increasing number of targeted therapies there is a strong need for short-term end-points to efficiently test such therapies for further pursuance. Minimal residual disease assessment may offer such an end-point since it is a strong independent prognostic factor. As proof of principle we examined this concept for *FLT3*-ITD status at diagnosis.

Design and Methods

We determined *FLT3*-ITD status in bone marrow samples from 196 patients with newly diagnosed acute myeloid leukemia. The frequencies of residual leukemic cells of these 196 patients were assessed in 267 follow-up bone marrow samples using immunophenotypic assessment of minimal residual disease.

Results

The median frequency of residual leukemic cells after the first cycle of chemotherapy was 8.5-fold higher in patients with *FLT3*-ITD than in those with wild type *FLT3*. Such a difference translates into differences in survival, even if other potentially outcome-modulating mutations, such as *NPM1*, *KIT*, *NRAS*, *KRAS*, *FLT3*-exon 20 and *PTPN11* are included in the analysis.

Conclusions

This study shows that it could be possible to study the efficacy of FLT3 inhibitors using the level of minimal residual disease as a short-term end-point.

Key words: acute myeloid leukemia, minimal residual disease, *FLT3*.

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Introduction

At present treatment of acute myeloid leukemia (AML) in adults is based on the assessment at diagnosis of a limited number of prognostic factors including cytogenetics. From a genetic point of view AML originates from a wide variety of acquired somatic mutations. The development of AML is now considered a two-step process: class 2 mutations such as t(8;21), *AML1-ETO* and inv(16) must work in concert with the so-called class 1 mutations. Examples of class 1 mutations are the activating mutations of receptor tyrosine kinases (*KIT* and *FLT3*), and mutations in the proto-oncogene *RAS* family members, *NRAS* and *KRAS*. These insights are being translated into the development of molecules that target these specific mutations directly or that act downstream in their disrupted signal transduction pathways. Numerous clinical trials using small molecule inhibitors, such as FLT3 inhibitors, in addition to conventional chemotherapy have been initiated.[1,2]

In order to have an effecient clinical trial design for the large variety of modulating agents and their combinations with other drugs, including classical chemotherapeutic agents, early efficacy read-out parameters are necessary. Until now, the achievement of complete remission, remission duration and overall survival have been used for this purpose. However, in the light of clone selection, shifts of (stem) cell subpopulations and multiple hit models, an earlier read-out is desirable.

The outgrowth of minimal residual disease (MRD) cells has previously been indicated to be responsible for the emergence of relapse.[3] Moreover, the frequencies of MRD cells in bone marrow, characterized immunophenotypically by an aberrant phenotype, were shown to have a prognostic impact after induction and intensification therapy.[4-7]

We hypothesized that the frequencies of MRD cells after the first cycle of chemotherapy could be used as an early read-out parameter in future clinical trials using FLT3 inhibitors. To test this hypothesis, we studied the relationship between the presence of *FLT3*-internal tandem duplications (ITD) and MRD cell frequency. The presence of class 1 mutations other than ITD in exon 14 of the *FLT3* gene (*NPM1, KIT, NRAS, KRAS, FLT3*-exon 20 and *PTPN11*) was accounted for in this analysis.

Design and Methods

Patients' samples

The bone marrow aspirates, peripheral blood samples and clinical data presented in this study were obtained from 288 AML patients, treated at the Hematology Department of the VU University Medical Center (Amsterdam, The Netherlands). Informed consent was obtained from all patients, with approval of the institutional review board. The diagnosis of AML was established on the bases of morphology and immunophenotype, according to the French-American-British (FAB) classification. Cytogenetic aberrations were scored according to Grimwade *et al.*[8]

Treatment characteristics and definitions

Patients were treated according to the Dutch-Belgian-Swiss (HOVON) protocols (*www.hovon.nl*). Patients 60 years of age or younger were treated according to the HOVON 29 (during 1998-2000) or HOVON 42 (during 2001-2006) protocol. Patients over 60 years of age were treated according to the HOVON 32 (during 1996-2000) or HOVON 43 (during 2000-2006) protocol.

All follow-up samples were obtained after full hematologic recovery of the bone marrow. Hematologic recovery was defined as the time point at which the white blood cell count (WBC) was greater than 1.0×10^9/L, the granulocyte count was greater than 0.5×10^9/L and the platelet count was greater than 100×10^9/L. Complete remission was determined morphologically and defined by the presence of less than 5% blasts in the bone marrow, combined with recovery of the peripheral blood cell counts. Early/toxic deaths were defined as all deaths occurring within 7 days after completion of the first induction cycle or death during therapy-induced bone marrow hypoplasia. Overall survival was defined as the time interval between inclusion in the study and death, relapse-free survival as the time interval between achievement of complete remission and relapse, and disease-free survival as the time interval between diagnosis and the first event (relapse or death, whether or not disease-related) for patients who had achieved complete remission.

Establishment of leukemia-associated phenotypes

MRD cell frequencies were determined using leukemia-associated phenotypes (LAP). LAP were established on the blasts at diagnosis applying a double step immunophenotypic labeling procedure using fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, peridin chlorophyll protein (PerCP)- and allophycocyanin (APC)-conjugated monoclonal antibodies, as previously described.[7,9,10]

In normal bone marrow these LAP are, by definition, absent or present in very low frequencies.[11] Since the penetrance of LAP positivity on leukemic blasts at diagnosis is most often not 100%, the actual LAP frequency at diagnosis has to be taken into account when the frequency of LAP-positive leukemic blasts in follow-up samples is calculated. Corresponding isotype controls were included to test the specificity of staining for all markers. In the present cohort every patient displayed one or more LAP, thus enabling MRD detection during follow-up.

Minimal residual disease study group and minimal residual disease cell frequency

Since the association between MRD cell frequencies and *FLT3*-ITD status was the main focus of this study, we restricted the description of clinical characteristics to the 196 patients displaying an aberrant blast immunophenotype at diagnosis, which enabled MRD assessment in follow-up samples. Details are shown in the second column of Table 1. Overall 157 out of these 196 patients achieved complete remission; 128 after the first cycle of consolidation chemotherapy, 23 after the second cycle and one after the third cycle. Complete

remission status in relation to chemotherapy cycle was unknown for three patients. Sixteen patients did not achieve complete remission after receiving two cycles of induction chemotherapy. Twenty patients experienced early/toxic death. Table 1 presents all these characteristics, subspecified for the patients included after the different MRD assessment times e.g. after first and second induction chemotherapy and after consolidation treatment. At diagnosis 179 bone marrow and 17 peripheral blood samples were obtained from the 196 patients. In patients whose peripheral blood was used for LAP assessment, the blast percentage was on average 57% (range, 4.6-94%). During follow-up 388 bone marrow samples were obtained at different time points, 175 after the first cycle of chemotherapy, 120 after the second cycle and 93 after the third cycle.

Table 1. Patients' characteristics.

	Total population			MRD assessment after		
		FLT3 wt	*FLT3* ITD	1st cycle	2nd cycle	3rd cycle
N.	196	138	36	111	96	60
Median WBCx10⁹/L	11.4	10.4	34.7	9.1	10.4	13.5
FAB classification						
M0	11	9	2	4	6	4
M1	21	13	6	13	11	7
M2	38	26	7	23	18	10
M3	9	7	2	7	6	6
M4	27	21	4	21	16	14
M5	42	26	10	20	20	11
M6	11	10	0	5	5	3
M7	0	0	0	0	0	0
RAEB-T/MDS	22	17	1	11	7	3
Unknown	15	9	4	7	7	2
Treatment protocol						
Age <60 years	131	91	24	81	74	49
Age ≥60 years	63	46	12	29	21	11
Unknown	2	1	0	1	1	0
Consolidation						
Autologous transplant	28	17	6	12	15	13
Allogeneic transplant	34	24	8	11	12	16
Third cycle	55	44	8	32	29	21
No consolidation	79	53	14	56	40	10
Cytogenetics						
Good	30	27	2	24	14	14
Intermediate	110	76	21	53	51	34
Poor	11	9	1	6	5	4
Unknown	45	26	12	28	19	8
CR status						
CR achieved	157	114	28	107	95	60
No CR achieved	16	12	2	2	1	0
CR status unknown	3	0	0	2	0	0
Early/toxic death	20	10	6	0	0	0

WBC: white blood cell count, FAB: French-American-British; RAEB-T: refractory anemia with excess blasts in transformation; MDS: myelodysplastic syndrome; CR: complete remission.

The total MRD study cohort thus included 196 patients, of whom 157 (80%) achieved morphological complete remission (Table 1). By definition the term MRD cell frequency applies to those patients who achieved complete remission (n=157). Based on this selection, MRD cell frequencies were determined after the first, second and third cycles of chemotherapy in 107/175, 95/120 and 60/93 samples, respectively.

Sample preparation

Genomic DNA or RNA was isolated from quickly frozen cell pellets obtained at diagnosis from all 288 patients included in the study. Genomic DNA was extracted using a QIAamp blood DNA extraction kit (Qiagen, Crawley, UK). Total RNA was extracted using RNABee solution (Tel-test Inc., Friendswood, TX, USA) according to the manufacturer's recommendations.

Polymerase chain reaction (PCR) primers and conditions for *FLT3*, *NPM1*, *KIT*, *NRAS*, *KRAS*, *FLT3*-exon 20 and *PTPN11* mutation analysis have been described[12-14] previously and are available on request.

Statistics

MRD cell frequency was assessed both as a continuous variable and, by applying cut-offs, also as a dichotomous variable. The cut-offs applied were the median MRD value, and various others decided on the basis of optimized hazard rates (HR). Kaplan-Meier survival curves were constructed for subgroups of patients based on MRD cut-offs. The present study included patients with an immunophenotypically defined (using CD45 blast cell gating[15]) MRD cell frequency of less than 5% blasts (Table 1). In two cases this contrasted with the morphologically defined status of complete remission.

The relationship between MRD cell frequency and overall, relapse-free and disease-free survival and also between *FLT3* status and these survival parameters were estimated for patients who were included in the MRD analysis. A Cox proportional hazard model was used for multivariate analysis of prognostic factors. Clinical pathological factors assessed in the multivariate analysis included FAB-class (categorical variable), cytogenetics (categorical variable) and WBC at diagnosis (expressed as a logarithm and applied as a continuous variable). Treatment protocol was included as a dichotomous variable: two protocols for younger patients (under 60 years old) and two protocols for elderly patients (60 years old or over). Age-related treatment protocols are dealt with separately since they had a different design. Type of consolidation treatment was assessed as a categorical variable, including four categories; third cycle of chemotherapy, allogeneic transplantation, autologous transplantation and no consolidation treatment.

The distribution of the clinical variables was compared using χ^2 analysis for categorical variables and the non-parametric Mann-Whitney U test for continuous variables. Multivariate analysis of the impact of multiple mutations on MRD cell frequencies was conducted using either linear regression (in the case that MRD was studied as a continuous variable) or logistic regres-

sion (when MRD cell frequency was studied as a dichotomous variable). Patients still in remission were censored at the time of last follow-up.

The reported p values are two-sided and $p<0.05$ was considered statistically significant in all analyses. Calculations were performed using the SPSS software package version 11.0.1 (SPSS, Chicago, IL, USA).

Results

FLT3-*ITD* status and survival

In total 265 samples from the 288 newly diagnosed AML patients were successfully analyzed for ITD in exon 14 of the *FLT3* gene. *FLT3*-ITD were found in 61/265 patients (23%). Failure of mutation analysis (23 patients) was due to either shortage of cells or low DNA yields. The percentage of *FLT3*-ITD in the total population (n=265) was comparable to the percentages observed in the subgroups of patients in the total MRD group (n=196) included in MRD assessment studies after the first (n=107), second (n=95) and third (n=60) cycles of chemotherapy, in which ITD were observed in 20.7%, 16.7%, 23.1% and 18.6% of the patients, respectively.

Assessment of the distribution of clinical characteristics across patients with *FLT3*-ITD (n=36) and those with wild type (wt) *FLT3* (n=138) in the MRD study revealed an unequal distribution between the two groups for log WBC (higher in the *FLT3*-ITD group, Table 1), favorable cytogenetics (lower in the *FLT3*-ITD group, Table 1) and intermediate cytogenetics (higher in the *FLT3*-ITD group, Table 1). Cases with unknown data were excluded from the calculations. No significant differences were found for most FAB types, age-related treatment protocol or type of consolidation regimen. Trends were visible for percentages of patients diagnosed as having refractory anemia with excess blasts in transformation or AML secondary to a myelodysplastic syndrome (13.0% in the *FLT3*-wt group compared to 3.0% in the *FLT3*-ITD group) and FAB class M5 (present in 30.3% of patients with *FLT3*-ITD compared to in 19.8% with *FLT3*-wt).

In the total MRD population of patients (n=196) in whom *FLT3*-ITD status was evaluated (n=171), a *FLT3*-ITD was associated with shorter overall survival (HR 1.7; 95%CI 1.06-2.72; p=0.027), relapse-free survival (HR 2.1; 95%CI 1.16-3.62; p=0.013) and disease-free survival (HR 1.9; 95%CI 1.13-3.21; p=0.015). The adverse prognostic impact of an ITD in the *FLT3* gene on overall, relapse-free and disease-free survival was maintained although the statistical significance was weaker because of the smaller numbers of patients in the subpopulations studied after the different courses of chemotherapy (HR after the first cycle 1.6/1.9/1.9; p=0.09/0.05/0.03; HR after the second cycle 1.7/2.0/1.9; p=0.11/0.05/0.05, HR after the third cycle 1.7/1.6/1.9; p=0.13/0.14/0.05).

Minimal residual disease cell frequency and survival

In agreement with our previous experience, MRD cell frequency in samples from 107 patients after the first cycle of induction chemotherapy predicted overall survival (HR 1.2; 95%CI 1.09-1.32; $p<0.0001$), relapse-free survival (HR 1.6; 95%CI 1.15-2.31; p=0.0006) and disease-free survival (HR 1.2; 1.08-1.34; p=0.001). The optimal cut-off in MRD cell frequency of 1.0% in the present cohort of patients revealed 3.7-fold and 4.1-fold higher risks of shorter overall survival (95%CI 1.63-8.30; p=0.002) and relapse-free survuval (95%CI 1.50-10.94; p=0.006), respectively, for patients with MRD cell frequencies of greater than 1.0%. The optimal cut-off of 1% remained significant in the subgroup of younger patients (p=0.02) and of borderline significance in the subgroup of elderly patients (p=0.08).

Based on the assessment of 96 patients after the second cycle of chemotherapy, MRD cell frequency, considered as a continuous variable, was also highly predictive of the patients' overall, relapse-free and disease-free survival (HR 1.23; 95%CI 1.059-1.431; p=0.007, HR 1.20; 1.035-1.392; p=0.016 and HR 1.17; 95%CI 1.033-1.340; p=0.014), respectively. The optimal cut-off 0.8% remained significant in both the younger and older groups of patients ($p<0.0001$ and p=0.024, respectively).

Among the 60 patients who were assessed after the third cycle of chemotherapy, a higher MRD cell frequency also correlated with poorer overall survival (HR 1.42; 95%CI 1.11-1.81; p=0.005), relapse-free survival (HR 1.36; 1.08-1.71; p=0.010) and disease-free survival (HR1.43; 95%CI 1.13-1.81; p=0.003). The cut-off in MRD cell frequency of 0.4% resulted in the most significant difference with a 3.6-fold higher relative risk of relapse for the patients in the high frequency group. This optimal cut-off of 0.4% remained significant for the younger patients (p=0.01) although not for the elderly patients (p=0.31) as could be expected on the basis of the low number of subjects in this group (n=11).

FLT3-*ITD status and minimal residual disease cell frequency*

FLT3-ITD status was not associated with remission rate (p=0.55). In the *FLT3*-ITD group 28/30 (Table 1, column 4) achieved complete remission, while in the *FLT3*-wt group 114/126 (Table 1, column 3) did so.

However, in patients entering complete remission, *FLT3*-ITD status was strongly associated with MRD cell frequency after the first cycle of chemotherapy (n=96, p=0.002). After the second course of chemotherapy the presence of an *FLT3*-ITD remained associated with higher MRD cell frequency although this association was much less pronounced (n=91, p=0.082). After the third cycle of chemotherapy the association was lost (n=59, p=0.69).

Median MRD cell frequencies in the total population of patients dropped during consecutive chemotherapy cycles (median MRD cell frequencies after the first, second and third cycles were 0.06, 0.04 and 0.03, respectively). Since 0.01% is the lowest detectable frequency of MRD cells for immunophenotypic assessment of MRD,[5,6] this reduces the differences in MRD cell frequencies between *FLT3*-wt and *FLT3*-ITD patients. It is likely that MRD cell frequencies would decrease further after additional cycles of chemotherapy.

Survival effects of minimal residual disease cell frequency in FLT3-*ITD and* FLT3-*wt patients*

The effect of MRD frequencies and their cut-offs on overall survival and relapse-free survival for patients with *FLT3*-ITD and *FLT3*-wt are displayed in Figure 1.

The distribution of MRD cell frequencies in *FLT3*-ITD and *FLT3*-wt samples after different cycles of chemotherapy is shown in Figure 2.

The results obtained for MRD after the first course of chemotherapy were analyzed in more detail: the median MRD cell frequencies in the two groups differed by a factor of 8.5: MRD% 0.34 (*FLT3*-ITD) versus 0.04 (*FLT3*-wt). Significantly more patients with *FLT3*-wt were found below the median cut-off for the *FLT3*-ITD group of 0.34% compared to above the cut-off (41/70 patients; χ^2 test 4.401). Similarly, significantly more *FLT3*-ITD cases were found above the median cut-off for the *FLT3*-wt patients of 0.04% (15/19 patients; χ^2 test 4.436). The 8.5-fold difference in MRD% translated into differences in median survival: among the total population of patients, the median overall and relapse-free survival of patients with MRD% of 0.01 (the lowest detectable MRD%) was, respectively 10.7 and 11.1 months, while the median overall and relapse-free survival of patients with MRD% that were 8.5-fold higher than the lowest detectable MRD% (range, 0.085-0.1) was 8.8 and 6.9 months, respectively.

This also held true for other 8.5-fold differences in ranges of MRD cell frequencies. This implies that the use of an inhibitor of tyrosine kinase activity could not only cause large differences in MRD cell frequency, but also differences in survival.

KIT, NRAS, KRAS, PTPN11, NPM1, FLT3 *exon 20 mutations and* FLT3-*ITD status*

Among other fractors, a poor response to therapy in part of the *FLT3*-wt group, may result from other

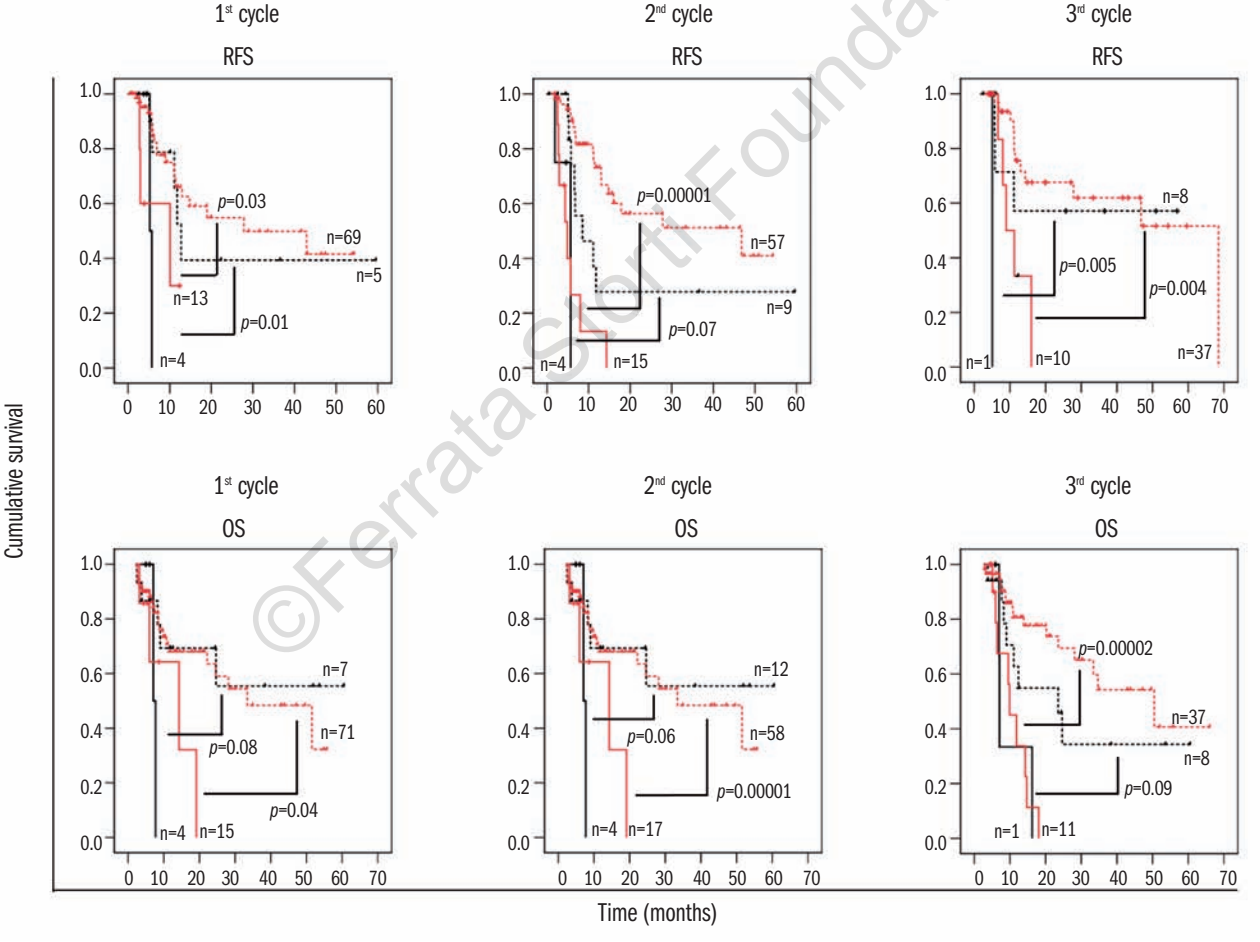

Figure 1. Relapse-free survival (RFS) and overall survival (OS) in *FLT3*-ITD and *FLT3*-wt patients based on chemotherapy cycle-specific cut-offs in MRD cell frequency. For each cycle of chemotherapy in the whole group of patients (*FLT3*-ITD + *FLT3*-wt) a cut-off in MRD frequency was determined that was best at dividing patients according to their survival (1.0 after the first cycle, 0.8 after the second cycle and 0.4 after the third cycle). Kaplan-Meier curves were constructed from these. Effects of chemotherapy cycle specific cut-offs in MRD frequency are shown for OS and RFS in patients with *FLT3*-ITD and *FLT3*-wt. Both *FLT3*-wt and *FLT3*-ITD patients with MRD frequencies higher than the cycle-specific cut-off had significantly or borderline significantly shorter OS and RFS compared to those patients with MRD frequencies lower than the cycle-specific cut-off. Black straight line: *FLT3*-ITD patients with MRD frequencies above the cut-off, black dotted line: *FLT3*-ITD patients with MRD frequencies below the cut-off, red straight line: *FLT3*-wt patients with MRD frequencies above the cut-off, red dotted line: *FLT3*-wt patients with MRD frequencies below the cut-off.

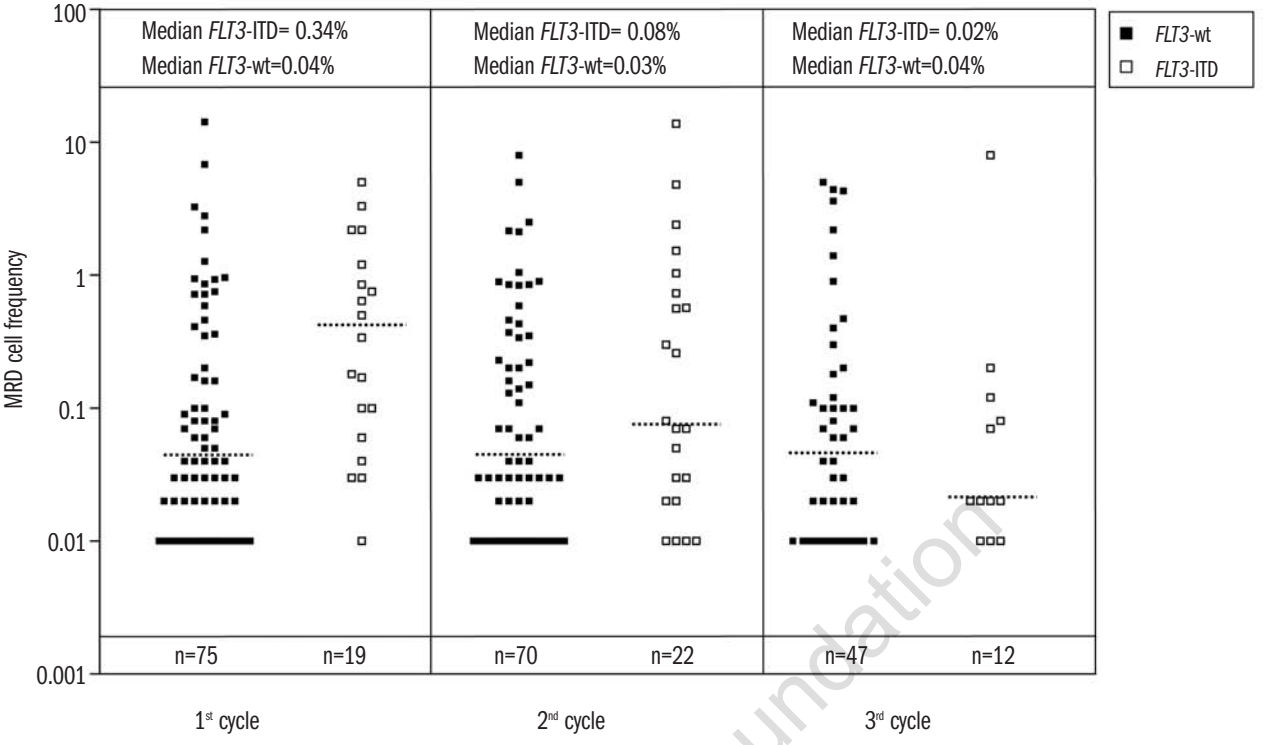

Figure 2. Distribution of MRD frequencies in *FLT3*-ITD and *FLT3*-wt patients. Dot plots show the distribution of MRD cell frequencies (y-axis) in *FLT3*-ITD (solid black squares) and *FLT3*-wt patients (open black squares) in morphological complete remission after the first, second and third cycles of chemotherapy (x-axis). The median MRD frequencies in *FLT3*-ITD and *FLT3*-wt patients after the first cycle of chemotherapy differed by a factor of 8.5: 0.34% in the *FLT3*-ITD patients versus 0.04% in the *FLT3*-wt patients. For the second and third cycles the corresponding figures are 0.08% versus 0.03% and 0.02% versus 0.04%, respectively. These differences in median MRD frequencies between *FLT3*-ITD and *FLT3*-wt patients were significant after the first and the second cycles of chemotherapy (p=0.05). Median MRD frequencies for *FLT3*-ITD patients after the second cycle dropped by a factor of 4.25 compared to the first cycle and after the third cycle by a factor of 4.0 compared to the second cycle. The corresponding figures for *FLT3*-wt patients were 1.3 and 0.75, respectively.

mutations. Although none of the investigated mutations besides *FLT3*-ITD had a significant impact on overall, relapse-free or disease-free survival on individual bases (*data not shown*), a combined regression analysis showed that both *KRAS* and *PTPN11* improved the overall prediction of the *FLT3*-status with respect to overall survival (p=0.03 χ^2 7.04 and p=0.03 χ^2 7.32, respectively). In the case of *KRAS* this contribution was possibly associated with the achievement of complete remission in the *FLT3*-wt patients, whereas *PTPN11* was found to be associated with early death (Table 2).

NPM1 mutations were more frequently observed among the patients with *FLT3*-ITD (Table 2). In agreement with the favorable prognosis of *NPM1* mutations in the *FLT3*-wt group,[16] we observed a lower MRD percentage in *FLT3*-wt patients with *NPM1* mutations than in *FLT3*-wt patients without *NPM1* mutations (0.015 and 0.06, respectively). In *FLT3*-ITD patients, the presence of *NPM1* mutations was associated with a higher MRD percentage; 1.20 versus 0.18 in *NPM1*-wt patients). In the total population of patients assessed after the first cycle of chemotherapy, the presence of an *NPM1* mutation was associated with a lower median MRD percentage (0.035 versus 0.07 in *NPM1*-wt patients).

Discussion

In this study we show that the presence of *FLT3*-ITD at diagnosis of AML is associated with an 8.5-fold higher frequency of MRD cells after the first course of chemotherapy compared to that found in *FLT3*-wt patients. This difference translated into differences in overall survival, relapse-free survival and disease-free survival. Partial or complete inhibition of the aberrations resulting from the ITD would, therefore, translate into significant survival differences. Since the effects were very pronounced after the first cycle of chemotherapy, the MRD cell percentage at this time point offers a fast read-out end-point to assess the efficacy of FLT3 inhibitors in terms of survival.

From a theoretical point of view a higher MRD cell frequency in *FLT3*-ITD patients after the first cycle of chemotherapy should result in higher MRD cell frequencies after the second and third cycles of chemotherapy, since MRD cell frequency after all cycles of chemotherapy strongly predicts survival.[5,6] The loss of association between MRD cell frequency and *FLT3*-status during consecutive cycles of chemotherapy most likely results from flow cytometric MRD detection limit (0.01%): in

many cases this detection limit has already been reached after the second and third cycles, not allowing further discrimination between patients. This loss in ability of discrimination is not insuperable; however, the first cycle of chemotherapy offers the earliest and, thereby, most favorable point in time for the assessment.

Since the association between MRD cell frequency and treatment efficacy is not limited to flow cytometry, MRD cell frequencies after the second and third cycles of chemotherapy may function as proper read-outs for trials of targeted inhibitors in AML in the future, provided that sensitive molecular techniques are available for the majority of patients.

In general, each course of chemotherapy reduces the amount of MRD in patients as a whole:[5,6] in the present study median MRD cell frequencies were 0.06, 0.04 and 0.03 after the first, second and third cycles of chemotherapy, respectively. However, Figure 2 shows the strong impact of the second and also the third cycles for the subgroup of patients with *FLT3*-ITD with 4.25 and 4-fold further reductions (total 17-fold) after the two extra courses. In contrast, among the group of patients with *FLT3*-wt, no reduction in median MRD cell frequency was seen after two extra courses of chemotherapy. From the large difference in MRD cell frequencies between *FLT3*-ITD and *FLT3*-wt patients it can be concluded that patients with a *FLT3*-ITD at diagnosis had a large benefit from the subsequent cycles of therapy.

For final proof of the utility of MRD cell frequency as a short-term read-out for the efficacy of targeted treatments, a large prospective study is needed. One may ask whether or not *FLT3*-wt patients should be included in the treatment group in such a study, since inhibitors may also exert effects in such patients as a result of non-specific effects. Clinical studies that randomize all patients between the use of an inhibitor versus no inhibitor would enable effectiveness of the inhibiors to be assessed both in patients with *FLT3*-wt and *FLT3*-ITD.

One of the major challenges and ultimate goals for the application of existing and new therapies is to be able to predict the response and survival of individual patients. For the area of overlap between MRD cell frequencies in wild type and ITD cases (Figure 2) this translates into two questions: (i) what is the cause of poor responses in some patients *FLT3*-wt?; and (ii) why do some *FLT3*-ITD patients perform quite well? With regard to the first question, an intriguing possibility is that, irrespective of the cellular characteristics in part of any group of AML patients, pharmacokinetic resistance appears to overrule cellular resistance.[10] With regards to the present study, this means that in the wild type group, but also in the ITD group, high levels of MRD may have resulted, in part, from pharmacokinetic resistance. Furthermore, we and others have recently shown that poor prognosis is not only a characteristic of patients with *FLT3*-ITD at diagnosis, but may also result from apparent post-diagnosis gains of *FLT3*-ITD in patients who had the wild type gene at diagnosis.[12,17] The second question is more difficult to answer. Part of the solution might be post-diagnosis losses of *FLT3*-ITD, since we and others have shown that such cases are associated with a better prognosis.[12,17] Whether such clone shifts can already be manifested during the first cycle of therapy is presently under investigation.

Table 2. Distribution of mutations specified for complete remission and *FLT3*-ITD status in patients after the first cycle of chemotherapy.

	CR=1 (n=138)	CR=1 *FLT3*-ITD (n=24)	CR=1 *FLT3*-wt (n=99)	CR=0 (n=16)	Early death (n=19)
FLT3 status at diagnosis					
Wild-type	99	0	99	12	10
Mutated	24	24	0	2	5
Unknown	15	0	0	2	4
% mutations	19.5	100.0	0.0	14.2	33.3
NPM1 status at diagnosis					
Wild-type	91	12	74	13	12
Mutated	36	10	23	2	4
Unknown	11	2	2	1	3
% mutations	28.3	45.5	23.7	13.3	25.0
KIT status at diagnosis					
Wild-type	87	18	69	13	12
Mutated	4	0	4	0	0
Unknown	47	6	26	3	7
% mutations	4.4	0.0	5.5	0.0	0.0
NRAS status at diagnosis					
Wild-type	76	17	59	10	9
Mutated	15	1	14	3	3
Unknown	47	6	26	3	7
% mutations	16.5	5.5	19.2	23.1	25.0
KRAS status at diagnosis					
Wild-type	83	18	65	13	12
Mutated	8	0	8	0	0
Unknown	47	6	26	3	7
% mutations	8.8	0.0	10.1	0.0	0.0
PTPN11 status at diagnosis					
Wild-type	90	18	72	12	10
Mutated	1	0	1	1	2
Unknown	47	6	26	3	7
% mutations	1.1	0.0	1.4	7.7	16.7

CR=1: in complete remission; CR=0: not in complete remission.

As common events take place in transduction and transcription pathways that are associated with malignant transformation of hematopoietic stem cells, levels of MRD cells may also be indicative of the efficacy of inhibitors that target mutations other than *FLT3*-ITD. Bearing in mind that MRD cell frequency is a very strong independent prognostic factor, it is likely that the present approach may also be applicable to other mutations. In fact, so far we have shown that other factors at diagnosis that have prognostic impact in terms of survival, i.e. apoptosis-related protein expression,[18] P-glycoprotein activity[19] and stem cell frequency,[20] translate into differences in MRD cell frequency. These factors may explain why some *FLT3*-wt patients have a poor survival. For the *FLT3*-ITD patients the additional effects of other mutations are less pronounced since most of the additional mutations are seen in the *FLT3*-wt patients. Furthermore, the additional mutations are more or less scattered over the *FLT3*-ITD group, irrespective of MRD cell frequency.

Overall, this study reveals excellent correlations between *FLT3*-ITD status, MRD frequency and patients' survival, enabling early evaluation of FLT3 inhibitor efficacy in a clinical setting.

Authorship and Disclosures

CJH, GJS, GJO and QW designed the research and wrote the manuscript; FD, CJH and PAM analyzed the data; MCH and AH performed the mutation analyses; AK and NF designed and performed the MRD cell frequency analysis; JB assisted in the statistical analysis. The authors reported no potential conflicts of interest.

References

1. Fiedler W, Serve H, Döhner H, Schwittay M, Ottmann OG, O'Farrell AM, et al. A phase 1 study of SU11248 in the treatment of patients with refractory or resistant acute myeloid leukemia (AML) or not amenable to conventional therapy for the disease. Blood 2005;105: 986-93.
2. Giles FJ, Stopeck AT, Silverman LR, Lancet JE, Cooper MA, Hannah AL, et al. SU5416, a small molecule tyrosine kinase receptor inhibitor, has biologic activity in patients with refractory acute myeloid leukemia or myelodysplastic syndromes. Blood 2003;102:795-801.
3. Kern W, Voskova D, Schoch C, Hiddemann W, Schnittger S, Haferlach T. Determination of relapse risk based on assessment of minimal residual disease during complete remission by multiparameter flow cytometry in unselected patients with acute myeloid leukemia. Blood 2004;104:3078-85.
4. Venditti A, Buccisano F, Del Poeta G Maurillo L, Tamburini A, Cox C, et al. Level of minimal residual disease after consolidation therapy predicts outcome in acute myeloid leukemia. Blood 2000;96:3948-52.
5. San Miguel JF, Martínez A, Macedo A, Vidriales MB, López-Berges C, González M, et al. Immunophenotyping investigation of minimal residual disease is a useful approach for predicting relapse in acute myeloid leukemia patients. Blood 1997;90:2465-70.
6. San Miguel JF, Vidriales MB, López-Berges C, Díaz-Mediavilla J, Gutiérrez N, Cañizo C, Ramos F, et al. Early immunophenotypical evaluation of minimal residual disease in acute myeloid leukemia identifies different patient risk groups and may contribute to postinduction treatment stratification. Blood 2001; 98:1746-51.
7. Feller N, van der Pol MA, van Stijn A Weijers GW, Westra AH, Evertse BW, et al. MRD parameters using immunophenotypic detection methods are highly reliable in predicting survival in acute myeloid leukaemia. Leukemia 2004;18:1380-90.
8. Grimwade D, Walker H, Oliver F, Wheatley K, Harrison C, Harrison G, et al. The importance of diagnostic cytogenetics on outcome in AML: analysis of 1,612 patients entered into the MRC AML 10 trial. The Medical Research Council Adult and Children's Leukaemia Working Parties. Blood 1998;92: 2322-33.
9. van der Pol MA, Pater JM, Feller N Westra AH, van Stijn A, Ossenkoppele GJ, et al. Functional characterization of minimal residual disease for P-glycoprotein and multidrug resistance protein activity in acute myeloid leukemia. Leukemia 2001;15:1554-63.
10. Feller N, Schuurhuis GJ, van der Pol MA, Westra G, Weijers GW, van Stijn A, et al. High percentage of CD34-positive cells in autologous AML peripheral blood stem cell products reflects inadequate in vivo purging and low chemotherapeutic toxicity in a subgroup of patients with poor clinical outcome. Leukemia 2003;17:68-75.
11. Feller N, Jansen-van der Weide MC, van der Pol MA, Westra GA, Ossenkoppele GJ, Schuurhuis GJ. Purging of peripheral blood stem cell transplants in AML: a predictive model based on minimal residual disease burden. Exp Hematol 2005; 33:120-30.
12. Cloos J, Goemans BF, Hess CJ, van Oostveen JW, Waisfisz Q, Corthals S, et al. Stability and prognostic influence of FLT3 mutations in paired initial and relapsed AML samples. Leukemia 2006;20:1217-20.
13. Goemans BF, Zwaan CM, Martinelli S, Harrell P, de Lange D, Carta C, et al. Differences in the prevalence of PTPN11 mutations in FAB M5 paediatric acute myeloid leukaemia. Br J Haematol 2005;130:801-3.
14. Goemans BF, Zwaan CM, Miller M, Zimmermann M, Harlow A, Meshinchi S, et al. Mutations in KIT and RAS are frequent events in pediatric core-binding factor acute myeloid leukemia. Leukemia 2005; 19:1536-42.
15. Lacombe F, Durrieu F, Briais A, Dumain P, Belloc F, Bascans E, et al. Flow cytometry CD45 gating for immunophenotyping of acute myeloid leukemia. Leukemia 1997; 11:1878-86.
16. Thiede C, Steudel C, Mohr B, Schaich M, Schäkel 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.
17. Tiesmeier J, Müller-Tidow C, Westermann A, Czwalinna A, Hoffmann M, Krauter J, et al. Evolution of FLT3-ITD and D835 activating point mutations in relapsing acute myeloid leukemia and response to salvage therapy. Leuk Res 2004;28:1069-74.
18. van Stijn A, Feller N, Kok A, van der Pol MA, Ossenkoppele GJ, Schuurhuis GJ. Minimal residual disease in acute myeloid leukemia is predicted by an apoptosis-resistant protein profile at diagnosis. Clin Cancer Res 2005;11:2540-6.
19. van der Pol MA, Feller N, Ossenkoppele GJ, Weijers GW, Westra AH, van Stijn A, et al. Minimal residual disease in acute myeloid leukemia is predicted by P-glycoprotein activity but not by multidrug resistance protein activity at diagnosis. Leukemia 2003;17: 1674-7.
20. van Rhenen A, Feller N, Kelder A, Westra AH, Rombouts E, Zweegman S, et al. High stem cell frequency in acute myeloid leukemia at diagnosis predicts high minimal residual disease and poor survival. Clin Cancer Res 2005;11: 6520-7.