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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 AML enter complete remission and at least half of these remission patients experience relapse.

Improvement of therapies is likely to come from increased duration of time to relapse especially for younger patients.

With the vastly increasing number of targeted therapies there is a strong need for short-term endpoints to efficiently test such therapies for further pursuance.

Objective

Minimal residual disease (MRD) assessment may offer such endpoint since it is a strong independent prognostic factor. As proof of principle we have examined this concept for FLT3-ITD status at diagnosis.

Design and methods

We determined FLT3-ITD status in bone marrow (BM) and periferal blood (PB) samples of 196 newly diagnosed AML patients. Frequencies of MRD cells of these 196 patients were assessed in 267 follow up BM and PB samples using immunophenotypically assessed MRD.

Results

Median MRD cell frequency after the first cycle of chemotherapy was 8.5 fold higher in patients with FLT3-ITD mutations compared to wild type. Such 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 enclosed in analysis.

Interpretation and conclusions

This study opens the possibility to study the efficacy of FLT3 inhibitors with MRD cell frequency as short-term endpoint.

INTRODUCTION

At present treatment of AML in adults is based on the assessment at diagnosis of a limited number of prognostic factors including cytogenetic analysis.
From a genetic point of view AML originates from a large diversity of acquired somatic mutations.
Development of AML is now considered a two-step process: class 2 mutations like 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 to the development of molecules that directly target these specific mutations or downstream targets in their disrupted signal transduction pathways. Numerous clinical trials using small molecule inhibitors, such as FLT3 inhibitors, additional to conventional chemotherapy have been initiated[1,2].
In order to have an effective clinical trial design for the large variety of modulating agents and combinations with others including classical chemotherapeutic agents, early efficacy read out parameters are obliged.
Until now, achieving complete remission, remission duration and overall survival have been used for that purpose. In the light of clone selection, shifts of (stem) cell subpopulations and multiple hit models, an earlier read out is desirable.
Outgrowth of minimal residual disease (MRD) cells has previously been indicated to be responsible for emergence of relapse[3]. Moreover frequencies of MRD cells in BM, characterized immunophenotypically by the presence of an aberrant phenotype, were shown to have prognostic impact after induction and intensification therapy [4,5,6 7.]
In this study we propose the use of frequencies of MRD cells after first cycle of chemotherapy as earlier read out parameter in future clinical trials using FLT3 inhibitors. For that purpose the relationship between the presence of FLT3-ITDs with MRD cell frequency was studied. In these analysis the presence of class 1 mutations other than ITDs in exon 14 of the FLT3 gene (NPM1, KIT, NRAS, KRAS, FLT3-exon 20 and PTPN11) was accounted for.

MATERIAL AND METHODS

Patient samples

Bone marrow (BM) aspirates, periferal blood (PB) 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 AML was established on 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 were treated according to HOVON 29 (during 1998-2000) and HOVON 42 (during 2001-2006). Patients >60 years were treated according to the HOVON 32 protocol (during 1996-2000) or according to the HOVON 43 protocol (during 2000-2006).
All follow-up samples were obtained after full hematological recovery of the bone marrow. Hematological recovery was defined as the time point were the white blood

cell count (WBC) >1.0*109/L, granulocytes >0.5*109/L and platelets >100*109/L. Complete remission (CR) was determined morphologically by the presence of less than 5% blasts in the bone marrow, combined with a 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 (OS) was defined as time interval between inclusion and death, relapse free survival (RFS) as the time interval between achievement of CR and relapse, disease free survival (DFS) was defined by the time interval between diagnosis and the first event (relapse or death either or not disease related) for patients who had achieved CR.

Establishment of LAP

MRD cell frequencies were determined using Leukemia Associated Phenotypes (LAPs). LAPs were established on the blasts at diagnosis applying a double step immunophenotypic labeling procedure using FITC-, PE-, PerCP- and APC-conjugated monoclonal antibodies (Moab's) as previously described[7,9,10].
In normal bone marrow these LAPs are per 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 in account when the frequency of LAP positive leukemic blasts in a follow-up samples is calculated. Corresponding isotype controls were included to test specificity of staining for all markers. In the present cohort every patient displayed one or more LAPs, thus enabling MRD detection during follow-up.

MRD patient group, MRD cell frequency and blast reduction

Since the association between MRD cell frequencies and FLT3-ITD status was the main focus of this paper, we restricted the description of clinical characteristics to the 196 patients displaying an aberrant immunophenotype at their blasts at diagnosis, which enabled MRD assessment in follow-up samples.
Details are shown in the second column of table 1. CR was achieved in 157 out of 196 patients; 128/157 after 1st cycle, 23/157 after 2nd cycle and 1/157 after 3rd cycle of consolidation chemotherapy. For 3 patients CR achievement in relation to chemotherapy cycle is unknown. Sixteen patients did not achieve CR after receiving two cycles of induction chemotherapy. Twenty patients experienced early/toxic death.
In table 1, all characteristics are subspecified for the patients included after the different MRD assessment times e.g. after 1st and 2nd induction chemotherapy and after consolidation treatment.
From the 196 patients 179 BM and 17 PB samples were obtained at diagnosis. In patients were periferal blood was used for LAP assessment the blast percentage was on average 57% (range 4.6-94%). During follow up 388 BM samples were obtained at different time points, 175 after 1st cycle, 120 after 2nd and 93 after 3rd cycle of chemotherapy. The total MRD study cohort thus included 196 patients, of whom 157 (80%) achieved morphological CR, (table 1).
By definition the term MRD cell frequency refers to those patients who achieved complete remission (n=157). Based on this selection, MRD cell frequencies were determined after 1st, 2nd and 3rd cycle of chemotherapy in respectively, 107/175, 95/120 and 60/93 samples.

Table 1. Patient characteristics

	Total population			MRD assessment after:		
		FLT3 WT	FLT3 mut	1st cycle	2nd cycle	3rd cycle
No.	196	138	36	111	96	60
Median WBC x 10^9/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 yrs	131	91	24	81	74	49
Age ≥60 yrs	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
3rd 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,
MDS; myelodysplastic syndrome; CR, complete remission

Sample preparation
Genomic DNA or RNA was isolated from quick 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) according to the manufacturer's recommendations.
PCR primers and conditions for FLT3, NPM1, KIT, NRAS, KRAS, FLT3-exon 20 and PTPN11 mutation analysis were previously described[12-14] and are available on request.

Statistics
MRD cell frequency was assessed both as continuous variable and by applying cut-offs, also as dichotomous variable. Cut-offs that were applied were median MRD value, and various cut-offs on bases of optimized Hazard Rates (HR).
Kaplan-Meier survival curves were constructed for patient subgroups based on MRD cut-offs.
The present study included patients with an immunophenotypically defined (using CD45 blast cell gating15) MRD cell frequency of <5% blasts (table 1). In 2 cases this contrasted with morphologically defined CR status.
The relationship between MRD cell frequency and OS, RFS and DFS and also between FLT3 status and OS, RFS and DFS were estimated for patients that were included in the MRD analysis.
For multivariate analysis of prognostic factors Cox proportional hazard model was used. Clinical pathological factors assessed in multivariate analysis include FAB-class (categorical variable), cytogenetics (categorical variable) and WBC at diagnosis (expressed as logarithm and applied as continuous variable).
Treatment protocol was included as dichotomous variable: 2 protocols for younger patients <60 years of age and 2 protocols for elderly patients ≥60 years of age. Age related treatment protocols are dealt with separately since they have an elementary different design.
Type of consolidation treatment was assessed as categorical variable, including 4 categories; third cycle of chemotherapy, allogeneic transplantation, autologous transplantation and no consolidation treatment.
Distribution of the clinical variables was compared using the Chi-square analysis for categorical variables and the non-parametric Mann Whitney U test for continuous variables.
Multivariate analysis on the impact of multiple mutations on MRD cell frequencies was estimated either by linear regression (in the case that MRD was studied as continuous variable) or by logistic regression (when MRD cell frequency was studied as dichotomous variable).
Patients still in remission were censored at the time of last follow-up.
The reported p-values are two-sided and p<.05 was considered significant in all analyses. Calculations were performed using the SPSS software package version 11.0.1 (SPSS, Chicago, IL).

RESULTS

FLT3-ITD status and survival
In total 265 samples from the 288 newly diagnosed AML patients were successfully analyzed for ITDs in exon 14 of the FLT3 gene.

FLT3-ITDs were found in 61/265 patients (23%). Failure of mutation analysis (23 patients) was either due to shortage of cells or low DNA yields.
The percentage of FLT3-ITDs in the total population (n=265) was comparable to those observed in the subselections of patients included in MRD assessment studies in the total MRD group (n=196), after 1st (n=107) 2nd (n=95) and 3rd (n=60) cycle of chemotherapy, where ITDs were observed in 20.7%, 16.7%, 23.1% and 18.6% of the patients, respectively.
Assessment of the distribution of clinical characteristics across FLT3-ITD (n=36) and FLT3-wt (n=138) patients 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). In the calculations the unknown cases were excluded. No significant differences were found for most FAB types, age related treatment protocol and type of consolidation regimen.
Trends were visible for percentage of patients diagnosed as RAEB-t or AML secondary to MDS (13.0% in the FLT3-wt compared to 3.0% in the FLT3-ITD group) and FAB class M5 (present in 30.3% of the patients in the FLT3-ITD compared to 19.8% in the FLT3-wt group)
In the total MRD patient population (n=196) in which FLT3-ITD status was evaluated (n=171), a FLT3-ITD was associated with shorter OS (HR 1.7; 95%CI 1.06-2.72; p=.027), RFS (HR 2.1; 95%CI 1.16-3.62; p=.013) and DFS (HR 1.9; 95%CI 1.13-3.21; p=.015).
The adverse prognostic impact of an ITD in the FLT3 gene on OS, RFS and DFS was maintained qua Hazard Ratio, though less significant due to lower numbers of patients, in the patient subpopulations studied after the different courses of chemotherapy (1st HR 1.6/1.9/1.9; p=.09/.05/.03; 2nd HR 1.7/2.0/1.9; p=.11/.05/.05, 3rd HR 1.7/1.6/1.9; p=.13/.14/.05).

MRD cell frequency and survival
In agreement with our previous experiences, MRD cell frequency in samples received from 107 patients after first cycle of induction chemotherapy predicted both OS (HR1.2; 95%CI 1.09-1.32; p<.0001), RFS (1.6; 95%CI 1.15-2.31; p=.0006) and DFS (HR1.2; 95%CI 1.08-1.34; p=.001).
The optimal cut-off in MRD cell frequency of 1.0% in the present patient cohort revealed a 3.7 fold and 4.1 fold higher risk on shorter OS (95%CI 1.63-8.30; p=.002) and RFS (95% CI 1.50-10.94; p=.006), for patients with MRD cell frequencies of >1.0%.
The optimal cut-off of 1% remained significant in the sub-group of younger patients (p=0.02) and borderline significant in the sub-group of elderly patients (p=0.08).
After 2nd cycle of chemotherapy (including 96 patients) MRD cell frequency as continuous variable was also highly predictive for patient OS, RFS and DFS (HR 1.23; 95%CI 1.059-1.431; p=.007, HR 1.20; 95%CI 1.035-1.392; p=.016 and HR 1.17; 95%CI 1.033-1.340; p=.014).
The optimal cut-off 0.8% remained significant in the younger as well as in the elderly patients (p<.0001 and p=.024).
In the 60 patients that were assessed after 3rd cycle of chemotherapy, a higher MRD cell frequency also correlated with impaired patient survival for OS (HR 1.42; 95%CI 1.11-1.81; p=.005), for RFS (HR 1.36; 95%CI 1.08-1.71; p=.010) and for DFS (HR1.43; 95%CI 1.13-1.81; p=.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=.01) though not for the elderly patients (p=.31) as could be expected on basis of the low number (n=11).

FLT3-ITD status and MRD cell frequency

FLT3-ITD status was not associated with remission rate (p=.55). In the FLT3-ITD group 28/30 (table 1, column 4) achieved CR, while in the FLT3-wt group 114/126 (table 1, column 3) did so.

However, in patients entering CR, FLT3-ITD status was strongly associated with MRD cell frequency after 1st cycle of chemotherapy (n=96, p=.002). After second course of chemotherapy the presence of an FLT3-ITD remained associated with higher MRD cell frequency although much less pronounced (n=91, p=.082). After third cycle of chemotherapy the association was lost (n=59, p=.69).

Median MRD cell frequencies in the total patient population drop during consecutive chemotherapy cycles (median MRD cell frequency after 1st 2nd and 3rd cycle are 0.06, 0.04 and 0.03, respectively). Since 0.01% is the lowest detectable MRD cell frequency for immunophenotypical 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 will reduce further after additional cycles of chemotherapy.

Survival effects of MRD cell frequency in FLT3-ITD and FLT3-wt patients

The effect of MRD frequencies and their cut-offs patients on overall survival (OS) and relapse free survival (RFS) both for FLT3-ITD and wt patients, are displayed in figure 1. The distribution of MRD cell frequencies in FLT3-ITD and wild type samples after different cycles of chemotherapy is shown in figure 2.

The results obtained for MRD after the first course of chemotherapy were analysed in more detail: median MRD cell frequencies in the two groups differ by a factor 8.5: MRD% 0.34 (FLT3-ITD) versus 0.04 (FLT3-wt). Significantly more wt patients are found below the median cut-off for the ITD group of 0.34% compared to above the cut-off (41/70 patients; Chi-square 4.401). Similarly, significantly more ITD cases are found above the median cut-off for the wt patients of 0.04% (15/19 patients; Chi square 4.436). The 8.5 fold difference in MRD% translates into difference in median survival: of the total patient population; median OS and RFS of patients with MRD % of 0.01 (lowest detectable MRD%) was, respectively 10.7 and 11.1 months, while median OS and RFS 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 holds true for other 8.5 fold differences in the ranges of MRD cell frequencies. This shows that the use of an inhibitor of tyrosine kinase activity potentially translates not only into large differences in MRD cell frequency, but also in survival differences.

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

Poor response to therapy in part of the FLT3-wt group, among other factors, may result from other mutations.

Although none of the investigated mutations other than FLT3-ITD had significant impact on either OS, RFS or DFS 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 OS (p=.03 chi square 7.04 and p=.03 chi square 7.32, respectively).

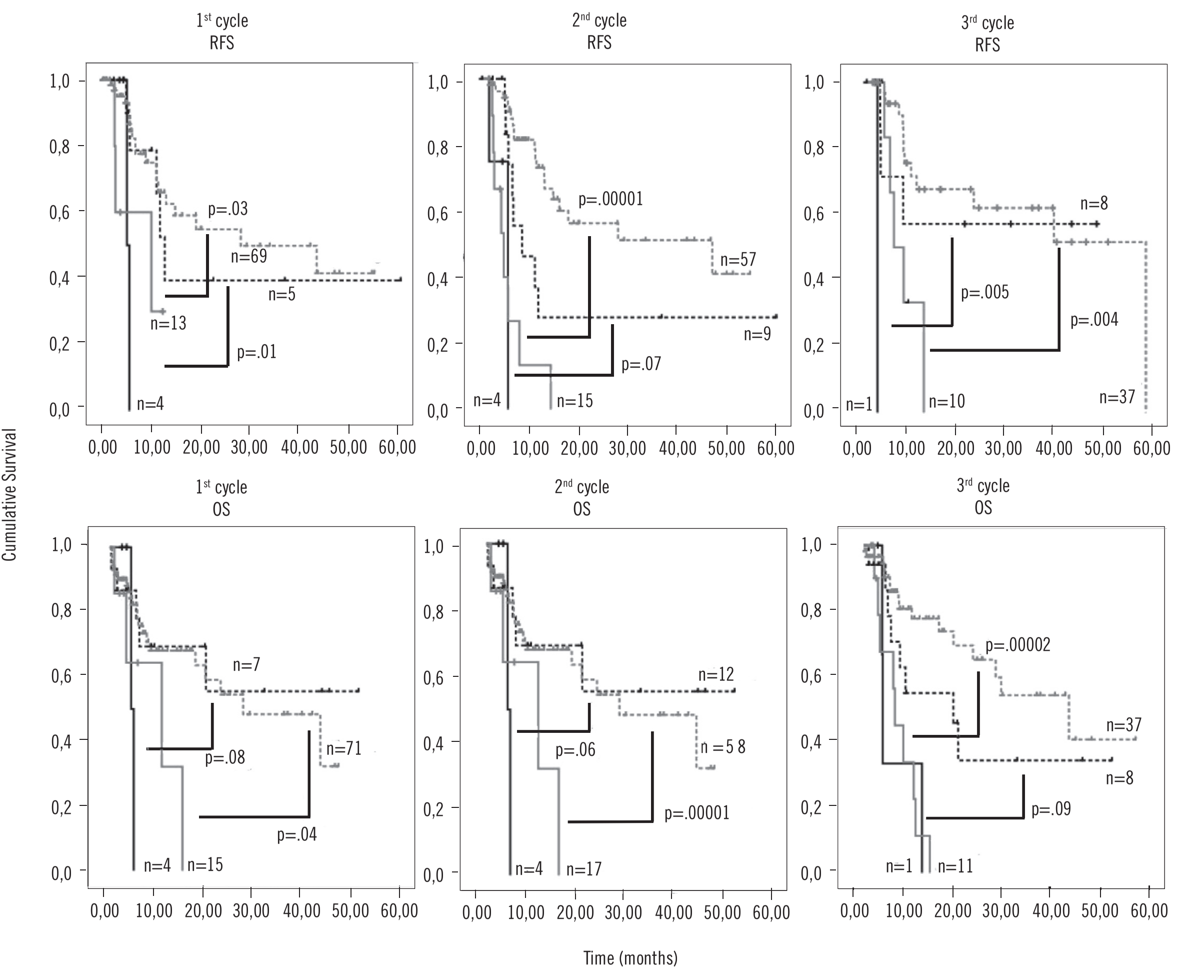

Figure 1. RFS and OS in FLT3-ITD and FLT3-wt patients based on chemotherapy cycle specific cut-offs in MRD cell frequency.
For each cycle of chemotherapy in the whole patient group (FLT3-ITD + wt) a cut-off in MRD frequency was determined that is most distinguishing for patient survival (1.0 after 1st cycle, 0.8 after 2nd cycle and 0.4 after 3rd cycle). Kaplan Meier curves were constructed from these. Effects of chemotherapy cycle specific cut-off in MRD frequency for Overall Survival (OS) and Relapse Free Survival (RFS) both in FLT3-ITD and wt patients. Both FLT3-wt and FLT3-ITD patients with MRD frequencies higher than the cycle specific cut-off, have 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,
Gray straight line: FLT3-wt patients with MRD frequencies above the cut-off,
Gray dotted line: FLT3-wt patients with MRD frequencies below the cut-off.

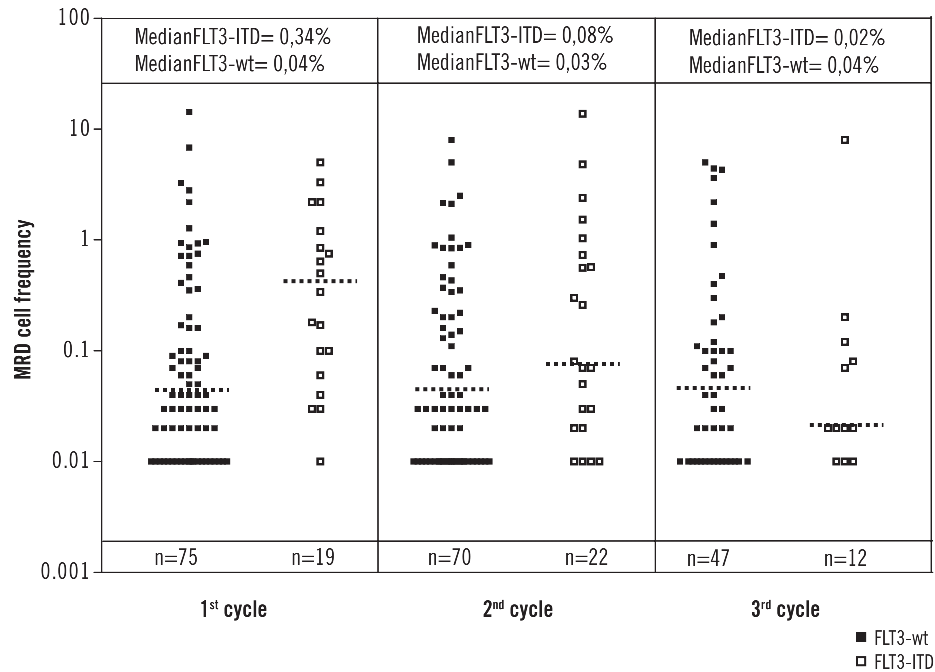

Figure 2. Distribution of MRD frequencies in FLT3-ITD and wild type samples
Dot plots show the distribution of MRD cell frequencies (y-axis) in FLT3-ITD (solid black squares) and FLT3-wt patients (open black squares) in morphological complete remission after first, second and third cycle of chemotherapy (x-axis). The median MRD frequencies in FLT3-ITD and FLT3-wt patients after first cycle of chemotherapy differ by a factor 8.5: 0.34% in the FLT3-ITD patients versus 0.04% in the FLT3-wt patients. For the 2nd and 3rd cycle these figures are 0,08%/0,03% and 0,02%/0,04%, respectively. These differences in median MRD frequencies between FLT3-ITD and FLT3-wt patients were significant after 1st and 2nd cycle of chemotherapy (p=0.05).
Median MRD frequencies for FLT3/ITD patients after the 2nd cycle have dropped with a factor 4.25 compared to the first cycle and after the 3rd cycle with a factor 4.0 compared to 2nd cycle. These figures were a factor 1,3 and 0,75, respectively for FLT3-wt patients.

In case of KRAS this contribution is possibly associated with achieving of CR in the FLT3-wt patients, whereas PTPN11 was found associated with early death (table 2). NPM1 mutations were more frequently observed in the FLT3-ITD patients (table 2). In agreement of the favorable prognosis of NPM1 mutations in the FLT3-wt group16, we observed a lower MRD percentage in FLT3-wt patients with NPM1 mutations compared to FLT3-wt patients without NPM1 mutations (respectively 0.015 and 0.06). In FLT3-ITD patients, the presence NPM1 mutations is associated with a higher MRD percentage; 1.20 versus 0.18 in NPM1 wild type patients.
In the total patient population after 1st cycle of chemotherapy, the presence of an NPM1 mutation was associated with a lower median MRD percentage; 0.035 versus 0.07 in NPM1 wild type patients.

DISCUSSION

In the present paper we show that the presence of FLT3-ITD at diagnosis AML, compared to wild type patients is associated with a 8.5 fold higher MRD cell frequency after the first course of chemotherapy.
These differences translate into differences in overall survival, relapse free survival and disease free survival.
Partial or complete inhibition of the aberrations resulting from the ITDs would thus translate into significant survival differences.
Since the effects were very pronounced after the first cycle of chemotherapy, the MRD cell percentage after the first therapy cycle offer a fast read-out endpoint to assess the efficacy of FLT3 inhibitors in terms of survival.
From a theoretical point of view, a higher MRD cell frequency in the FLT3-ITD patients after first cycle of chemotherapy should result in a higher MRD cell frequency after second and third cycle 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 flowcytometric MRD detection limit (0.01): after the second and third cycle in many cases this detection limit has been reached, not allowing further discrimination between patients.
This loss in ability to distinguish is not insuperable, however, since first cycle of chemotherapy offers the earliest and thereby most favorable point in time.
Since the association between MRD cell frequency and treatment efficacy is not limited to flowcytometry, MRD cell frequencies after second and third cycle may function as proper read outs for targeted inhibitors in AML in future, provided sensitive molecular techniques will be available for the majority of patients.
In general, each course of chemotherapy reduces the amount of MRD in the total group[5,6]: in the present study median MRD cell frequency values were 0.06, 0.04 and 0.03 after first, second and third cycle, respectively.
However, figure 2 shows the high impact of the second and also the third cycle for the subgroup of patients with FLT3-ITDs: a 4.25 and 4 fold further reduction (total 17 fold) after the two extra courses.
In the wild type group 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 large benefit from the subsequent therapy cycles.
For final prove of MRD cell frequency as short term read out for targeted treatment 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 wt patients as a result of nonspecific effects.

Clinical studies that randomize all patients between the use of an inhibitor versus no inhibitor would offer the possibility to assess the effectiveness both in patients with wild type FLT3 and FLT3-ITD.
One of the major challenges and ultimate goals for application of existing and new therapies is to enable prediction of response and survival for individual patients.
In the area of overlap between MRD cell frequencies between wild type and ITD cases (figure 2) this translates into two questions; 1. what is the cause of poor responses in part of the patients with wild type FLT3 and 2. why do part of FLT3-ITD patients perform quite well?

Table 2. Distribution of mutations specified for complete remission (CR) and FLT3 status in patients after 1st cycle of chemotherapy

	CR=1 (n=138)	CR=1 FLT3-mut (n=24)	CR=1 FLT3-wt (n=99)	CR=0 (n=16)	Early death (n=19)
FLT3 status at diagnosis					
WT	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					
WT	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
CKIT status at diagnosis					
WT	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					
WT	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					
WT	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					
WT	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 CR; CR=0: not in CR

With regard to the first question an intriguing possibility would be that, irrespective of cellular characteristics in part of any AML patient group pharmacokinetic resistance appears to overrule cellular resistance (Feller Leukemia 2003). For the present study it means that in the wild type group, but also in the ITD group high MRD may in part have resulted from pharmacokinetic resistance.
Apart from that 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-ITDs in patients who were wild type 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 already manifest during the first cycle of therapy is presently under investigation.

As common themes take place in transduction and transcription pathways that are associated with malignant transformation of the hematopoietic stem cell, levels of MRD cells may as well be implicative for the efficacy of inhibitors that target mutations other than FLT3-ITDs.
Keeping in mind that MRD cell frequency is a very strong independent prognostic factor, it is likely that the present approach may also be applicable for other mutations. In fact, up to now we have shown that other diagnosis factors that have prognostic impact in terms of survival, i.e. apoptosis related protein expression[18], P-glycoprotein activity[19] and stem cell frequency20 translate into differences in MRD cell frequency.
These factors may explain why part of FLT3-wt patients have poor survival. For the FLT3-ITD patients the additional effects of other mutations are less pronounced since firstly most of the additional mutations are seen in the FLT3-wt patients. Also the additional mutations are more or less scattered over the FLT3-ITD patient group irrespective of MRD cell frequency.
Overall, this study reveals excellent correlations between FLT3-ITD status, MRD frequency and patient survival, which enables rapid evaluation of FLT3 inhibitor efficacy in a clinical setting.

AUTHORSHIP STATEMENTS

C.J.H, G.J.S, G.J.O and Q.W designed the research and wrote the manuscript. F.D, C.J.H and P.A.M analysed the data. M.C.H and A.H performed the mutation analysis. A.K and N.F designed and performed the MRD cell frequency analysis. J.B assisted in the statistical analysis.

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