Gene mutations and response to treatment with all-*trans* retinoic acid in elderly patients with acute myeloid leukemia. Results from the AMLSG Trial AML HD98B

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ABSTRACT

Background

In a previous randomized trial, AML HD98B, we showed that administration of all-*trans* retinoic acid in addition to intensive chemotherapy improved the outcome of older patients with acute myeloid leukemia. The objectives of this study were to evaluate the prognostic impact of gene mutations and to identify predictive genetic factors for the all-*trans* retinoic acid treatment effect.

Design and Methods

Data from mutation analyses of the *NPM1*, *CEBPA*, *FLT3*, and *MLL* genes were correlated with outcome in patients 61 years and older treated within the AML HD98B trial.

Results

The frequencies of mutations were: *NPM1*, 23%; *CEBPA*, 8.5% (analysis restricted to patients with a normal karyotype); *FLT3* internal tandem duplications (ITD), 17%; *FLT3* tyrosine kinase domain mutations, 5%; and *MLL* partial tandem duplications, 4.5%. The genotype mutant *NPM1* was positively and adverse cytogenetics as well as higher white blood cell count negatively correlated with achievement of complete remission. In Cox regression analysis, a significant interaction between the genotype mutant *NPM1* without *FLT3*-ITD and treatment with all-*trans* retinoic acid was identified, in that the beneficial effect of all-*trans* retinoic acid on relapse-free and overall survival was restricted to this subgroup of patients. Other significant factors for survival were age, adverse cytogenetics, and logarithm of white cell count.

Conclusions

In elderly patients with acute myeloid leukemia, *NPM1* mutations are associated with achievement of complete remission, and the genotype 'mutant *NPM1* without *FLT3*-ITD' appears to be a predictive marker for response to all-trans retinoic acid given as an adjunct to intensive chemotherapy *(ClinicalTrials.gov Identifier: NCT00151242)*.

Key words: acute myeloid leukemia, all-*trans* retinoic acid, nucleophosmin-1 mutation, predictive factor.

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Introduction

There has been a long-standing interest in the clinical use of all-*trans* retinoic acid (ATRA) in the treatment of acute myeloid leukemia (AML) subtypes other than acute promyelocytic leukemia (APL). Based on promising *in vitro* data,[1-6] several clinical trials evaluated ATRA in combination with intensive chemotherapy in non-APL AML.[7-12] Initial encouraging data came from a phase II trial combining low-dose cytarabine with ATRA in 33 patients ineligible for intensive therapy.[7] However, the results from subsequent randomized studies have been contradictory, with the majority reporting negative results. Estey *et al*. studied 215 patients older than 71 years with high-risk myelodysplastic syndrome or AML.[8] Although no effect of ATRA could be shown in multivariable analysis, univariate analysis revealed significantly better overall survival in the treatment arms containing ATRA. The British Medical Research Council (MRC) performed three randomized trials, one in younger patients receiving first-line treatment (MRC AML12, n=1097),[9] one in medically unfit patients (MRC AML14, n=207),[10] and one in high-risk, refractory, or relapsed patients (MRC AML-HR, n=362).[11] In none of these trials was there a significant effect of ATRA on any end-point analyzed. In contrast, in our trial of AML patients aged 61 years and older (AML HD98B, n=242), patients randomized to the ATRA arm had a significantly higher complete remission rate and better event-free and overall survival.[12] We hypothesized that the beneficial effect of ATRA may be restricted to a specific biological subgroup of AML.

Recently, somatic mutations in the *NPM1*, *CEBPA*, *FLT3*, and *MLL* genes have been identified in AML. In younger adult patients, these mutations have been shown to be of prognostic and predictive relevance.[13,14] In particular, the genotypes 'mutant *NPM1* without *FLT3*-ITD' and 'mutant *CEBPA*' have emerged as significant factors for achievement of complete remission as well as better relapse-free survival and overall survival. To date, little is known about the impact of these gene mutations in older patients with AML.

The objectives of this study were to evaluate the association of mutations in *NPM1*, *CEBPA*, *FLT3*, and *MLL* with clinical outcomes in the patients who had been entered into the treatment trial AML HD98B of the German-Austrian AML Study Group (AMLSG),[12,15,16] and to identify predictive factors for the beneficial treatment effect of ATRA.

Design and Methods

Patients and treatment

Between March 1998 and July 2004, 377 patients were prospectively enrolled into the AMLSG AML HD98B treatment trial.[12,15,16]

Patients 61 years or older with *de novo* AML or refractory anemia with excess of blasts in transformation as defined by the French–American–British classification system,[17] secondary AML with a preceding history of myelodysplasia of at least 3 months before the diagnosis of AML, or therapy-related AML following treatment of a primary malignancy were eligible for the trial. Patients with APL were excluded. Details of the treatment plan have already been published.[12,16] Briefly, patients received two induction cycles of idarubicin, standard-dose cytarabine, and etoposide with or without ATRA, followed by one consolidation cycle of intermediate-dose cytarabine and mitoxantrone with or without ATRA. During induction therapy, ATRA was given at a dose of 45 mg/m^2 on days 3 through 5, and at 15 mg/m^2 on days 6 through 28. During first consolidation, ATRA was given at 15 mg/m^2 on days 3 through 28. For second consolidation therapy, patients were randomized to either intensive therapy with idarubicin and etoposide or 12 monthly courses of outpatient maintenance therapy with the same agents given orally.[16] Allogeneic hematopoietic stem-cell transplantation was allowed for patients with an HLA-identical family donor at the discretion of the local investigator. For conditioning, a combination of fludarabine, cyclophosphamide, idarubicin, and etoposide was recommended.[18]

Following a pilot phase that included 19 patients, 242 patients were randomized for ATRA, 122 into the experimental arm and 120 into the standard arm. The planned interim analysis in 2001 revealed a trend for a better complete remission rate in the experimental arm. However, the difference was not statistically significant and, according to protocol, randomization for ATRA was stopped. An additional 116 patients were assigned to the standard arm to achieve the sample size required for second randomization. A diagram summarizing the randomized patient population is provided in the original report.[12]

Written informed consent was obtained from all patients at study entry. The study was approved by the local Ethics Review Committees of the participating institutions.

Cytogenetic and molecular genetic analysis

Leukemia samples were studied centrally in the Laboratory for Cytogenetic and Molecular Diagnostics at the University Hospital of Ulm. Data from conventional chromosome analysis were available for 316 of the 377 (84%) patients and have been published elsewhere.[15] To improve diagnostic accuracy, all specimens were also analyzed by fluorescence *in situ* hybridization using a DNA probe set for the detection of the following AML-associated cytogenetic aberrations: inv(3)/t(3;3), t(8;21), t(9;22), t(11q23), t(15;17), inv(16)/t(16;16), +4q, del(5q), del(7q), +8q, +11q, abn(12p), del(13q)/+13q, del(17p), del(20q), +21q, +22q, and del(Xq).[19]

For the present study, diagnostic samples were analyzed for mutations in the *NPM1, CEBPA, FLT3* (internal tandem duplication [*FLT3*-ITD] and tyrosine kinase domain mutations at codons D835 and I836 [*FLT3*-TKD]), and *MLL* (partial tandem duplication [*MLL*-PTD]) genes using previously reported methods.[14,20-23] *CEBPA* mutation analysis was restricted to patients with a normal karyotype.

Criteria for treatment outcomes

Response to induction therapy was assessed after two courses of chemotherapy. In accordance with standard criteria,[24] complete remission was defined as less than 5% bone marrow blasts, an absolute neutrophil count of 1.5×10^9/L or more, a platelet count of 100×10^9/L or more, no blasts in the peripheral blood, and no extramedullary leukemia. Therapeutic failures were classified as either refractory disease or early/hypoplastic death (death less than 7 days after completion of the first course of induction therapy/death during the remainder of double induction therapy). Relapse was defined as more than 5% bone marrow blasts unrelated to recovery from the preceding course of chemotherapy or new extramedullary leukemia in patients with previously documented complete remission. End-points for overall survival, measured from the date of study entry, were death (failure) and alive at last follow-up (censored). End-points for relapse-free survival, measured from the date of achievement of complete remission, were death in complete remission or relapse (failure) and alive in complete remission at last follow-up (censored).

Statistical analyses

The median duration of follow-up was calculated according to the method of Korn.[25] Analyses in relation to clinical outcome were restricted to the upfront randomized patients according to their randomization. A logistic regression model was used to analyze associations between presenting features as well as treatment with ATRA and achievement of complete remission. The Kaplan-Meier method was used to estimate the distribution of overall survival; confidence interval (CI) estimation was based on the cumulative hazard using Greenwood's formula for standard error (SE) estimation. Survival distributions were compared using the log-rank test. A Cox model was used to identify prognostic variables. In addition to the molecular markers (*NPM1, MLL, FLT3*-ITD, and *FLT3*-TKD mutations), cytogenetics, age, white blood cell count (WBC), platelet count, bone marrow blast count, presence or absence of hepato-splenomegaly, and type of AML were added as explanatory variables. In multivariable models cytogenetics was categorized into three groups, core binding factor (CBF), normal karyotype and all other aberrations into the group of adverse cytogenetics. For multivariable analyses, we performed a missing value imputation in the subset of patients with at least one molecular marker analyzed.[26] The frequency of missing data for the single co-variates was below 20%. We estimated missing data for co-variates by using 50 multiple imputations in chained equations incorporating predictive mean matching.[26] All statistical analyses were performed with the statistical software environment R, version 2.4.1, using the R package Design, version 2.0-12.[27] p values of less than 0.05 were considered to indicate statistical significance.

Table 1. Mutation frequencies of the *NPM1*, *MLL*, *CEBPA*, and *FLT3* genes in elderly acute myeloid leukemia patients.

Gene	Mutation frequency, n. of patients (%) All patients	Patients with a normal karyotype
NPM1	60/254 (24%)	53/124 (43%)
MLL-PTD	11/250 (4%)	4/106 (4%)
CEBPA	-	10/117 (8.5%)
FLT3-ITD	51/295 (17%)	31/130 (24%)
FLT3-TKD	13/279 (5%)	6/128 (5%)

PTD: partial tandem duplication; ITD: internal tandem duplication; TKD: tyrosine kinase domain mutation.

Results

Molecular markers and baseline characteristics

Molecular markers were analyzed in all available diagnostic peripheral blood and/or bone marrow samples (*NPM1*, n=252; *CEBPA*, n=117 [restricted to normal karyotype AML]; *FLT3*-ITD, n=295; *FLT3*-TKD, n=279; *MLL*-PTD, n=250). The mutation status of all four genes (excluding *CEBPA*) was available for 206 patients (55%), and at least one marker could be analyzed in 302 of the 377 (80%) patients.

The frequencies of the mutations for all patients and for the subgroup of patients with cytogenetically normal AML are given in Table 1. *NPM1* mutations and *FLT3*-ITD were significantly more frequent in cytogenetically normal AML ($p<0.0001$ and $p=0.005$, respectively), whereas no such association was found for *FLT3*-TKD ($p=0.50$) or *MLL*-PTD ($p=0.75$).

In cytogenetically normal AML, *MLL*-PTD showed no overlap with *NPM1* or *CEBPA* mutations, whereas three of ten patients with mutant *CEBPA* showed a concurrent *NPM1* mutation. Both types of *FLT3* mutations were more frequently associated with mutant *NPM1* (ITD, $p<0.0001$; TKD $p=0.04$), whereas no such association was present with *MLL*-PTD. Of ten patients with mutant *CEBPA*, two had a *FLT3*-ITD and none had a *FLT3*-TKD mutation.

Pretreatment characteristics of all patients and the subgroup of patients randomized for ATRA are given in Table 2.

Induction therapy

Of the 242 patients randomized for ATRA, 114 (47%) achieved a complete remission, 92 (38%) had refractory disease, and 36 (15%) died. Logistic regression analysis of patients for whom at least one molecular marker was analyzed (n=206) revealed that mutant *NPM1* (odds ratio [OR], 3.17; 95% CI, 1.37-7.35; $p=0.02$), logarithm of WBC (OR, 0.58; 95% CI, 0.35-0.96; $p=0.03$) and adverse cytogenetics (OR, 0.46; 95% CI, 0.21-0.99; $p=0.05$) were significantly associated with achievement of complete remission. The molecular markers *FLT3*-ITD, *FLT3*-ITD, and *MLL*-PTD, clinical characteristics such as age, platelet count, percentage of bone marrow blasts, and type of AML, as well randomization to ATRA had no significant impact.

Table 2. Presenting clinical and laboratory findings in elderly acute myeloid leukemia patients.

Characteristics	All patients for ATRA n=377	Patients randomized n=242
Sex (male/female)	206/171	128/114
FAB type (%)		
M0	16 (4)	13 (5.5)
M1	46 (12)	30 (12.5)
M2	78 (21)	47 (19.5)
M4	59 (16)	36 (15)
M5	24 (6)	20 (8)
M6	3 (1)	3 (1)
M7	2 (0.5)	1 (0.5)
s-AML	83 (22)	52 (21.5)
t-AML	37 (10)	27 (11)
Missing	29 (7.5)	13 (5.5)
Cytogenetics (%)		
inv(16)/t(16;16); t(8;21)	22 (6)	14 (6)
Normal karyotype	145 (38)	98 (40)
Others	149 (40)	91 (38)
Missing	61 (16)	39 (16)
Lymphadenopathy (%)	47/341 (14)	36/232 (16)
Hepato-splenomegaly (%)	169/315 (54)	121/219 (55)
Gingival hyperplasia (%)	14/352 (4)	8/225 (4)
	Median (range)	**Median (range)**
Age (years)	67 (61-84)	67 (61-84)
WBC count ($\times 10^9$/L)	6.75 (0.4-303)	6.0 (0.4-303)
Missing	n=15	n=2
Platelet count ($\times 10^9$/L)	58 (4-672)	61 (4-445)
Missing	n=15	n=2
Hemoglobin level (g/L)	91 (38-141)	93 (47-141)
Missing	n=15	n=2
Bone marrow blasts (%)	68 (0-100)	70 (0-100)
Missing	n=51	n=20
Peripheral blood blasts (%)	29 (0-100)	29 (0-100)
Missing	n=70	n=39

FAB: French-American-British; s-AML: AML after a preceding myelodysplastic syndrome; t-AML: AML after chemo- and/or radiation therapy; WBC: white blood cells.

Table 3. Cox proportional hazard models for relapse-free and overall survival on an as randomized basis.

Relapse-free survival	HR (95% CI)	p
[ATRA] x [mutant *NPM1* without *FLT3*-ITD][1]	0.27 (0.07-0.95)	0.04
Log_{10}(WBC)	1.98 (1.37-2.87)	0.0003
Overall survival	**HR (95% CI)**	**p**
[ATRA] x [mutant *NPM1* without *FLT3*-ITD][1]	0.28 (0.11-0.75)	0.01
Log_{10}(WBC)	1.33 (1.05-1.68)	0.02
Adverse cytogenetics[2]	1.62 (1.13-2.32)	0.009

HR: hazard ratio; CI: confidence interval; ATRA: all-trans retinoic acid; ITD: internal tandem duplication; WBC: white blood cell count; [1]Interaction term of the two factors [ATRA] and [mutant NPM1 without FLT3-ITD]; [2]adverse cytogenetics denotes karyotypes other than normal cytogenetics, inv(16)/t(16;16), and t(8;21).

Survival analyses according to treatment with all-trans *retinoic acid*

The median follow-up time for survival was 68.5 months. Patients randomized to ATRA had a significantly better relapse-free survival (p=0.006) and overall survival (p=0.003), with 4-year relapse-free survival and overall survival rates of 20.9% (95% CI, 12.5-30.8%) and 10.8% (95% CI, 6.1-17%), respectively, as compared to 4.8% (95% CI, 1.6-10.9%) and 5% (95% CI, 2-10%), respectively, in the standard treatment arm of the study.

Survival analyses according to molecular markers and treatment with all-trans *retinoic acid*

The evaluation of predictive factors for the beneficial treatment effect of ATRA was performed on an intention-to-treat basis. In univariable survival analyses, an interaction was found between genotypes and the effect of randomization to ATRA. A significant difference in both relapse-free and overall survival in favor of the ATRA arm was evident in patients with the genotype 'mutant *NPM1* without *FLT3*-ITD', whereas relapse-free and overall survival of patients with all other combinations of these two genetic markers were dismal, irrespective of whether they had been randomized to ATRA or not (Figure 1). For Cox regression analysis, we included an interaction term to account for the observed interaction between genotypes and randomization to ATRA. Cox regression models for relapse-free survival and overall survival, using the genotype 'mutant *NPM1* without *FLT3*-ITD' revealed a significant interaction between this marker constellation and randomization to ATRA. The hazard ratios and 95% CI of the significant factors are given in Table 3. Since both *NPM1* mutation and *FLT3*-ITD were significantly associated with cytogenetically normal AML, we also performed a univariable analysis in this subset of patients. Again, a significant difference in both relapse-free and overall survival in favor of the ATRA arm was evident in patients with the genotype 'mutant *NPM1* without *FLT3*-ITD' (Figure 2).

Discussion

In the randomized AML HD98B trial of the AMLSG, we previously showed that ATRA given after intensive chemotherapy significantly improved the outcome of older patients with non-APL AML.[12] Data from the current correlative study suggest that this effect was accounted for by patients whose leukemic cells harbor mutant *NPM1*, and, more specifically, exhibit the genotype 'mutant *NPM1* without *FLT3*-ITD'. Mutant *NPM1*, in addition to cytogenetics, emerged as a strong favorable prognostic factor for achievement of complete remission. The updated survival analyses reported here confirm our previous observation that ATRA significantly improved survival in older AML patients.[12] Randomized trials from three groups evaluating ATRA as an adjunct to intensive chemotherapy in non-APL

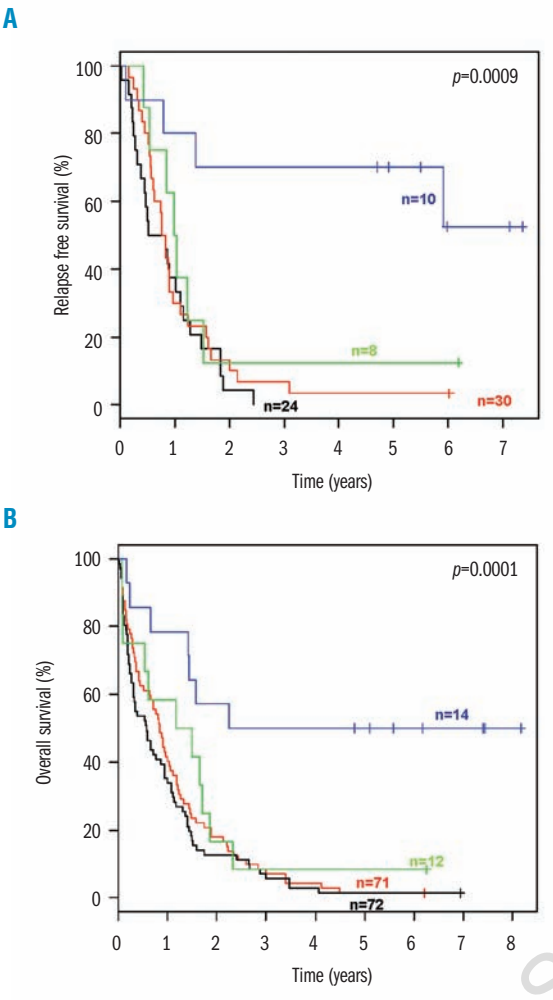

Figure 1. Relapse-free and overall survival according to treatment with all-*trans* retinoic acid (ATRA) and genotype on an intention-to-treat basis. (A) Relapse-free survival. (B) Overall survival. Blue, mutant *NPM1* without *FLT3*-ITD and treatment with ATRA; green, mutant *NPM1* without *FLT3*-ITD and no treatment with ATRA; red, all other combinations of the two markers and treatment with ATRA; black, all other combinations of the two markers and no treatment with ATRA.

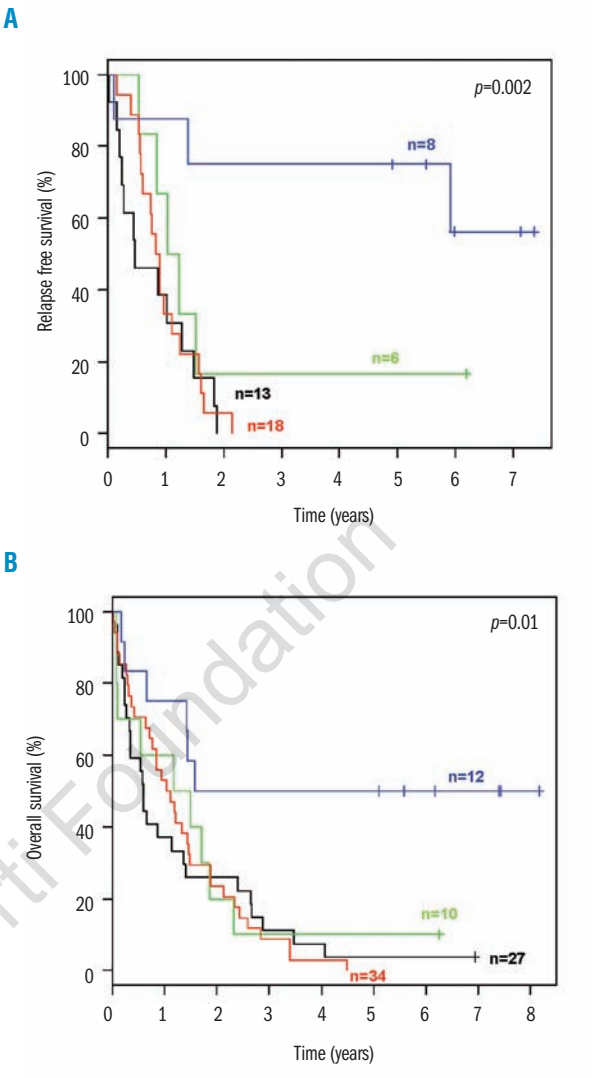

Figure 2. Relapse-free and overall survival according to treatment with all-*trans* retinoic acid (ATRA) and genotype in patients with cytogenetically normal karyotype on an intention-to-treat basis. (A) relapse-free survival. (B) Overall survival. Blue, mutant *NPM1* without *FLT3*-ITD and treatment with ATRA; green, mutant *NPM1* without *FLT3*-ITD and no treatment with ATRA; red, all other combinations of the two markers and treatment with ATRA; black, all other combinations of the two markers and no treatment with ATRA.

AML have been published.[8-12] However, in contrast to the positive results of our trial, the results of all other studies had been negative.[8-11] The selection of patients, the choice of the chemotherapeutic agents that were used in combination with ATRA, and the schedule of ATRA administration are variables that differed partly between the trials and thus might explain the discrepant results. In our view, one issue deserves special attention. Data from *in vitro* experiments with AML blasts have suggested that the addition of ATRA to cytotoxic agents, such as cytarabine or idarubicin, increases the killing of clonogenic cells. Importantly, these studies indicated that the schedule of ATRA administration may play a crucial role because synergistic effects on cell viability were only observed when ATRA was administered after exposure to the cytotoxic drug.[1-5] Consistent with these observations, Tabe *et al.* recently showed that pretreatment of the APL cell line NB4 with ATRA upregulates the transmembrane drug transporter ABCB1 (also known as MDR1) and induces doxorubicin resistance.[28] In the study by Estey *et al.*,[8] ATRA was started 2 days prior to chemotherapy, and in the MRC trials,[9-11] ATRA was started simultaneously with chemotherapy. In contrast, in the AML HD98B trial, ATRA was started on the third day of chemotherapy, a time point when a significant proportion of the cytotoxic drugs had already been administered.

In exploratory analyses, we were able to attribute the beneficial impact of ATRA on relapse-free survival and overall survival to a specific, genetically defined sub-

group of patients. Univariable and multivariable analyses pointed to an interaction between ATRA treatment and the genotype 'mutant *NPM1* without *FLT3*-ITD', with the beneficial effect of ATRA being restricted to this subgroup of patients. Patients with the genotype 'mutant *NPM1* without *FLT3*-ITD' who had been randomized to ATRA had a significantly better outcome compared to patients with the same genotype who had not been randomized to ATRA (Figures 1 and 2).

The exact molecular mechanism through which ATRA may exert its effects in AML with mutant *NPM1* remains elusive. However, recent studies have suggested a link between NPM1 and retinoic acid-mediated transcriptional regulation under physiological conditions as well as in myeloid leukemogenesis. First, NPM1 seems to function as a transcriptional co-repressor during retinoic acid-induced cell differentiation.[29] Second, the *NPM1* gene is involved in a chromosomal translocation, t(5;17)(q35;q21), which is present in a rare variant of APL, and the resulting ATRA-sensitive NPM1-RARA fusion protein has been shown to possess aberrant transcriptional regulatory activity.[30-32] Third, Martelli *et al.*[33] showed, in the *NPM1* mutant cell line OCI-AML3 and in primary *NPM1* mutant leukemias propagated in NOD-SCID mice, that pharmacological doses of ATRA induce cell cycle arrest and apoptosis by selectively downregulating the mutant NPM1 protein.

In summary, our data suggest that mutant *NPM1*, and more specifically the genotype mutant *NPM1* without *FLT3*-ITD, is a predictive factor for response to ATRA given as an adjunct to intensive chemotherapy in older patients with AML. These findings are currently being validated prospectively in our AMLSG 07-04 treatment protocol in which 920 younger adults 18 to 60 years of age with AML are randomized to intensive combination chemotherapy with or without ATRA. In addition, the results of our study support the concept of systematic molecular genetic studies in AML patients, not only for the evaluation of biomarkers for prognostication, but also for the identification of predictive factors for response to novel therapies.

Appendix

The following co-investigators of the AML HD98B trial contributed between one and nine patients to this study: Axel Glasmacher, University of Bonn; Hans-G. Mergenthaler, Klinikum Stuttgart; Christoph Nerl, Klinikum München-Schwabing; Hans Pralle, University of Giessen; Manfred Hensel, University of Heidelberg; Joachim Preiss, Caritas-Klinik St. Theresia Saarbrücken; Hans Salwender, Klinikum Hamburg-Altona; Hans-G. Biedermann, Kreiskrankenhaus Trostberg; Stephan Kremers, Caritas-Krankenhaus Lebach; Frank Griesinger, University of Göttingen.

Authorship and Disclosures

RFS, KD, SF, HD: designed the research and wrote the paper; AB: reviewed biometrical analyses; DS, SG: analyzed results; LB, MH, BK, SK, AC, AA: performed experiments; MK, KG, FH, FV, HK, EK, JF: co-investigators who contributed more than nine patients, participated in designing the clinical study, and reviewed and approved the paper. The authors reported no potential conflicts of interest.

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