Novel molecular biomarkers and their clinical consequences in acute myeloid leukemia

Neue molekulare Biomarker und deren klinische Konsequenzen in der Akuten Myeloischen Leukämie

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Zusammenfassung:

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Die Akute Myeloische Leukämie (AML) ist eine sowohl zytogenetisch als auch molekulargenetisch äußerst heterogene Erkrankung, die durch die klonale Proliferation myeloider Vorläuferzellen sowie eine Ausreifungsblockade charakterisiert ist. Trotz des in den letzten Jahren zugenommenen Wissens über die Biologie dieser Erkrankung und Weiterentwicklung von Therapiemethoden bleibt das Gesamtüberleben der Patienten mit AML überwiegend schlecht. Damit für mehr Patienten eine Heilung der AML möglich werden kann, sind ein tieferes Verständnis über die funktionellen Zusammenhänge in der Leukämogenese, eine bessere Risikostratifizierung und neue Therapieoptionen erforderlich.
Diese Habilitationsschrift fasst Publikationen zusammen, die neue molekulare Biomarker und deren klinischen Einfluss in der AML untersucht haben. Der Fokus liegt auf der Erfassung molekularbiologischer Veränderungen bei Diagnose einer AML oder im Krankheitsverlauf, die die Risikostratifizierung der Patienten verbessern kann. Außerdem gestattet die Arbeit Einblicke in die mit diesen molekularen Markern verbundene Biologie der AML, sowie mögliche neue Therapieoptionen.
Der erste bis dritte Abschnitt der Arbeit fokussiert sich auf Genmutationen und Genexpressionen in der AML. Es wird dargelegt, wie das Vorhandensein bestimmter Fusionstranskripte (hier *CBFB-MYH11*), rekurrenter Mutationen allein (hier im *DNMT3A* Gen) sowie als Teil genetischer Risikoklassifikationssysteme (hier die genetischen Risikogruppen des European LeukemiaNet) und die abberrante Expression AML-assoziierter Gene (hier *BAALC*, *ERG* und *MN1*) Beiträge zur Prognoseabschätzung der AML bieten.
Darüber hinaus hat sich in den letzten Jahren die entscheidende Rolle von MicroRNAs in der Pathophysiologie der AML herausgestellt. Heute weiß man, dass für die Leukämieentstehung und die Aggressivität der Erkankung schon die Dysregulation einer einzelnen microRNA entscheidend sein kann. Im vierten Abschnitt wird auf den progostischen Einfluss der Expressionslevel zweier MicroRNAs – *miR-181a* und *miR-29b* – und deren klinische und biologische Konsequenzen eingegangen. Außerdem wird dargestellt, wie verschiedene therapeutische Interventionen zu günstigen Änderungen des Expressionsniveaus dieser beiden neuen Biomarker und so zu potentiell neuen Therapiestrategien in der AML führen können.
Weiterhin wächst die Erkenntnis, dass in der AML so gennannte Leukämie-initiierende Zellen für Therapieresistenz und Rezidive verantwortlich zu sein scheinen. Der letzte Abschnitt dieser Habilitationsschrift fokussiert sich auf das CD34+/CD38- Zellkompartiment, welches einen Großteil der Leukämie-initiiernden Zellen enthält. Es wird gezeigt, dass die Bestimmung der Größe des CD34+/CD38- Zellkompartiments bei Diagnose geeignet ist, AML Patienten mit einem erhöhten Rezidivrisiko nach allogener Stammzelltransplantation zu identifizieren.
Zusammenfassend zeigt die Arbeit verschiedene Ansätze, wie neue molekulare Biomarker zu einer besseren Risikostratifizierung und einem tieferen Verständnis der AML zugrunde liegenden Biologie führen können. Des Weiteren beschreibt sie Möglichkeiten der therapeutischen Intervention und weist insgesamt auf die klinischen Implikationen dieser neuen Biomarker hin.

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1. INTRODUCTION / EINFÜHRUNG IN DIE THEMATIK

1.1. Diagnosis

Acute myeloid leukemia (AML) is the most frequently diagnosed acute leukemia in adults, often affecting older individuals with a median age at diagnosis in the late 60's.[1-5] AML originates from the clonal expansion of myeloid progenitor cells that lost their ability to mature. Subsequently, healthy hematopoietic stem cells (HSCs) are displaced and unable to provide sufficient formation of peripheral blood cells. At diagnosis, AML patients present with a variety of non-specific symptoms, which mostly are a result of peripheral cytopenia (*i.e.* anemia, thrombocytopenia and neutrophilopenia) and dysfunctional leukocytes, including fatigue, weekness, bleeding, and infections.[1-5]

AML diagnosis requires the myeloid blast count to exceed 20% in bone marrow or peripheral blood, with the exception of AML with t(15;17), t(8;21), inv(16) or t(16;16), which represent AML defining cytogenetic changes.[4,5] Flow cytometric analysis for surface markers (cluster of differentiation, CD) may aid in establishing the diagnosis of AML, as well as in defining mixed-phenotype acute leukemias.[5,6] While traditionally, the morphologic characterisation within the French American British Classification (FAB) was commonly used,[7] over the last years the prognostic importance of cytogenetic and moleculargenetic characterisation became clearer, proofed to be more objective and consequently replaced the FAB classification.[5] The majority of AML cases arise as *de novo* disease. However, AML might also develop as secondary disease originating from other hematologic disorders (e.g. myelodysplastic syndromes, myeloproliferative neoplasia) or after cytotoxic therapy of unrelated neoplasms (therapy-related AML).[1-5] AML is a clinically highly heterogeneous disease, with a variable disease course and prognosis, which is thought to be based on differences in clinical features such as patient age, performance status and comorbidities, as well as leukemia-specific features including cytogenetics or presence of moleculargenetic and epigenetic changes.[1-5]

1.2 Treatment and prognosis

Current outcomes with standard treatment approaches remain highly unsatisfactory. Today, only up to aproximatly one third of adult patients under the age of 60 years and only about 15% of patients over the age of 60 years achieve long-term survival.[5] However, older or medically unfit patients not eligible for intensive chemotherapy, in general have median survival times of only about 5 - 10 months.[5] Treatment of AML has changed little over the past decades. Curative treatment approaches remain to include the administration of the nucleoside analog cytarabine in combination with anthracyclines as induction therapy, followed by repeated cycles of high-dose cytarabine and/or an autologous or allogeneic hematopoietic stem cell transplantation (HSCT).[5,8,9] It is only very recently that a deeper understanding of AML biology started to provide us with novel therapeutic strategies, *e.g.* the introduction of tyrosine kinase inhibitors.[10] However, today only few of these agents are available in clinical routine and most are still being tested in clinical trials.[5] For frontline treatment in older and medically unfit AML patients the administration of hypomethylating agents such as decitabine or acazitidine is often recommended and used. These treatments result in survival benefits but rarely produce long lasting remissions.[5]

After achievement of a complete remission (CR) following induction chemotherapy, allogeneic HSCT is an established form of consolidation therapy for patients at high risk of relapse with a suitable donor available.[8,9] The therapeutic effects of an allogeneic HSCT are also based on an immunologic graft-versus-leukemia (GvL) reaction.[11] Furthermore, with the introduction of non-myeloablative conditioning regimes that greatly reduced the toxicity of high doses of chemotherapy and/or radiation and have a greater reliance on GvL effects, allogeneic HSCT also became available for older and medically constrained patients.[11,12]

1.3 Current risk assessment

Over the past years, the cytogenetic and molecular characterization of AML to determine phenotype aggressiveness has steadily improved.[5,13,14] Still, optimisation of risk-adapted treatment strategies are of high importance and remain an everyday challenge for clinicians.

Today, numerous prognosticators facilitate decisions on therapy de-escalation or intensification, enrolment in studies testing new agents, and the decision of appropriateness of an allograft procedure in first CR with its subsequent treatment-related morbidity, mortality, and financial costs.[5]

Cytogenetic abberations are among the strongest known prognostic parameters in AML.[5,15] About 60% of AML patients show cytogenetic aberrations and for some of these abnormalities a strong prognostic impact on CR achievement, cumulative incidence of relapse (CIR) and overall survival (OS) has been shown.[5,15] About 25% of younger adult AML patients present with cytogenetic aberrations known to associate with a favorable prognosis. These include the translocation t(15;17)(q22;q21) in acute promyelocytic leukemia (APL) and the inv(16)(p13q22) / t(16;16)(p13;q22) and t(8;21)(q22;q22) in the core-binding factor (CBF) leukemias.[5,15] These individuals can expect CR rates over 90% and long-term survival in up to 65% of cases. On the other hand, in about 20% of AML adverse-risk cytogenetics, *e.g.* monosomies of chromosome 5 (-5), abnormalities of 3q [abn(3q)] and monosomal or complex karyotypes, are found. In patients harboring these adverse-risk cytogenetics, CR rates in 65% and long-term survival in only 10% of cases can be exprected. Finally, in approximately 40-50% of newly diagnosed AML, no chromosomal aberrations can be found and despite their homogenous genetic appearance (46, XX or XY, normal cytogenetics, CN) clinical outcome in these patients proved highly heterogeneous.[15]

The desire to further dissect the heterogenous outcome of CN-AML patients and to improve risk-stratification in this large AML subgroup let to the identification of important recurrent mutations in AML. Among these are mutations in the nucleophosmin-1 (*NPM1*) gene, which is one of the most commonly mutated genes in AML. *NPM1* mutations are present in up to 20% - 50% of AML cases, depending on the subgroups investigated.[16-23] In the latest update of the World Health Organization (WHO) classification for AML, *NPM1* mutations represent a distinct entity and commonly indicate a more favorable prognosis.[5,15-23] Approximately one third of AML

patients carry an internal tandem duplications in the *FLT3* gene (*FLT3*-ITD), resulting in a constitutive activation of several downstream FLT3 pathways. The presence of a *FLT3*-ITD, especially with a higher mutated-to-wildtype allelic ratio (≥ 0.5), is associated with a dismal prognosis, with a high risk of relapse even after allogeneic HSCT.[24-29] The prognostic impact along with the observation that FLT3 is frequently overexpressed in a large subset of AML cases has let to the development of FLT3-targeting strategies, and today several FLT3 kinase inhibitors are being tested in clinical trials.[5,30,10] Among the most studied proteins in AML is the CCAAT/enhancer-binding protein alpha (C/EBPα), a transcription factors that is important for myeloid differentiation. In AML, the encoding gene *CEBPA* frequently exhibits mutations, deregulation of expression or other functional alterations. *CEBPA* mutations lead to an altered C/EBPα function, affecting expression of downstream genes and consequently deregulated myelopoiesis.[31] Detecting *CEBPA* mutations at diagnosis identifies patients with better outcomes, especially in the double mutated patients, and particularly in the molecular high-risk group (defined by presence of a *FLT3*-ITD and *NPM1* wild-type), thus improving molecular risk-based classification of AML.[32-38]

The growing knowledge of molecular changes *e.g.* cytogenetics, recurrent mutations, or the aberrant expression of coding and non-coding genes not only allows us to improve risk stratification, but may also enable re-definition of risk-adapted treatment strategies in AML patients. It also helps us to better understand the underlying pathogenesis that leads to the development of AML. Current research also focuses on how we can exploit this knowledge to develop novel targeted therapies for each patient that finally may result in improved cure rates of the disease.

In 2010 an international expert panel of the European LeukemiaNet (ELN) provided updated evidence and expert opinion-based recommendations for the diagnosis and management of AML.[39] The standardized reporting system integrated molecular genetic data of the three genes mentioned above (*i.e. NPM1*, *CEBPA*, *FLT3*-ITD) into the known diagnostic information of

cytogenetics (Table 1). Subsequently, also a prognostic impact of the suggested standardized reporting system was demonstrated for AML patients focusing on chemotherapy consolidation.[40,41]

Table 1 (Adapted from Döhner *et al.*)[39]: Cytogenetic and molecular genetic risk classification in AML according to the 2010 ELN reporting system

Genetic group	Subsets
Favorable	t(8;21)(q22;q22); *RUNX1-RUNX1T1* inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11* Mutated *NPM1* without *FLT3*-ITD (normal karyotype) Mutated *CEBPA* (normal karyotype)
Intermediate-I	Mutated *NPM1* and *FLT3*-ITD (normal karyotype) Wild-type *NPM1* and *FLT3*-ITD (normal karyotype) Wild-type *NPM1* without *FLT3*-ITD (normal karyotype)
Intermediate-II	t(9;11)(p22;q23); *MLLT3-MLL* Cytogenetic abnormalities not classified as favorable or adverse
Adverse	inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1* t(6;9)(p23;q34); *DEK-NUP214* t(v;11)(v;q23); *MLL* rearranged –5 or del(5q) –7 abnl(17p) Complex karyotype*

* Three or more chromosome abnormalities in the absence of one of the WHO designated recurring translocations or inversions, that is, t(15;17), t(8;21), inv(16) or t(16;16), t(9;11), t(v;11)(v;q23), t(6;9), inv(3) or t(3;3); indicate how many complex karyotype cases have involvement of chromosome arms 5q, 7q, and 17p

Since then, additional frequent and recurrent mutations have been identified (*e.g.* in the *DNMT3A*, *IDH1* and *IDH2* genes) in AML.[42-48] The continuous development of next-generation sequencing and other methods to detect genetic and epigenetic changes allowed to further characterize the biological AML background. However, the genetic profiling of AML is heterogeneous and only a few mutations (*e.g. FLT3*, *NPM1*, *DNMT3A*) are present in more than a quarter of AML patients.[5]

Very recently, the ELN recommandations for the diagnostic work-up in AML have been updated and now include screening for mutations in *NPM1*, *CEBPA,* and *RUNX1* genes, mutations in *FLT3* (activating mutations of FLT3 may also benefit from tyrosine kinase inhibition), and

mutations in *TP53* and *ASXL1*, since they consistently have been associated with poor prognosis (Table 2).[5,49-60]

Table 2 (Adapted from Döhner *et al.*)[5]: 2017 European LeukemiaNet risk stratification by genetics[a]

Risk Category[b]	Genetic Abnormality
Favorable	t(8;21)(q22;q22); *RUNX1-RUNX1T1* inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11* Mutated *NPM1* without *FLT3*-ITD or with *FLT3*-ITD$^{low\ (c)}$ Biallelic mutated *CEBPA*
Intermediate	Mutated *NPM1* and *FLT3*-ITD$^{high\ (c)}$ Wild-type *NPM1* without *FLT3*-ITD or *FLT3*-ITD$^{low\ (c)}$ (without adverse-risk genetic lesions) t(9;11)(p22;q23); *MLLT3-KMT2A*[d] Cytogenetic abnormalities not classified as favorable or adverse
Adverse	t(6;9)(p23;q34.1); *DEK-NUP214* t(v;11q23.3); *KMT2A* rearranged t(9;22)(q34.1;q11.2); *BCR-ABL1* inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); *GATA2*,*MECOM* (*EVI1*) -5 or del(5q); -7; -17/abn(17p) Complex karyotype,[e] monosomal karyotype[f] Wild-type *NPM1* and *FLT3*-ITD^{high} Mutated *RUNX1*[g] Mutated *ASXL1*[g] Mutated *TP53*[h]

[a] Frequencies, response rates and outcome measures should be reported by risk category, and, if sufficient numbers are available, by specific genetic lesions indicated.
[b] Prognostic impact of a marker is treatment-dependent and may change with new therapies.
[c] Low, low allelic ratio (<0.5); high, high allelic ratio (>0.5); semi-quantitative assessment of *FLT3*-ITD allelic ratio (using DNA fragment analysis) is determined as ratio of the area under the curve (AUC) "*FLT3*-ITD" divided by AUC "*FLT3*-wild type"; recent studies indicate that acute myeloid leukemia with *NPM1* mutation and *FLT3*-ITD low allelic ratio may also have a more favorable prognosis and patients should not routinely be assigned to allogeneic hematopoietic-cell transplantation.
[d] The presence of t(9;11)(p21.3;q23.3) takes precedence over rare, concurrent adverse-risk gene mutations.
[e] Three or more unrelated chromosome abnormalities in the absence of one of the World Health Organization-designated recurring translocations or inversions, i.e., t(8;21), inv(16) or t(16;16), t(9;11), t(v;11)(v;q23.3), t(6;9), inv(3) or t(3;3); AML with *BCR-ABL1*.
[f] Defined by the presence of one single monosomy (excluding loss of X or Y) in association with at least one additional monosomy or structural chromosome abnormality (excluding core-binding factor AML).
[g] These markers should not be used as an adverse prognostic marker if they co-occur with favorable-risk AML subtypes.
[h] *TP53* mutations are significantly associated with AML with complex and monosomal karyotype.

1.4 AML-associated gene expression

Apart from recurrent mutations at diagnosis, expression levels of some AML-associated genes *e.g.* brain and acute leukemia, cytoplasmatic (*BAALC*), *ETS*-related gene (*ERG*), and

meningioma-1 (*MN1*) have been shown to have strong, independent prognostic impact in AML patients.

High expression of the gene *BAALC* consistently associated with worse outcomes in younger and older patients with AML.[61-63] This gene maps to chromosome band 8q22.3, is highly expressed in CD34-positive bone marrow progenitor cells and downregulated during hematopoietic lineage-specific maturation.[61,62] Although it's function in hematopoiesis is still not fully understood, there ist evidence that BAALC blocks myeloid differentiation and may contribute to leukemogenesis when co-occuring with a second molecular alteration providing a proliferation advantage.[64] The gene *ERG*, located at chromosome band 21q22.3, was first shown to be involved in leukemogenesis in the rare, but recurrent in AML t(16;21)(p11;q22).[65] Moreover, *ERG* overexpression was demonstrated in AML patients with complex karyotypes with cryptic amplification of chromosome 21.[66] It was also found overexpressed in part of patients with CN-AML where it contributes to poor prognosis in younger and older CN-AML patients.[67-71] The *MN1* gene is localized at human chromosome band 22q12 and encodes a transcriptional coregulator.[72] *MN1* is involved in myeloid malignancies as a fusion partner of the *ETV6* gene in the recurrent translocation t(12;22)(p13q11)[73] and has been shown to be overexpressed in subsets of AML.[74,75] Different studies have shown that high *MN1* expression levels are prognosticators for poor outcome in younger and older CN-AML patients.[76-78]

The exploration of diagnostic expression levels of these AML associated genes provides important additional information for risk stratification and treatment strategies. However, the differential expression of these genes may also contribute to AML leukemogenesis. For example, Heuser *et al.*[79] demonstrated that *MN1* upregulation is involved in development of resistance mechanisms against all-trans retinoic acid (ATRA), an agent commonly used in APL treatment. Furthermore, they showed MN1 leukemogenecity to cooperate with a MEIS1/AbdB-like HOX-protein complex, representing a possible therapeutic target in high MN1 expressing leukemias.[80]

1.5 microRNAs in AML

Besides the aberrant expression of coding genes the differential expression of microRNAs (miRs) – small, non-coding RNAs – have been identified to play important roles in the initiation and progression of various malignancies, including AML.[81,82] MiRs are essential for many biological processes by controlling the expression of a number of genes involved in commitment and differentiation of HSCs, proliferation, apoptosis, immune response, and leukemogenesis.[81,82]

Over the past years, progress has been made in understanding the significance of expression levels of some miRs in AML. MiR expression profiles associated with outcome and certain molecular aberations in AML which have shed light on AML biology and also aid in refining AML risk stratification.[81,82] As an example Marcucci *et al.* identified a miR signature that is associated with clinical outcome in a subgroup of patients with high-risk molecular features of AML (those who have *FLT3*-ITD, wild-type *NPM1*, or both).[82] These data suggested that there is a functional relationship between miR expression and disease biology in AML patients that may render miR expression levels suitable prognostic markers and will help to further identify important players in AML biology.

However, a more comprehensive understanding of miR expression within the complex regulatory networks that are disrupted in AML cells is necessary for the development of better prognostic systems and novel therapeutic strategies employing miR modulators to improve patients' outcomes.

1.6 Leukemia-initiating cells

Another focus of current research in AML lies on the cells of AML origin. AML is a disease developing from the clonal expansion of early progenitor cells or HSCs that lost the ability to mature[1] – often termed leukemia initiating cells – which are believed to exists within the CD34+/CD38- stem cell compartment.[83-86] Leukemia initiating cells are postulated to survive chemotherapy as measurable (minimal) residual disease and cause AML relapse; their non-

proliferative state might be one reason for their resistance to chemotherapy.[87-90] Some studies have suggested that a high burden of leukemia initiating cells at diagnosis of AML patients treated with chemotherapy increased their relapse probability and associated with inferior outcomes.[91-93]

1.7 Measurable residual disease

Additionally to risk stratification at diagnosis, recently, some studies have shown a strong prognostic influence of the presence of measurable residual disease (MRD) in AML at different time points during treatment.[94-96] There is considerable effort in developing reliable techniques to quantify remaining leukemia cells in CR defined by cytomorphological criteria to better predict the likelihood of AML relapse. Some study groups cassess MRD by multiparameter flow cytometric analysis detection of aberrant surface antigen expression on malignant cells.[94] Other established methods are quantitative real time polymerase chain reaction (PCR) assays detecting disease-specific fusion genes or mutations.[95,96] Recent data indicates that the detection of genes also expressed in healthy individuals at low levels but upregulated in AML patients may further extend MRD evaluation possibilities.[96] However, today it remains to be determined which molecular markers at which time points and detected by which techniques are most suitable for residual disease detection and consequently for AML treatment guidance. Currently, this remains a very active field of translational research.

2. OWN CONTRIBUTION / EIGENER BEITRAG

2.1 Purpose and objective of the presented work

As described above AML is a highly heterogeneous disorder, characterized by the clonal expansion of AML progenitors arrested at various stages of myeloid differentiation, and by the progressive accumulation of multiple chromosomal, moleculargenetic, and epigenetic alterations. Prognosis of patients with AML is strongly influenced by the type of chromosomal and moleculargenetic alteration, as well as by aberrant (coding and non-coding) gene expression. Today outcomes of most AML patients remain poor. Thus, there is urgent need to identify prognostic biomarkers for risk stratification, to identify the underlying AML biology and to develop new therapeutic targets to subsequently improve AML patients' survival.

The studies included in this habilitation focus on molecular prognostic biomarkers, their clinical consequences, how theses markers contribute to a better risk stratification in newly diagnosed AML as well as how they may be exploited for MRD assessment and novel treatment approaches in AML.

The frist two paragraphes include publications focusing on recurrent cytogenetic and molecular changes and also the aberrant expression of AML associated genes, specifically of *BAALC*, *ERG,* and *MN1,* as prognostic markers at AML diagnosis. These studies expanded the knowledge on risk stratification in AML and highlighted some of the associated AML-biology. The third part shows that AML associated gene expression, *i.e. BAALC*, also functions as MRD marker if measured in complete remission. The fourth part focuses on a differential expression of miRs – specifically of *miR-181a* and *miR-29b* - as prognostic markers in AML, and reveal biological, clinical, and therapeutic implications of the identified aberrant miR expression levels. Finally, the included paper in the last paragraph demonstrates that the burden of leukemia initiating cells at diagnosis - defined by the bone marrow CD34+/CD38- cell population - impacts on outcome in patients undergoing allograft procedures, elucidating the importance of treatment approaches targeting leukemia initiating cells.

In summary, twelve publications are included in this work:

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2.2 Cytogenic aberations and recurrent molecular changes at diagnosis as prognostic factors in AML

The first paper in this paragraph concentrates on the common genetic alteration inv(16)(p13q22) / t(16;16)(p13;q22) in AML that results in various *CBFB-MYH11* fusion transcripts. In AML, the biologic and prognostic implications of different inv(16) fusion-products remained unclear. This work analyzed *CBFB-MYH11* fusion types in inv(16) / t(16;16) patients and found that no patient with non-type A fusion carried a *KIT* mutation, whereas about a third of type A fusion patients did. Among the latter, *KIT* mutations conferred adverse prognosis. Furthermore, non-type A fusions associated with distinct clinical and genetic features and a unique gene-expression profile.

The next study determined the frequency of *DNMT3A* mutations, their associations with clinical and molecular characteristics and outcome, as well as the associated gene- and miR-expression signatures in CN-AML. Missense mutations affecting arginine codon 882 (R882-*DNMT3A*) were more common than those affecting other codons (non-R882-*DNMT3A*). Furthermore, *DNMT3A* mutations were age dependent: while *DNMT3A*-R882 mutations were associated with adverse prognosis in older AML patients, non-R882-*DNMT3A* mutations were associated with adverse prognosis in younger AML patients.

The last paper included in this paragraph analyzed the prognostic impact of the 2010 ELN genetic groups in AML patients receiving allogeneic HSCT after non-myeloablative conditioning. While the prognostic utility of the four ELN 2010 genetic groups (favorable, intermediate-I, intermediate-II and adverse) in AML patients consolidated with chemotherapy had already been described, their impact after allogeneic HSCT remained to be elucidated. The data presented here suggest that the ELN 2010 genetic groups may have a reduced prognostic impact for patients undergoing allogeneic HSCT after non-myeloablative conditioning as compared to those receiving a chemotherapy-based consolidation in first CR.

Manuscripts included in this paragraph:

- **Schwind S**,* Edwards CG,* Nicolet D, Mrózek K, Maharry K, Wu YZ, Paschka P, Eisfeld AK, Hoellerbauer P,Becker H, Metzeler KH, Curfman J, Kohlschmidt J, Prior TW, Kolitz JE, Blum W, Pettenati MJ, Dal Cin P, Carroll AJ, Caligiuri MA, Larson RA, Volinia S, Marcucci G, Bloomfield CD:

inv(16)/t(16;16) acute myeloid leukemia with non-type A *CBFB-MYH11* fusions associate with distinct clinical and genetic features and lack *KIT* mutations. *Blood*. 2013; 121:385-91. *shared first-author

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Regular Article

MYELOID NEOPLASIA

inv(16)/t(16;16) acute myeloid leukemia with non–type A *CBFB-MYH11* fusions associate with distinct clinical and genetic features and lack *KIT* mutations

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Key Points

- Patients with inv(16) non–type A *CBFB-MYH11* fusions lack *KIT* mutations and have distinct clinical and cytogenetic features.
- inv(16) non–type A fusions have a distinct gene-expression profile with up-regulation of genes associated with apoptosis, differentiation, and cell cycle.

The inv(16)(p13q22)/t(16;16)(p13;q22) in acute myeloid leukemia results in multiple *CBFB-MYH11* fusion transcripts, with type A being most frequent. The biologic and prognostic implications of different fusions are unclear. We analyzed *CBFB-MYH11* fusion types in 208 inv(16)/t(16;16) patients with de novo disease, and compared clinical and cytogenetic features and the *KIT* mutation status between type A (n = 182; 87%) and non–type A (n = 26; 13%) patients. At diagnosis, non–type A patients had lower white blood counts ($P = .007$), and more often trisomies of chromosomes 8 ($P = .01$) and 21 ($P < .001$) and less often trisomy 22 ($P = .02$). No patient with non–type A fusion carried a *KIT* mutation, whereas 27% of type A patients did ($P = .002$). Among the latter, *KIT* mutations conferred adverse prognosis; clinical outcomes of non–type A and type A patients with wild-type *KIT* were similar. We also derived a fusion-type–associated global gene-expression profile. Gene Ontology analysis of the differentially expressed genes revealed—among others—an enrichment of up-regulated genes involved in activation of caspase activity, cell differentiation and cell cycle control in non–type A patients. We conclude that non–type A fusions associate with distinctclinical and genetic features, including lack of *KIT* mutations, and a unique gene-expression profile. (*Blood.* 2013;121(2): 385-391)

Introduction

Approximately 5%-7% of acute myeloid leukemia (AML) patients have an inv(16)(p13q22) or t(16;16)(p13;q22) [hereafter referred to as inv(16)/t(16;16)].[1-3] This cytogenetic group is usually associated with high complete remission (CR) rates and a relatively favorable outcome, especially when treated with repetitive cycles of high-dose cytarabine as consolidation therapy.[4,5] However, 30%-40% of these patients experience relapse.[6-10] We and others reported that the presence of a *KIT* mutation confers worse outcome in inv(16)/t(16;16) patients.[10-12]

Molecularly, inv(16)/t(16;16) results in the juxtaposition of the myosin, heavy chain 11, smooth muscle gene (*MYH11*) at 16p13 and the core-binding factor, β subunit gene (*CBFB*) at 16q22, and creation of the *CBFB-MYH11* fusion gene.[13,14] Because of the variability of the genomic breakpoints within *CBFB* and *MYH11*, more than 10 differently sized *CBFB-MYH11* fusion transcript variants have been reported.[15,16] More than 85% of fusions are type A, and 5%-10% each are type D and type E fusions.[15-20] Fusion types B, C, and F-K have been reported mostly in single cases.[15-20]

To our knowledge, only one study examined the biologic and clinical significance of different *CBFB-MYH11* fusions, but did not characterize the *KIT* mutation status.[18] Here, we report the frequency of *CBFB-MYH11* fusion transcripts, their associations with

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cytogenetic and clinical characteristics, *KIT* mutation status, and the fusion transcripts impact on prognosis in a relatively large cohort of patients with de novo inv(16)/t(16;16) AML. Furthermore, to gain insights into the biologic and functional differences of the distinct fusion types, we derived a fusion-type specific genome-wide gene-expression profile.

Methods

Patients and treatment

Two hundred eight patients aged 17-74 years with inv(16)/t(16;16) de novo AML, who were enrolled on Cancer and Leukemia Group B (CALGB; n = 206) or Southwest Oncology Group (SWOG; n = 2) frontline treatment protocols (for details please see supplemental Methods, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article) and had pretreatment material available, were analyzed for the *CBFB-MYH11* fusion type. Of these patients 147 patients enrolled on CALGB protocols that required ≥ 3 cycles of high-dose cytarabine-based consolidation treatment were eligible for outcome analyses. All patients provided written Institutional Review Board–approved informed consent for participation in these studies in accordance with the Declaration of Helsinki.

Cytogenetics, determination of fusion type, and *KIT* mutation status

For all 208 patients, pretreatment cytogenetic analyses of bone marrow (BM) or blood were performed by CALGB-approved institutional cytogenetic laboratories as part of CALGB 8461, and the results were reviewed centrally.[21] Three patients did not have mitoses on karyotype analysis, but were RT-PCR positive for *CBFB-MYH11*, and thus included in this study.

The *CBFB-MYH11* fusion types were determined for all 208 patients centrally in the Clinical Laboratory Improvement Amendments–certified Molecular Pathology Laboratory at The Ohio State University, as previously described.[22] The presence of mutations in *KIT* exons 8 and 17 was also determined centrally in pretreatment BM or blood, as previously described.[11]

Gene-expression profiling

For gene-expression profiling, total RNA was extracted from pretreatment BM or blood mononuclear cells. Gene-expression profiling was performed using the Affymetrix U133 plus 2.0 microarray (Affymetrix; ArrayExpress accession: E-MTAB-1356) as previously reported.[23,24] Briefly, summary measures of gene expression were computed for each probe-set using the robust multichip average method, which incorporates quantile normalization of arrays. Expression values were logged (base 2) before analysis. A filtering step was performed to remove probe-sets that did not display significant variation in expression across arrays. In this procedure, a χ^2 test was used to test whether the observed variance in expression of a gene was significantly larger than the median observed variance in expression for all genes, using $\alpha = .01$ as the significance level. A total of 6747 genes passed the filtering criterion.

Normalized expression values were compared between type A and non–type A fusion inv(16)/t(16;16) patients and a univariable significance level of .001 was used to identify differentially expressed genes (all genes had false detection rate ≤ 0.05).

Gene Ontology (GO) analysis to assess enrichment of genes associated with distinct biologic processes for up- and down-regulated genes in non–type A inv(16)/t(16;16) patients compared with type A inv(16)/t(16;16) patients was conducted using a hypergeometric test and Cytoscape.[25] *P* values were corrected for multiple testing using the false detection rate according to Benjamini-Hochberg.

Definition of clinical end points and statistical analysis

The main objective of this study was to evaluate the frequency of distinct *CBFB-MYH11* fusion transcripts (we applied the nomenclature of fusion transcripts according to van Dongen et al[17]), their associations with cytogenetic and clinical characteristics, and *KIT* mutation status, and their prognostic impact in a relatively large set of patients with de novo AML and inv(16)/t(16;16). The differences among patients in their baseline cytogenetics, *KIT* mutation status, demographic and clinical features according to their fusion transcript type were tested using the Fisher exact and Wilcoxon rank-sum tests for categorical and continuous variables, respectively.

A subset of 147 patients who were enrolled on protocols requiring at least 3 cycles of high-dose cytarabine-based postremission treatment, were eligible for outcome analyses. These patients had similar pretreatment characteristics to the total set of 208 patients studied (supplemental Table 1). Material to determine the pretreatment *KIT* mutation status was available for 141 of these 147 patients. CR was defined as recovery of morphologically normal BM and blood counts (ie, neutrophils ≥ 1.5×10^9/L and platelets > 100×10^9/L), and no circulating leukemic blasts or evidence of extramedullary leukemia for more than one month. CR rates were compared using the Fisher exact test. Cumulative incidence of relapse (CIR) was measured from the date of CR until relapse. Patients alive without relapse were censored, whereas those who died without relapse were counted as a competing cause of failure. Overall survival (OS) was measured from the date of study entry until date of death. Patients alive at last follow-up were censored for OS. Event-free survival (EFS) was measured from the date of study entry until induction failure, relapse or death, regardless of cause; patients alive and in CR were censored at last follow-up. Estimates of CIR were calculated, and the Gray k-samples test[26] was used to evaluate differences in relapse rates. Estimated probabilities of OS and EFS were calculated using the Kaplan-Meier method, and the log-rank test evaluated differences between survival distributions. The Holm step-down procedure and Sidak adjustment were used to adjust *P* values for the multiple comparisons analyses of fusion type by *KIT* status for CR and survival analyses, respectively.[27] The dataset was locked on September 24, 2012.

For the gene-expression profiling, summary measures of gene expression were computed, normalized, and filtered. The inv(16)/t(16;16) fusion-type-associated signature was derived by comparing gene expression between type A and non–type A patients with wild-type *KIT*. Univariable significance levels of .001 for gene-expression profiling were used to determine the probe-sets that constituted the signature.

All analyses were performed by the Alliance for Clinical Trials in Oncology Statistics and Data Center.

Results

Frequency and associations of inv(16)/t(16;16) fusion types with clinical characteristics and *KIT* mutation status in de novo inv(16)/t(16;16) AML patients

In our study, 182 (87%) patients with inv(16)/t(16;16) AML had a type A fusion, whereas 26 (13%) harbored a non–type A fusion. Eighteen (9%) patients harbored a type E fusion, 6 (3%) a type D fusion, and 2 (1%) harbored other fusion types (Table 1; supplemental Figure 1). There was no significant difference in non–type A fusion frequencies between patients with inv(16) and those with t(16;16) (13% vs 6%; $P = .70$).

Pretreatment characteristics of our patients are presented in Table 2. Non–type A patients had lower white blood counts (WBC; $P = .007$) at diagnosis. Most patients, 60% (n = 124), had inv(16) or t(16;16) as a sole chromosome abnormality, whereas 40% (n = 81) had ≥ 1 secondary abnormality. Non–type A patients more often had a secondary abnormality than type A patients (58% vs 37%; $P = .07$; Table 2). Non–type A patients more frequently had +8 ($P = .01$) and +21 ($P < .001$) than type A patients. However, none of the non–type A patients had +22, whereas 19%

Table 1. Frequencies of *CBFB-MYH11* fusion types among 208 patients with inv(16)/t(16;16) AML in our study and 162 patients reported by Schnittger et al[18]

	This study (n = 208)*		Schnittger et al[18] (n = 162)†		
Fusion type	No.	%	No.	%	P‡
Type A	182	87	128	79	.03
Type E	18	9	8	5	.22
Type D	6	3	16	10	.007
Other types	2§	1	10‖	6	.006

AML indicates acute myeloid leukemia.

*All patients were diagnosed with de novo AML.

†One hundred thirty-eight patients were diagnosed with de novo AML and 24 patients with treatment-related AML. Within the de novo AML cohort, 83% had type A fusions; frequencies for other fusion types within this cohort were not provided.

‡*P* values are from the Fisher exact test.

§Both fusions were type I (n = 2).

‖These were the following fusion types: Avar (n = 1), Bvar (n = 1), F (n = 1), G (n = 2), H (n = 1), J (n = 2), and S/L (n = 2).

of the type A patients did ($P = .02$, Table 2). Forty-eight (24%) of inv(16)/t(16;16) patients harbored *KIT* mutations. Interestingly, they were detected exclusively in type A patients, with none of the non–type A patients carrying a *KIT* mutation (27% vs 0%; $P = .002$, Table 2).

Genome-wide gene-expression profiling

To gain further insights into the biology of inv(16)/t(16;16) AML with different fusion types, we derived a genome-wide gene-expression signature. To avoid bias associated with the unequal distribution of *KIT* mutations between type A and non–type A fusion inv(16)/t(16;16) patients, and because *KIT* mutations have been shown to be associated with a distinct gene-expression profile,[28] we compared non–type A patients (n = 15) with those with type A fusion and wild-type *KIT* (n = 86). We observed the differential expression of 121 genes between non–type A and type A inv(16)/t(16;16) patients (Figure 1). Of these genes, 51 were up-regulated in non–type A patients (supplemental Table 2) and 70 were down-regulated (supplemental Table 3).

Among the up-regulated genes in non–type A inv(16)/t(16;16) patients, we found genes involved in differentiation, eg, *GFI1* that encodes a transcriptional repressor contributing to myeloid differentiation[29,30]; epigenetics, eg, *DNMT3B* that encodes one of the isoforms of DNA methyltransferases mediating DNA methylation and gene silencing[31]; and apoptosis, eg, *CYCS* that encodes the small heme protein cytochrome C that is associated with cellular apoptosis.[32,33] Among the down-regulated genes in non–type A inv(16)/t(16;16) patients, we found genes involved in kinase pathways, eg, *CD9* that encodes a member of the transmembrane 4 superfamily that has been shown to physically interact with the aforementioned tyrosine kinase receptor KIT,[34] and *CD52*, that encodes a surface protein with not fully elucidated function, but that is expressed on neutrophils and hematologic stem cells and targeted by alemtuzumab.[35] We also observed lower expression of *MYH9*, a gene frequently linked to inheritable thrombocytopenia,[36] and of *SPARC*, a gene found to be also down-regulated in AML with *MLL*-rearrangements.[37]

To focus on the functional differences of the different inv(16)/t(16;16) fusion types, we performed a Gene Ontology (GO) analysis and found an enrichment of genes involved in activation of caspase activity, positive regulation of cell differentiation, G0/G1 transition and G2/M transition in the up-regulated genes of non–type A inv(16)/t(16;16) patients (supplemental Table 4). Among the genes down-regulated in non–type A fusion inv(16)/t(16;16) patients, the GO analysis revealed enrichment of biologic processes related to actin cytoskeleton, ruffles, uropod, phosphoinositide binding, barbed-end actin filament capping, blood vessel endothelial cell migration, syncitium formation by plasma membrane fusion and tissue regeneration (supplemental Table 5). These results suggest a potentially less aggressive phenotype of non–type A inv(16)/t(16;16) AML.

Prognostic impact of the inv(16)/t(16;16) fusion type on clinical outcome

A subset (n = 147) of the 208 patients received high-dose cytarabine-based treatment and thus was eligible for outcome analyses. The CR rates ($P = 1.00$), CIR ($P = .14$), and OS

Table 2. Clinical and cytogenetic characteristics and *KIT* mutation status according to *CBFB-MYH11* fusion type in 208 patients with de novo AML and inv(16)/t(16;16)

Characteristic	Non–type A fusion* (n = 26)	Type A fusion (n = 182)	P
Age, y			.75
Median	41	41	
Range	22-62	17-74	
Sex, no. of males (%)	14 (54)	113 (62)	.52
Race, no. (%)			.56
White	20 (77)	149 (82)	
Nonwhite	6 (23)	33 (18)	
Hemoglobin, g/dL			.42
Median	8.9	8.8	
Range	6.6-13.0	3.1-14.8	
Platelet count, × 10^9/L			.33
Median	46	42	
Range	15-208	7-272	
WBC, × 10^9/L			.007
Median	21.9	33.8	
Range	1.4-87.2	0.4-500.0	
Percentage of blood blasts			.26
Median	43	52	
Range	3-93	0-97	
Percentage of BM blasts			.59
Median	53	58	
Range	22-93	2-89	
FAB (centrally reviewed), no. (%)			.04
M1	3 (14)	2 (1)	
M2	0 (0)	8 (5)	
M4	4 (19)	21 (14)	
M4Eo	14 (67)	121 (78)	
M5	0 (0)	3 (2)	
Cytogenetic characteristics‡			
sole inv(16)/t(16;16), no. (%)	10 (42)	114 (63)	.07
+8, no. (%)	7 (29)	18 (10)	.01
+13, no. (%)	2 (8)	3 (2)	.11
+21, no. (%)	6 (25)	1 (1)	< .001
+22, no. (%)	0 (0)	35 (19)	.02
***KIT*, no. (%)§**			.002
Mutated	0 (0)	48 (27)	
Wild-type	24 (100)	130 (73)	

FAB indicates French-American-British classification; and WBC, white blood count.

*Type E (n = 18), type D (n = 6), type I (n = 2).

‡Patients may have multiple secondary abnormalities and thus can be classified in more than 1 category; 3 patient samples had no mitoses.

§Six patients (2 with non–type A and 4 with type A fusions) had no material available to study *KIT* mutations and thus have an unknown *KIT* mutation status.

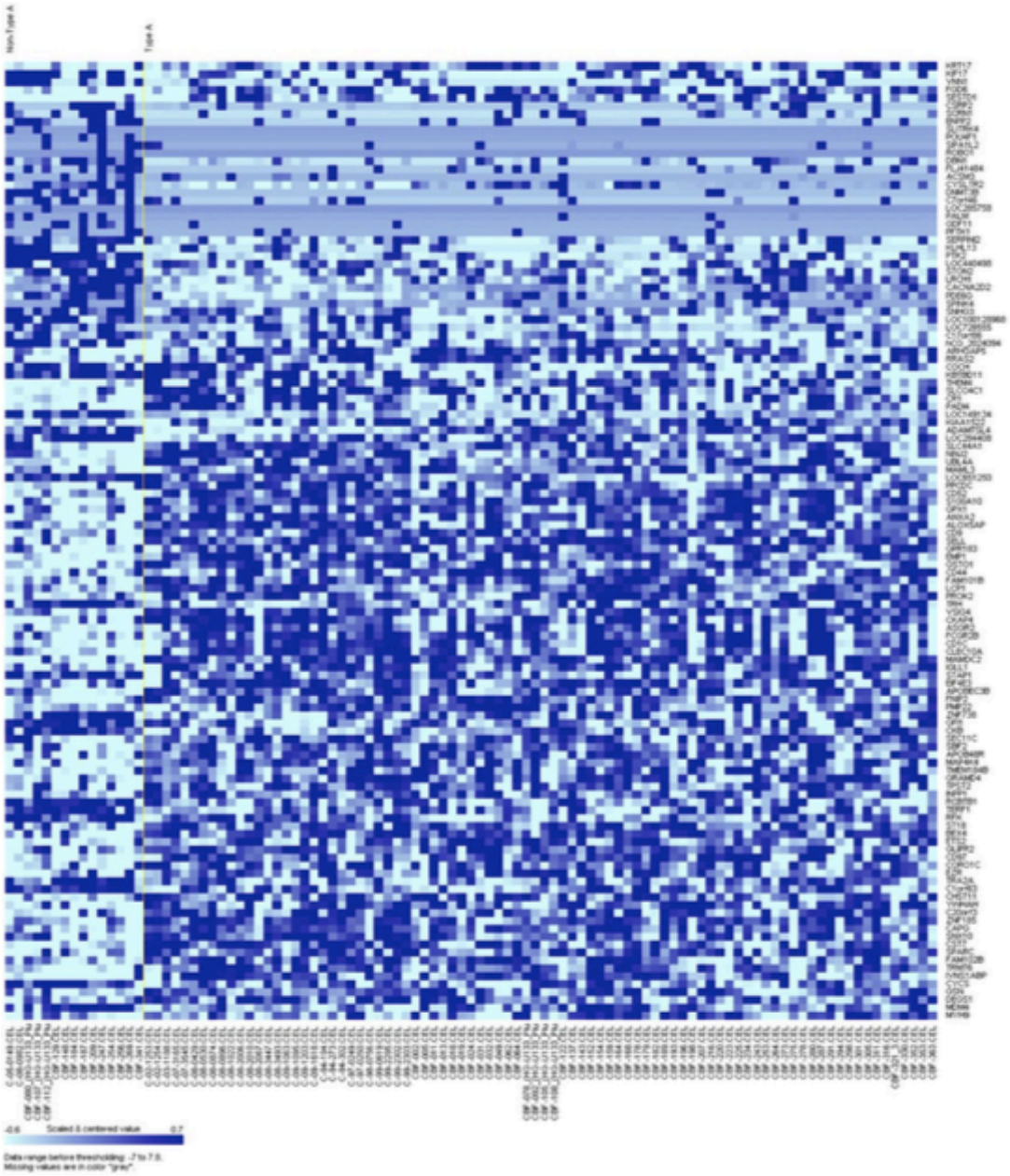

Figure 1. Heat map of the derived gene-expression signature associated with the *CBFB-MYH11* fusion type (non–type A vs type A with wild-type *KIT*) in patients with de novo AML and inv(16)/t(16;16). Rows represent gene names and columns represent patients. Genes are ordered by hierarchical cluster analysis. Expression values of the genes are represented by color, with dark blue indicating higher expression and light blue indicating lower expression.

($P = .36$; supplemental Figure 2A) of non–type A patients (n = 19) and type A patients (n = 128) did not differ significantly (supplemental Table 6). However, non–type A patients tended to have longer EFS than type A patients ($P = .05$; 72% vs 50% at 5 years; supplemental Figure 2B).

Because non–type A fusions and *KIT* mutations were mutually exclusive, we wondered whether the difference in EFS could be attributed to the different distribution of *KIT* mutations. Therefore, we compared the outcome of patients with non–type A fusions with that of type A fusion patients who had wild-type *KIT* (n = 85). In

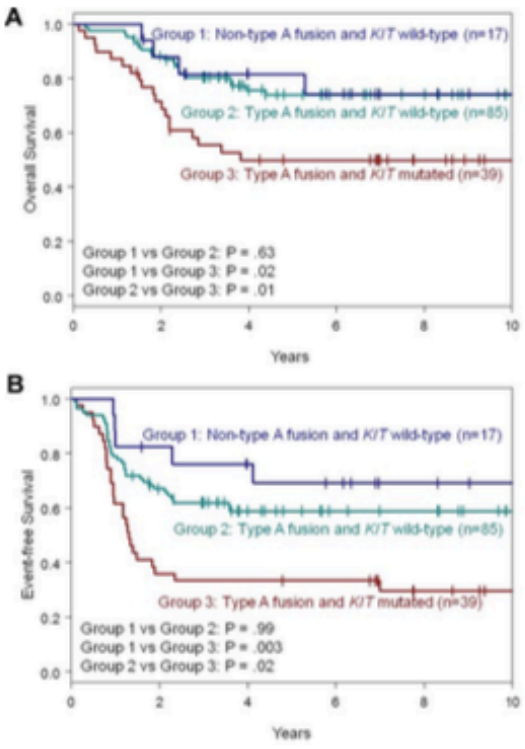

Figure 2. Survival of patients with de novo AML and inv(16)/t(16;16) according to *CBFB-MYH11* fusion type (non–type A vs type A) and *KIT* mutation status. (A) OS. (B) EFS. All *P*-values from pairwise comparisons are adjusted for multiple comparisons.

this analysis, non–type A patients behaved similarly to type A patients with wild-type *KIT* (Figure 2; supplemental Table 7). We did not find significant differences in CR rates ($P = 1.00$), CIR ($P = .60$), OS ($P = .63$; Figure 2A) or EFS ($P = .99$; Figure 2B), suggesting it was the presence or absence of *KIT* mutations that affected clinical outcome rather than the type of fusion transcript. Indeed, type A patients with mutated *KIT* had a shorter OS ($P = .01$; 50% vs 74% at 5 years; Figure 2A) and EFS ($P = .02$; 33% vs 59% at 5 years; Figure 2B) than type A patients with wild-type *KIT*. Likewise, both OS ($P = .02$; 50% vs 82% at 5 years; Figure 2A) and EFS were shorter ($P = .003$; 33% vs 69% at 5 years; Figure 2B) for *KIT*-mutated type A patients compared with non–type A patients. Thus, *KIT* mutations remain an important prognosticator in type A inv(16)/t(16;16) patients.

Discussion

AML patients with inv(16)/t(16;16) usually have favorable outcome. The resulting *CBFB-MYH11* fusion gene results in various transcripts.[15,16] However, the biologic and clinical significance of these different fusion types require further evaluation. In the presented study, 87% of de novo inv(16)/t(16;16) patients had a type A fusion, 13% harbored a non–type A fusion (18 had type E, 6 type D and 2 type I; Table 1, supplemental Figure 1). Schnittger et al, who also included treatment-related AML (t-AML) cases, reported a lower type A frequency of 79%[18] ($P = .03$; Table 1). Since in the study by Schnittger et al treatment-related inv(16)/t(16;16) less often have type A fusions,[18] we compared only de novo cases and found no significant difference in type A frequency between the 2 studies (87% vs 83%; $P = .28$). Although type E frequencies were similar (9% vs 5%; $P = .22$), type D (3% vs 10%; $P = .007$) and all other types combined (1% vs 6%; $P = .006$; Table 1) were more frequent in the Schnittger et al study.[18] This finding may also be related to the inclusion of t-AML cases by Schnittger et al, who did not report on the individual non–type A frequencies in their de novo cases.[18]

Consistent with the study by Schnittger et al,[18] non–type A patients in our study also had lower WBC. With respect to additional cytogenetic aberrations, in our study non–type A patients more often had a secondary abnormality than type A patients. While non–type A patients more frequently had +8 and +21 than type A patients, none of the non–type A patients had +22. Schnittger et al found that non–type A patients harbored +8, +21 and +22 less frequently,[18] although a comparison of the individual trisomy frequencies with our data was not possible because they combined all trisomies into 1 subset. Because *KIT* mutations have been associated with inferior outcome in inv(16)/t(16;16) AML we analyzed the frequency of *KIT* mutations, and found that *KIT* mutations could not be detected in non–type A patients. This unexpected finding may have implications for treatment and risk-stratification of inv(16)/t(16;16) patients. Recently, mutated *KIT* was found to cooperate with the *CBFB-MYH11* fusion toward leukemogenesis in mice.[38] Our data suggest that this cooperation might be limited to type A fusion transcripts and that other cooperative events occur in inv(16)/t(16;16) AML with non–type A fusions.

To gain further biologic insights into the biology of inv(16)/t(16;16) AML with different fusion types, we performed a microarray analysis to assess differences in the genome-wide gene expression between patients with non–type A and type A fusion transcripts with wild-type *KIT*. We observed that patients with non–type A fusion showed an up-regulation of genes involved in the activation of caspase activity, cell differentiation and cell cycle control in addition to increased expression of other genes that have been previously linked to myeloid leukemogenesis, including *GFI1* or *DNMT3B*.[29-31] In addition, we observed that non–type A patients presented with down-regulation of *CD9*, a gene involved in mechanisms of activation of the receptor tyrosine kinase KIT, which is often found mutated or aberrantly expressed in inv(16)/t(16;16) AML,[34] and *MYH9* that has been previously linked with inheritable thrombocytopenia.[36] How the differential expression of these genes ultimately impact on the leukemia phenotype, clinical characteristics and outcome of non–type A inv(16)/t(16;16)–patients remains unknown and should be studied in preclinical models to test the hypothesis that novel treatment strategies can be tailored to the type of fusion transcript in inv(16)/t(16;16) AML. It is interesting, however, that the GO analysis suggested a less aggressive phenotype for non–type A inv(16)/t(16;16)–leukemia, given the activation of genes involved in cell differentiation, cell cycle regulation and apoptosis and conversely the down-regulation of genes potentially involved in angiogenesis and cell migration.

In conclusion, non–type A *CBFB-MYH11* fusion transcripts occur in a subset (13%) of inv(16)/t(16;16) patients. Although the fusion type does not impact on outcome of inv(16)/t(16;16) patients, the presence of non–type A fusions is associated with distinct clinical and genetic characteristics, as well as a distinct global gene-expression profile. *KIT* mutations, not found in non–

type A patients but occurring in more than one-fourth of type A patients, conferred adverse prognosis among the latter. The biologic and therapeutic implications of these findings remain to be investigated, especially in the context of tyrosine kinase inhibitors targeting KIT being used in current clinical trials for inv(16)/t(16;16) patients (eg, NCT01238211 and NCT00416598).

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Authorship

Contribution: S.S., C.G.E., G.M., and C.D.B. designed the study; S.S., C.G.E., K. Mrózek, G.M., and C.D.B analyzed the data, and wrote the manuscript; S.S., Y.-Z.W., P.P., A.-K.E., P.H., H.B., K.H.M., J.C., and T.W.P performed the laboratory-based research; D.N., S.V., K. Maharry, and J.K. performed the statistical analyses; J.E.K., W.B., M.J.P., P.D.C., A.J.C., M.A.C., R.A.L., G.M., and C.D.B. were involved directly or indirectly in the care of patients and/or sample procurement; and all authors read and agreed on the final version of the manuscript.

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ORIGINAL REPORT

Age-Related Prognostic Impact of Different Types of *DNMT3A* Mutations in Adults With Primary Cytogenetically Normal Acute Myeloid Leukemia

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ABSTRACT

Purpose
To determine the frequency of *DNMT3A* mutations, their associations with clinical and molecular characteristics and outcome, and the associated gene- and microRNA-expression signatures in primary cytogenetically normal acute myeloid leukemia (CN-AML).

Patients and Methods
Four hundred fifteen previously untreated adults were analyzed for *DNMT3A* mutations and established prognostic gene mutations and expression markers. Gene- and microRNA-expression profiles were derived using microarrays.

Results
Younger (< 60 years; n = 181) and older (≥ 60 years; n = 234) patients had similar frequencies of *DNMT3A* mutations (35.3% *v* 33.3%). Missense mutations affecting arginine codon 882 (R882-*DNMT3A*) were more common (n = 92; 62%) than those affecting other codons (non–R882-*DNMT3A*). *DNMT3A*-mutated patients did not differ regarding complete remission rate, but had shorter disease-free survival (DFS; $P = .03$) and, by trend, overall survival (OS; $P = .07$) than *DNMT3A*–wild-type patients. In multivariable analyses, *DNMT3A* mutations remained associated with shorter DFS ($P = .01$), but not with shorter OS. When analyzed separately, the two *DNMT3A* mutation types had different significance by age group. Younger patients with non–R882-*DNMT3A* mutations had shorter DFS ($P = .002$) and OS ($P = .02$), whereas older patients with R882-*DNMT3A* mutations had shorter DFS ($P = .005$) and OS ($P = .002$) after adjustment for other clinical and molecular prognosticators. Gene- and microRNA-expression signatures did not accurately predict *DNMT3A* mutational status.

Conclusion
DNMT3A mutations are frequent in CN-AML, and their clinical significance seems to be age dependent. *DNMT3A*-R882 mutations are associated with adverse prognosis in older patients, and non–R882-*DNMT3A* mutations are associated with adverse prognosis in younger patients. Low accuracy of gene- and microRNA-expression signatures in predicting *DNMT3A* mutation status suggested that the role of these mutations in AML remains to be elucidated.

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INTRODUCTION

Acute myeloid leukemia (AML) is a genetically heterogeneous disease characterized by nonrandom cytogenetic aberrations[1,2] and, at the submicroscopic level, recurrent gene mutations and changes in gene expression.[3] Cytogenetic and molecular alterations not only define distinct biologic entities, but are also relevant for disease classification and treatment guidance.[4]

Cytogenetically normal (CN) AML, comprising 45% to 50% of adults with primary disease,[5] is one of the best molecularly characterized cytogenetic groups. Some gene mutations recurrent in CN-AML are strong, independent prognosticators (eg, *FLT3* internal tandem duplications [*FLT3*-ITD],[6,7] *CEBPA*,[8,9] and *WT1*[10,11] mutations), whereas others affect outcome in distinct molecular or clinical subsets of CN-AML (eg, *NPM1*,[12,13] *TET2*,[14] and *IDH1/IDH2*[15,16] mutations) or are of uncertain significance (eg, *FLT3*-tyrosine kinase domain mutations [*FLT3*-TKD][17,18]). More intense treatment may modify the prognostic weight of some molecular markers in CN-AML, such as *MLL*

partial tandem duplication (*MLL*-PTD)[19,20] or *FLT3*-ITD.[21] Additionally, altered expression of genes (eg, high *BAALC*,[22,23] *ERG*,[23,24] and *MN1*[25-27] levels) and microRNAs (eg, low *miR-181a* level[28]) identify high-risk CN-AML patients.

The *DNMT3A* gene encodes one of the three DNA methyltransferase (DNMT) isoforms. Among these, DNMT1 is the most abundant and preferentially replicates existing DNA methylation patterns, whereas DNMT3A and DNMT3B are responsible for establishing de novo DNA methylation. The process of DNA methylation consists of an enzymatic addition of a methyl group at the carbon 5 position of cytosine in the context of cytosine-guanine dinucleotides. When occurring in the promoter region of a coding gene, it generally results in gene silencing. In AML, all three DNMT enzymes are reportedly overexpressed in malignant blasts compared with normal bone marrow (BM) cells and contribute to leukemogenesis by mediating tumor suppressor gene silencing.[29] Somatic *DNMT3A* mutations in AML were first described by Yamashita et al[30] and subsequently by other groups.[31,32] Ley et al[31] first reported that *DNMT3A* mutations conferred worse outcome in AML. However, the patients analyzed were heterogeneous for biologic and clinical characteristics and treatment received, and the prognostic value of *DNMT3A* mutations was not fully evaluated within the context of other known molecular prognosticators.[31] Recently, Thol et al[33] reported that *DNMT3A* mutations are associated with shorter overall survival (OS) in cytogenetically diverse patients with AML who are younger than 60 years and with lower complete remission (CR) rates and shorter OS in a CN-AML subset. However, this study included patients with secondary disease and those who received allogeneic stem-cell transplantation (SCT) and did not analyze the prognostic impact of different types of *DNMT3A* mutations.[33]

To our knowledge, our study is the first to investigate the prognostic impact of *DNMT3A* mutations in a large population of patients diagnosed exclusively with primary CN-AML, comprehensively characterized for other molecular prognosticators, and receiving intensive chemotherapy. Additionally, we analyzed the differential impact of *DNMT3A* mutations by age group (younger [< 60 years] *v* older [≥ 60 years]) and mutation type (missense mutations at codon R882 [hereafter called R882-*DNMT3A*] *v* mutations at other locations [denoted non–R882-*DNMT3A*]). Furthermore, to gain insights into the biologic role of *DNMT3A* mutations in CN-AML, we derived genome-wide *DNMT3A* mutation-associated gene- and microRNA-expression signatures.

PATIENTS AND METHODS

Patients, Treatment, and Cytogenetic Studies

Pretreatment BM or blood samples were obtained from 415 patients with primary CN-AML, 18 to 83 years of age (181 younger and 234 older), who received intensive first-line therapy on Cancer and Leukemia Group B trials.[34-42] Patients received cytarabine-daunorubicin-based induction chemotherapy; most younger patients received consolidation with high-dose chemotherapy and autologous SCT. Per protocol, no patient received allogeneic SCT during first CR. For details regarding treatment protocols and sample collection, see the Data Supplement. The diagnosis of normal cytogenetics was based on centrally reviewed analysis of ≥ 20 metaphases in BM specimens.[43] All patients provided written informed consent; study protocols were in accordance with the Declaration of Helsinki and approved by local institutional review boards.

Mutational Analyses

For *DNMT3A* mutational analysis, the sequences of exons 18, 19, 21, 22, and 24 to 26 (GenBank reference NM_175629) were analyzed from genomic DNA by polymerase chain reaction and direct sequencing. Patients were also characterized for *FLT3*-ITD,[7,44] *FLT3*-TKD,[17] *MLL*-PTD,[20,45] mutations in *NPM1*,[13] *CEBPA*,[8] *WT1*,[10,11] *TET2*[14] and *IDH1*/*IDH2*,[15] and expression levels of *ERG*[23,24] and *BAALC*,[22,23] as previously reported. Molecular analyses were performed at The Ohio State University.

Microarray Experiments

Gene-expression profiling was performed using oligonucleotide microarrays (Affymetrix, Santa Clara, CA), and microRNA-expression profiling was performed using a custom microarray, as previously reported.[13,14,46] Expression signatures were identified by comparing *DNMT3A*-mutated and *DNMT3A*–wild-type (*DNMT3A*-wt) patients, and analyses to predict *DNMT3A* mutation status were performed (Data Supplement).

Statistical Analyses

Baseline characteristics were compared between *DNMT3A*-mutated and *DNMT3A*-wt patients using Fisher's exact test for categorical and the Wilcoxon rank sum test for continuous variables. Clinical end points were defined according to published recommendations (Data Supplement).[47] For time-to-event analyses, survival estimates were calculated using the Kaplan-Meier method, and groups were compared using the log-rank test. In addition to analyzing all *DNMT3A*-mutated cases as a combined group, we also evaluated the prognostic significance of R882-*DNMT3A* and non–R882-*DNMT3A* mutations separately, in the entire cohort and in the younger and older groups.

In models considering both age groups, we adjusted for an age-group effect (≥ 60 years *v* < 60 years). We constructed multivariable logistic regression models to analyze factors influencing achievement of CR and multivariable Cox proportional hazards models for factors associated with survival end points (Data Supplement). All analyses were performed by the Alliance for Clinical Trials in Oncology Statistics and Data Center.

RESULTS

Prevalence and Spectrum of DNMT3A Mutations in Primary CN-AML

Excluding known single-nucleotide polymorphisms, 148 nonsynonymous sequence variations (mutations) in *DNMT3A* were found in 142 (34.2%) of 415 patients (Data Supplement). The frequencies of these mutations were similar in younger (35.3%) and older (33.3%) patients. Six patients had two mutations each, and four mutations appeared homozygous. Ninety-two mutations (62%) were missense changes in codon R882, leading to an amino acid exchange from arginine to histidine (R882H, n = 49), cysteine (R882C, n = 36), proline (R882P, n = 3), serine (R882S, n = 3), or glycine (R882G, n = 1). R882-*DNMT3A* missense mutations were the most common mutation type among both younger (26%) and older (19%) patients. The remaining non–R882-*DNMT3A* mutations (n = 56; 38%) included 22 nonsense, frameshift, and splice-site mutations found in 22 different patients. These mutations are predicted to either trigger nonsense-mediated RNA decay or result in a truncated protein and thus are likely to impair protein function.[48] Two of these 22 patients concomitantly had an R882-*DNMT3A* mutation (for outcome analyses, these patients were included in the R882-*DNMT3A* mutation group), and two others concomitantly had another non–R882-*DNMT3A* missense mutation. Furthermore, there were 32 missense mutations not affecting codon R882 and two short in-frame deletions. All 32 non-R882 missense mutations were predicted to be "disease causing" by the MutationTaster software,[49] a

Table 1. Clinical and Molecular Characteristics of 415 Patients With Primary Cytogenetically Normal Acute Myeloid Leukemia According to *DNMT3A* Mutation Status

Characteristic	*DNMT3A* Mutated (n = 142) No.	%	*DNMT3A* Wild Type (n = 273) No.	%	*P**
Age, years					.46
Median	61		62		
Range	22-82		18-83		
Age, years					.68
< 60	64	45	117	43	
≥ 60	78	55	156	57	
Female sex	72	51	135	49	.84
Race					.49
White	124	89	247	91	
Nonwhite	16	11	25	9	
Hemoglobin, g/dL					.80
Median	9.4		9.4		
Range	4.8-14.5		4.6-15		
Platelet count, $\times 10^9$/L					.55
Median	66		61		
Range	4-481		7-850		
WBC, $\times 10^9$/L					< .001
Median	43.4		22.4		
Range	0.9-434.1		0.9-450		
Percentage of blood blasts					.83
Median	58		57		
Range	0-97		0-99		
Percentage of bone marrow blasts					.03
Median	70		66		
Range	4-97		7-96		
FAB category					< .001
M0	1	1	7	4	
M1	29	27	54	27	
M2	18	17	71	36	
M4	33	31	41	21	
M5	26	24	21	11	
M6	0	0	3	2	
NPM1					< .001
Mutated	107	75	146	53	
Wild type	35	25	127	47	
FLT3-ITD					.01
Present	62	44	85	31	
Absent	80	56	188	69	
CEBPA					< .001
Mutated	7	5	58	21	
Single mutated	4		26		
Double mutated	3		32		
Wild type	135	95	215	79	
ELN genetic group†					.10
Favorable	60	42	140	51	
Intermediate-I	82	58	133	49	
FLT3-TKD					1.00
Present	10	7	21	8	
Absent	128	93	245	92	
WT1					.37
Mutated	10	7	28	10	
Wild type	132	93	245	90	

(continued in next column)

Table 1. Clinical and Molecular Characteristics of 415 Patients With Primary Cytogenetically Normal Acute Myeloid Leukemia According to *DNMT3A* Mutation Status (continued)

Characteristic	*DNMT3A* Mutated (n = 142) No.	%	*DNMT3A* Wild Type (n = 273) No.	%	*P**
TET2					.54
Mutated	31	22	67	25	
Wild type	110	78	202	75	
MLL-PTD					1.00
Present	7	6	15	6	
Absent	116	94	228	94	
IDH1					.07
R132	22	16	26	10	
Wild type	118	84	246	90	
IDH2					
Mutated	24	17	51	19	.79
Codon R140	18		44		
Codon R172	6		7		
Wild type	116	83	221	81	
ERG expression group‡					.39
High	56	55	92	49	
Low	45	45	94	51	
BAALC expression group‡					.38
High	45	47	104	53	
Low	51	53	93	47	

Abbreviations: FAB, French-American-British classification; ELN, European LeukemiaNet; *FLT3*-ITD, internal tandem duplication of the *FLT3* gene; *FLT3*-TKD, tyrosine kinase domain mutation in the *FLT3* gene; *MLL*-PTD, partial tandem duplication of the *MLL* gene.

*P values for categorical variables are from Fisher's exact test; P values for continuous variables are from the Wilcoxon rank sum test.

†The ELN favorable genetic group includes patients with mutated *CEBPA* and/or mutated *NPM1* without *FLT3*-ITD.[4] The ELN intermediate-I risk group comprises the remaining patients with CN-AML who had wild-type *CEBPA* and wild-type *NPM1* with or without *FLT3*-ITD or mutated *NPM1* with *FLT3*-ITD.

‡The median expression value was used as a cut point.

computational algorithm that evaluates the disease-causing potential of gene mutations on the basis of evolutionary conservation and structural protein features.

Associations of DNMT3A *Mutations With Pretreatment Clinical and Molecular Characteristics*

No differences in age, sex, or race were observed between patients with and without *DNMT3A* mutations. However, *DNMT3A*-mutated patients had higher WBC counts ($P < .001$) and BM blasts percentages ($P = .03$) and harbored *NPM1* mutations ($P < .001$) and *FLT3*-ITD ($P = .01$) more often and *CEBPA* mutations ($P < .001$) less often than those with *DNMT3A*-wt (Table 1).

Because AML biology and treatment regimens differ between younger and older patients, and it is unclear whether R882-*DNMT3A* and non–R882-*DNMT3A* mutations are functionally and clinically equivalent, we performed subgroup analyses taking age and *DNMT3A* mutation types into account. Younger R882-*DNMT3A*–mutated patients more often had *NPM1* mutations ($P = .02$) and *FLT3*-ITD ($P = .03$) and less often had *CEBPA* mutations ($P < .001$), *WT1* mutations ($P = .02$), and low *ERG*

Table 2. Age Group–Adjusted Analysis of Outcomes of Patients With Primary Cytogenetically Normal Acute Myeloid Leukemia According to *DNMT3A* Mutation Status

End Point	*DNMT3A*-mut (n = 142)	R882-*DNMT3A* (n = 92)	non–R882-*DNMT3A* (n = 50)	*DNMT3A*-wt (n = 273)	*P* (*DNMT3A*-mut *v* *DNMT3A*-wt)	*P* (R882-*DNMT3A* *v* *DNMT3A*-wt)	*P* (non–R882-*DNMT3A* *v* *DNMT3A*-wt)
Complete remission					.42	.85	.21
Odds ratio	1.22	1.05	1.62	Reference group			
95% CI	0.75 to 1.96	0.61 to 1.83	0.76 to 3.43				
Disease-free survival					.03	.03	.19
Hazard ratio	1.34	1.42	1.30	Reference group			
95% CI	1.02 to 1.75	1.03 to 1.96	0.88 to 1.90				
Overall survival					.07	.05	.39
Hazard ratio	1.25	1.32	1.16	Reference group			
95% CI	0.98 to 1.57	1.01 to 1.74	0.83 to 1.63				

Abbreviations: mut, mutated; wt, wild type.

expression ($P = .04$) than *DNMT3A*-wt patients (Data Supplement). Younger patients with non–R882-*DNMT3A* mutations were more frequently *NPM1*-mutated ($P = .02$) and showed trends toward a higher frequency of *FLT3*-ITD ($P = .11$) and lower frequency of *CEBPA* mutations ($P = .07$; Data Supplement). Among older patients, those with R882-*DNMT3A* mutations showed trends toward a higher frequency of *NPM1* mutations ($P = .09$) and *FLT3*-ITD ($P = .15$) and lower frequency of *CEBPA* mutations ($P = .14$; Data Supplement). Older patients with non–R882-*DNMT3A* mutations were more likely *NPM1*-mutated ($P = .003$) and, by trend, *WT1*-mutated ($P = .07$) and less likely *CEBPA*-mutated ($P = .05$; Data Supplement).

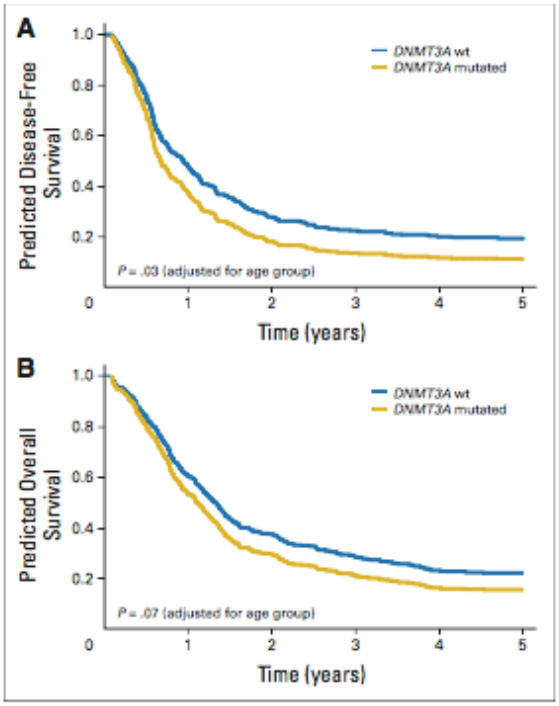

Fig 1. Age group–adjusted clinical outcome for patients with and without *DNMT3A* mutations. (A) Disease-free survival. (B) Overall survival. The curves are adjusted for age group. wt, wild type.

Association of DNMT3A *Mutation Status With Clinical Outcome*

When younger and older patients were considered together in analyses adjusted for age group, *DNMT3A* mutations were not associated with the probability of CR attainment ($P = .42$; Table 2). With a median follow-up of 7.5 years (range, 2.3 to 12.4 years) for patients alive, those harboring *DNMT3A* mutations had shorter disease-free survival (DFS; $P = .03$) and a trend toward shorter OS ($P = .07$) than *DNMT3A*-wt patients (Table 2; Fig 1). In a multivariable analysis for DFS (Table 3), *DNMT3A* mutations were associated with a 47% increased risk of relapse or death ($P = .01$), once adjusted for *FLT3*-ITD, *WT1* mutations, *MLL*-PTD status, and age group. In contrast, once adjusted for other clinical and molecular prognosticators, there was no association of *DNMT3A* mutation status with OS.

Association of Different DNMT3A *Mutation Types With Clinical Outcome*

We tested the association of the two types of *DNMT3A* mutations with outcome of younger and older patients separately because these age groups were treated on Cancer and Leukemia Group B protocols that differ in chemotherapy intensity (Data Supplement). Neither type of *DNMT3A* mutation had an impact on the probability of achieving CR in younger or older patients.

In younger patients, R882-*DNMT3A* mutations were not significantly associated with DFS or OS (Table 4, Figs 2A and 2B). In contrast, patients harboring non–R882-*DNMT3A* mutations had a significantly shorter DFS ($P = .007$; 3-year rates, 20% *v* 49%; Fig 2A) and a trend toward shorter OS ($P = .09$; 3-year rates, 29% *v* 52%; Fig 2B) than *DNMT3A*-wt patients (Table 4). In a multivariable analysis for DFS (Table 3), patients with non–R882-*DNMT3A* mutations had an almost three-fold increased risk of relapse or death ($P = .002$), once adjusted for *FLT3*-ITD and mutations in *NPM1*, *CEBPA*, and *WT1*. Likewise, in a multivariable model for OS (Table 3), the risk of death of non–R882-*DNMT3A*–mutated patients was more than twice that of *DNMT3A*-wt patients ($P = .02$) after adjustment for *FLT3*-ITD, *NPM1*, *CEBPA*, and *WT1* mutation status.

Table 3. Multivariable Analyses for Outcome in Patients With Primary Cytogenetically Normal Acute Myeloid Leukemia

Group	Disease-Free Survival			Overall Survival		
	HR	95% CI	*P*	HR	95% CI	*P*
All patients						
DNMT3A, mutated *v* wild type	1.47	1.08 to 2.00	.01	*DNMT3A* mutation status was not significantly associated with OS upon adjusting for other variables		
FLT3-ITD, ITD *v* no ITD	1.82	1.34 to 2.48	< .001			
WT1, mutated *v* wild type	2.17	1.26 to 3.72	.005			
MLL-PTD, present *v* absent	1.95	1.12 to 3.39	.02			
Age group, older *v* younger	2.53	1.89 to 3.39	< .001			
Patients, age < 60 years						
DNMT3A, non-R882-mutated *v* wild type	2.78	1.45 to 5.38	.002	2.24	1.17 to 4.30	.02
NPM1, mutated *v* wild type	0.52	0.29 to 0.95	.03	0.38	0.23 to 0.64	< .001
FLT3-ITD, ITD *v* no ITD	1.79	1.07 to 3.02	.03	1.70	1.06 to 2.76	.03
CEBPA, double-mutated *v* single-mutated or wild type	0.21	0.09 to 0.50	< .001	0.15	0.06 to 0.35	< .001
WT1, mutated *v* wild type	4.88	2.37 to 10.04	< .001	5.91	3.20 to 10.90	< .001
Patients, age ≥ 60 years						
DNMT3A, R882-mutated *v* wild type	1.85	1.20 to 2.84	.005	1.76	1.24 to 2.49	.002
NPM1, mutated *v* wild type	0.54	0.36 to 0.80	.002	0.48	0.35 to 0.66	< .001
FLT3-ITD, ITD *v* no ITD	2.00	1.34 to 2.98	< .001	1.80	1.31 to 2.47	< .001
Age, each 10 year increase	0.98	0.93 to 0.99	.02			

Abbreviations: *FLT3*-ITD, internal tandem duplication of the *FLT3* gene; HR, hazard ratio; *MLL*-PTD, partial tandem duplication of the *MLL* gene.

In older patients, R882-*DNMT3A* mutations were associated with significantly shorter DFS ($P = .006$; 3-year rates, 3% *v* 21%; Fig 2C) and OS ($P = .01$; 3-year rates, 4% *v* 24%; Fig 2D), whereas non–R882-*DNMT3A* mutations were not (Table 4, Figs 2C and 2D). In a multivariable model for DFS (Table 3), R882-*DNMT3A* mutations remained associated with an 85% increased risk of relapse or death ($P = .005$) after adjustment for *NPM1* mutation and *FLT3*-ITD status and age. Similarly, in a multivariable model for OS (Table 3), R882-*DNMT3A* mutations were associated with a 76% increased risk of death ($P = .002$) once adjusted for *NPM1* mutation and *FLT3*-ITD status.

Gene- and microRNA-Expression Signatures Associated With DNMT3A *Mutations*

To gain insights into the biology of *DNMT3A*-mutated CN-AML, we studied mutation-associated gene-expression signatures in a subset of patients (n = 278) with available material. Clinical and

Table 4. Outcome of Patients With Primary Cytogenetically Normal Acute Myeloid Leukemia, According to Age Group and Type of *DNMT3A* Mutation

End Point	*DNMT3A*-mut	R882-*DNMT3A*	non–R882-*DNMT3A*	*DNMT3A*-wt	*P* (*DNMT3A*-mut *v* *DNMT3A*-wt)	*P* (R882-*DNMT3A* *v* *DNMT3A*-wt)	*P* (non–R882-*DNMT3A* *v* *DNMT3A*-wt)
Patients < 60 years of age, no.	64	47	17	117			
Complete remission rate, %	81	79	88	96 (82)	1.00	.66	.74
Disease-free survival					.16	.68	.007
Median, years	1.1	1.3	0.7	2.9			
% Disease-free at 3 years	37	43	20	49			
95% CI	24 to 49	27 to 58	5 to 42	39 to 58			
Overall survival					.36	.78	.09
Median, years	1.4	3.5	1.3	3.6			
% Alive at 3 years	45	51	29	52			
95% CI	33 to 57	36 to 64	11 to 51	43 to 61			
Patients ≥ 60 years of age, no.	78	45	33	156			
Complete remission rate, %	73	71	76	66	.30	.59	.31
Disease-free survival					.11	.006	.91
Median, years	0.7	0.7	1.0	1.0			
% Disease-free at 3 years	11	3	20	21			
95% CI	4 to 20	1 to 14	7 to 37	14 to 30			
Overall survival					.10	.01	.96
Median, years	1.0	0.9	1.1	1.3			
% Alive at 3 years	12	4	24	24			
95% CI	6 to 21	1 to 13	11 to 39	17 to 31			

Abbreviations: mut, mutated; wt, wild type.

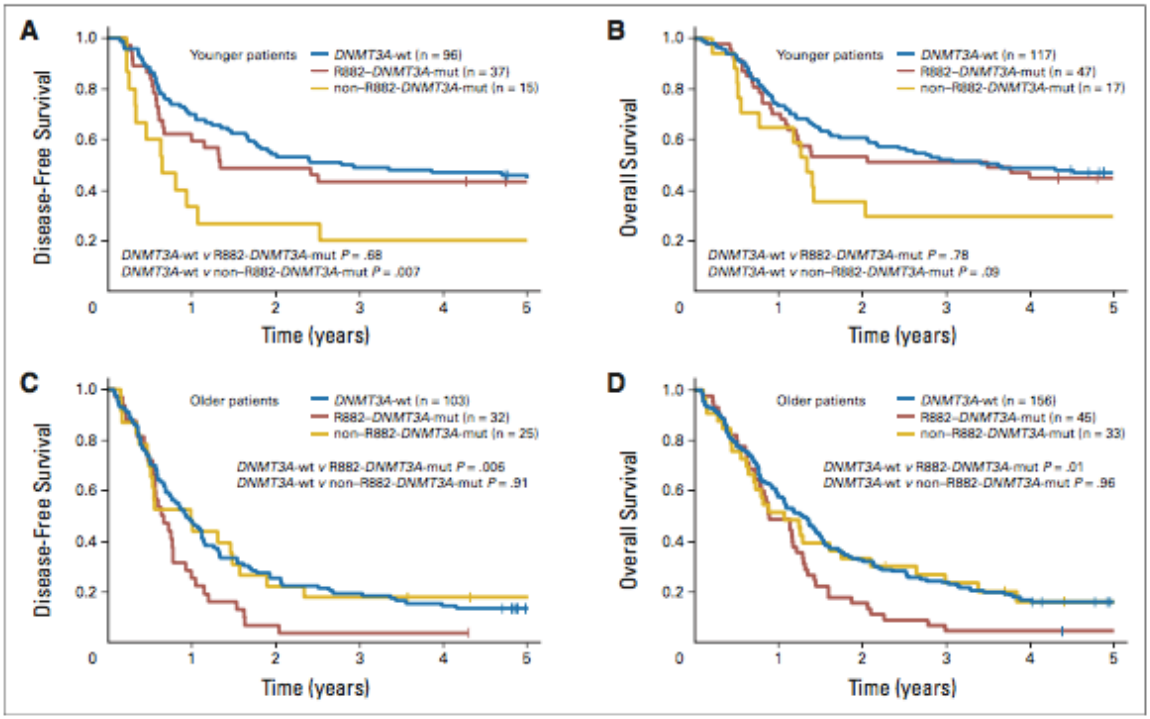

Fig 2. Kaplan-Meier survival curves according to *DNMT3A* mutation type (R882-*DNMT3A* v non–R882-*DNMT3A* mutations v *DNMT3A* wild type). (A) Disease-free survival and (B) overall survival of younger (< 60 years) patients. (C) Disease-free survival and (D) overall survival of older (≥ 60 years) patients. mut, mutated; wt, wild type.

molecular characteristics and outcome of this subset were similar to those of patients not analyzed.

A gene-expression signature associated with *DNMT3A* mutations comprised 1,886 differentially expressed probe sets: 1,323 were upregulated and 563 downregulated in *DNMT3A*-mutated patients (Data Supplement). The most upregulated known gene was *VCAN*, encoding a protein involved in cell adhesion, proliferation, migration, and angiogenesis; the most downregulated gene was *ALAS2*, involved in the heme biosynthetic pathway. However, the signature had an overall cross-validated accuracy of only 67% for predicting *DNMT3A* mutation status (62% sensitivity; 70% specificity), thereby suggesting the contributing effect of other associated molecular aberrations.

When we attempted to derive gene-expression signatures associated with specific types of *DNMT3A* mutations, no significant signature separated patients harboring non–R882-*DNMT3A* mutations (n = 32) from those with *DNMT3A*-R882 mutation (n = 60).

For microRNA profiling, younger and older patients were analyzed separately to avoid confounding batch effects. Testing for differentially expressed microRNAs revealed no signature associated with *DNMT3A* mutations in the younger group. In contrast, we derived a signature consisting of 12 microRNAs associated with *DNMT3A* mutations in older patients (Data Supplement), with four microRNA probes upregulated, including a member of the *miR-10* family reportedly associated with *NPM1* mutations,[13] and eight microRNA probes downregulated, including *miR-181c*, a member of the *miR-181* family associated with *CEBPA* mutations.[8] However, these features might reflect confounding as a result of the significant positive association of *DNMT3A* mutations with *NPM1* mutations and the negative association with *CEBPA* mutations. This microRNA-expression signature had an overall accuracy of only 58% for predicting *DNMT3A* mutation status (49% sensitivity; 62% specificity).

DISCUSSION

Advanced sequencing technologies have allowed analysis of the whole genome of AML blasts. Application of these technologies has recently identified two novel recurrent gene mutations in CN-AML, first *IDH1* mutations[50] and, more recently, *DNMT3A* mutations.[31] As this approach becomes broadly used, it is likely that previously unrecognized mutations in AML will continue to emerge. Because these mutations have the potential to contribute to myeloid leukemogenesis and become prognostic factors and/or therapeutic targets, it is imperative to rapidly test their biologic and clinical impact on patients with AML. However, from previously discovered mutated or aberrantly expressed genes in CN-AML, we have learned that only rarely is testing for a single genetic alteration sufficient for accurate outcome prediction and treatment guidance.[13] Instead, the clinical impact of most molecular markers is influenced by other, concurrent molecular

aberrations.[12,14-16,21] Therefore, to fully understand the clinical significance of emerging molecular markers, such as *DNMT3A* mutations, they need to be evaluated in large series of patients homogeneous for age and type of disease (primary *v* secondary or treatment-related AML), similarly treated and fully characterized for established prognostic markers. To our knowledge, our study analyzed *DNMT3A* mutations in the largest CN-AML patient cohort to date and is first to report subgroup analyses and multivariable models considering different types of *DNMT3A* mutations in distinct age groups.

We found that *DNMT3A* mutations were among the most common mutations in CN-AML, occurring in 34% of patients, with a similar frequency among younger and older patients, and were significantly associated with *NPM1* mutations, *FLT3*-ITD, and wild-type *CEBPA*. Regarding prognostic significance, we showed that *DNMT3A* mutations had worse DFS and OS, after adjustment for age. Moreover, we observed that the prognostic significance of *DNMT3A* mutations depended both on age and the type of mutation (R882-*DNMT3A v* non–R882-*DNMT3A*) considered concurrently (see Fig 2 and also Data Supplement). In younger patients, only non–R882-*DNMT3A* mutations were associated with worse clinical outcome, whereas R882-*DNMT3A* mutations had no prognostic significance. Conversely, in older patients, only R882-*DNMT3A* mutations, not non–R882-*DNMT3A* mutations, were independently associated with worse outcome. The reasons why the prognostic significance of different *DNMT3A* mutation types varies in younger and older patients are currently unknown. One could postulate that this is related to their association with other prognosticators. Thus, in older patients, the potentially negative prognostic significance of non–R882-*DNMT3A* mutations might have been somewhat offset by a high incidence (79%) of accompanying *NPM1* mutations, known to favorably affect prognosis of older patients.[13] However, two thirds of older patients with the prognostically adverse R882-*DNMT3A* mutations also harbored *NPM1* mutations, and slight differences in frequencies of other molecular markers between patients harboring R882-*DNMT3A* and non–R882-*DNMT3A* mutations, both in the older and younger age groups, do not seem sufficient to account for the differential association of the two *DNMT3A* mutation types on treatment outcome.

Our results differ somewhat from those reported by Ley et al,[31] who found a strong, independent association of *DNMT3A* mutations with OS, and those by Thol et al,[33] who studied only patients younger than 60 years and who found that in the CN-AML subgroup, *DNMT3A* mutations were associated with a lower CR rate and shorter OS in multivariable analyses. These discrepancies may be related to differences in the patient populations analyzed with respect to their size, cytogenetics, molecular markers, age, disease type, and treatment. Furthermore, previous studies did not include older patients[33] or included only a small proportion of older patients and did not present data on CR rates, DFS, or multivariable analyses for patients with CN-AML.[31] Therefore, a direct comparison of the findings across studies is not possible.

We also report the first gene- and microRNA-expression signatures associated with *DNMT3A* mutations. However, the accuracy of the gene-expression signature in predicting *DNMT3A* mutational status was only 67%. These results are consistent with an unsupervised analysis of gene-expression array data reported by Ley et al,[31] where no patient cluster was clearly linked to *DNMT3A* mutation status. Similarly, a microRNA-expression signature derived in older patients with CN-AML comprised microRNAs strongly associated with other markers (ie, *NPM1* mutations and wild-type *CEBPA*) and was not accurate in predicting *DNMT3A* mutational status. These results suggest that *DNMT3A* mutations have no strong impact on genome-wide gene- and microRNA-expression profiles in CN-AML. The signatures we identified might at least partially reflect the association of *DNMT3A* mutation status with other molecular markers that are themselves associated with characteristic gene- and microRNA-expression signatures.

The mechanisms through which *DNMT3A* mutations contribute to leukemogenesis are not yet characterized. Although two studies[31,33] found no differences in global DNA methylation or changes in gene methylation patterns in *DNMT3A*-mutated patients, other reports[30,32] suggested that most of the *DNMT3A* mutations decrease the enzymatic activity of the encoded protein. Uncovering how *DNMT3A* mutations affect DNA methylation and epigenetic regulation of gene expression may have ramifications for treatment selection because DNA hypomethylating agents, such as decitabine, are increasingly used for up-front or salvage therapies in older patients with AML,[51] and response to these drugs may be affected by alterations in *DNMT3A* function.[52]

In summary, testing for the mutations in *DNMT3A* may provide a new tool for refining age-related risk classification of CN-AML. The strongest prognostic significance was found in older patients harboring R882-*DNMT3A* mutations, whereas non–R882-*DNMT3A* mutations were associated with relapse risk in younger patients. The gene- and microRNA-expression signatures were not accurate in predicting *DNMT3A* mutational status likely because they are affected by other, concurrent molecular markers. Thus the contribution of the *DNMT3A* mutations to myeloid leukemogenesis requires further investigation, as does the usefulness of *DNMT3A* mutations for risk stratification both in patients with CN-AML and in other cytogenetic and molecular subsets of AML.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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LETTER TO THE EDITOR

Prognostic impact of the European LeukemiaNet standardized reporting system in older AML patients receiving stem cell transplantation after non-myeloablative conditioning

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AML is a biologically heterogeneous disease with highly diverse patient outcomes. A reporting system has been developed by the European LeukemiaNet (ELN) to standardize AML classification.[1] The prognostic impact of the four ELN genetic groups (favorable, intermediate-I, intermediate-II and adverse) has mainly been shown in two studies.[2,3] While patients within the favorable genetic group had the best outcome and patients within the adverse genetic group had the worst outcome, the prognosis for younger patients (< 60 years) within the intermediate-II genetic group was superior compared to the intermediate-I genetic group. In older patients (≥60 years), the outcome was comparable between the two intermediate genetic groups.[2–4] These studies focused predominantly on patients who did not receive a hematopoietic stem cell transplantation (HCT) in first CR.[1–3]

HCT is an important treatment option for AML patients for whom a suitable donor is available.[5,6] To date, only the study by Röllig *et al.*[2] has analyzed the prognostic impact of the ELN genetic groups in a subgroup of 165 patients under 56 years receiving myeloablative conditioning (12 Gy TBI or busulfan (16 mg/kg) followed by cyclophosphamide (120 mg/kg)) HCT.

Table 1. Characteristics of 159 older AML patients treated with NMA-HCT

	All patients	*ELN genetic groups*			
		Favorable	*Intermediate-I*	*Intermediate-II*	*Adverse*
All patients, *n* (%)	159	28 (18)	35 (22)	39 (25)	57 (36)
Age, median, years (range)	66 (60–76)	66 (60–74)	67 (61–75)	68 (60–74)	65 (60–76)
Female, *n* (%)	73 (46)	16 (57)	12 (34)	15 (38)	29 (51)
Male, *n* (%)	86 (54)	12 (43)	23 (66)	24 (62)	28 (49)
Hemoglobin, g/dL (range)	8.5 (5.3–14.1)	8.4 (6.1–12.5)	9.0 (6.4–14.1)	8.8 (5.8–11.7)	8.2 (5.3–13.3)
Platelet count, $\times 10^9$/L (range)	64 (1–305)	75 (3–207)	80 (1–178)	72 (2–305)	41 (2–201)
WBC, $\times 10^9$/L (range)	4.5 (0.7–385)	10.9 (1–160)	7.5 (0.9–295)	2.5 (0.7–385)	3.7 (0.7–78)
Blood blasts, % (range)	20 (0–97)	23 (2–92)	15 (0–97)	26 (4–97)	17 (0–82)
BM blasts, % (range)	52 (3–95)	56 (3–88)	51 (20–95)	55 (21–95)	52 (21–92)
Therapy cycles to achieve CR1, *n* (range)	1 (1–4)	1 (1–2)	1 (1–4)	1 (1–3)	2 (1–3)
Diseases status					
De novo, *n* (%)	81 (51)	18 (64)	21 (60)	11 (28)	31 (54)
Following MDS/MPN, *n* (%)	61 (38)	8 (29)	11 (32)	25 (64)	17 (30)
Therapy-related, *n* (%)	17 (11)	2 (7)	3 (6)	3 (8)	9 (16)
Median year of Tx (range)	2009 (2003–2013)	2008 (2003–2013)	2008 (2003–2013)	2009 (2000–2014)	2009 (2001–2014)
Donor status					
Matched-related, *n* (%)	23 (14)	6 (21)	6 (17)	6 (15)	5 (9)
Matched-unrelated, *n* (%)	89 (56)	18 (64)	21 (60)	20 (51)	30 (53)
Mismatched-unrelated, *n* (%)	47 (30)	4 (15)	8 (23)	13 (34)	22 (37)
Remission status					
CR, *n* (%)	114 (72)	24 (85)	30 (86)	27 (70)	33 (58)
CR1, *n* (%)	89 (56)	20 (71)	22 (63)	19 (49)	28 (49)
CR2/CR3, *n* (%)	25 (16)	4 (14)	8 (23)	8 (21)	5 (9)
CRi, *n* (%)	24 (15)	3 (11)	2 (6)	6 (15)	13 (23)
PR, *n* (%)	21 (13)	1 (4)	3 (9)	6 (15)	11 (19)

Abbreviations: BM = bone marrow; CRi = CR with incomplete recovery; ELN = European LeukemiaNet; HCT = hematopoietic stem cell transplantation; MDS = myelodysplastic syndrome; MPN = myeloproliferative neoplasm; NMA = non-myeloablative; Tx = transplantation; WBC = WBC count.

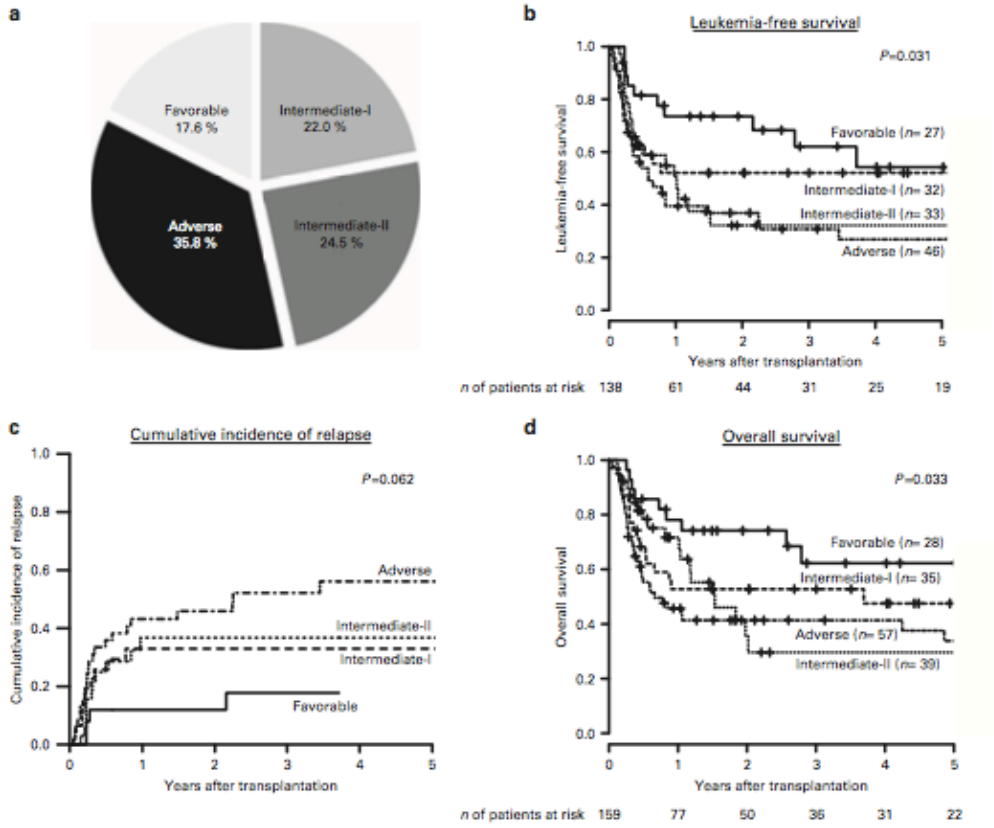

Figure 1. Distribution of the ELN genetic groups (**a**) and leukemia-free survival (**b**), cumulative incidence of relapse (**c**), and overall survival (**d**) according to the ELN genetic groups of 159 older AML patients (⩾60 years) after NMA-HCT.

For these patients, the authors observed no significant outcome differences between the favorable, intermediate-I and intermediate-II genetic groups, but a worse prognosis of the adverse genetic group. Another study by Oran *et al.*[7] analyzed the prognostic impact of a modified ELN classification system—lacking information on *NPM1* or *CEBPA* gene mutation status—in 423 AML patients (including 101 patients ⩾60 years) who underwent HCT in first CR after various conditioning regimens. Here, the modified favorable risk and modified intermediate-II risk genetic groups had the best outcomes for both younger and older patients.

In the last decade, non-myeloablative (NMA) conditioning regimens for HCT have been used increasingly, since they allow patients to benefit from HCT, who are not eligible to receive myeloablative conditioning due to advanced age or comorbidities. NMA regimens are mainly immunosuppressive with low toxicity, and their therapeutic action is based almost exclusively on an immunological GvL effect. However, to our knowledge, studies analyzing the prognostic impact of the ELN standardized reporting system in AML patients receiving NMA-HCT have not been previously reported.

Here, we analyzed 159 older AML patients (⩾60 years) with a median age of 66 years (range 60–76 years) diagnosed between May 2000 and December 2012 at our institution. All patients received NMA-HCT (2 Gy TBI with or without fludarabine) after cytarabine-based induction cycles (for further details, see Supplementary Information). Written informed consent for participation in these studies was obtained in accordance with the Declaration of Helsinki. Basic clinical and biological characteristics of the analyzed patients are shown in Table 1 and Supplementary Table S1. The median follow-up for patients alive was 2.9 years. Bone marrow or peripheral blood from the day of diagnosis was used for analyses. Cytogenetics were determined using standard techniques for banding and FISH. We analyzed the presence of *FLT3*-ITD (internal tandem duplication),[8] and the mutation status of *NPM1*,[9] as previously described, and that of the *CEBPA* gene (see Supplementary Information and Supplementary Table S2) and classified the patients into four genetic groups according to ELN recommendations.[1]

Patient distribution according to the ELN genetic groups was: 17.6% favorable ($n=28$), 22.0% intermediate-I ($n=35$), 24.5% intermediate-II ($n=39$) and 35.8% adverse ($n=57$; Figure 1a). The ELN genetic group distribution of our cohort was comparable to previously published data, for example, by Mrózek *et al.*[3] for older patients ($P=0.59$; 20%, 19%, 30% and 31%, respectively, according to ELN genetic groups).

We observed a leukemia-free survival of 42.5% (95% confidence interval 34.4–52.6%), a cumulative incidence of relapse of 37.9% (95% confidence interval 29.1–46.6%) after 3 years, a median overall survival after NMA-HCT of 2.0 years and a 3-year overall

survival of 46.5% (95% confidence interval 38.6–84.8%). These data are comparable with data from previous publications for TBI-based NMA conditioning regimens (see Supplementary Information).[10,11]

We found a significantly different leukemia-free survival ($P = 0.031$, Figure 1b), a trend for a different cumulative incidence of relapse ($P = 0.062$, Figure 1c) and a significantly different overall survival ($P = 0.033$, Figure 1d) by grouping AML patients according to the ELN classification system. Non-relapse mortality was not significantly different between the ELN genetic groups ($P = 0.56$, Supplementary Figure S4). However, pairwise comparisons of overall survival among the ELN genetic groups yielded a significant difference only between the favorable and intermediate-II ($P = 0.01$), and between the favorable and adverse ($P = 0.005$) genetic groups. Notably, with the caveat of limited group sizes, no significant outcome differences were observed between the intermediate-I, intermediate-II and adverse genetic groups, suggesting that the ELN genetic grouping may have a reduced prognostic impact in NMA-HCT-treated AML patients compared to patients receiving a chemotherapy-based consolidation in first CR.[2,3] Interestingly, Röllig *et al.*[2] detected no clear outcome differences between favorable, intermediate-I and intermediate-II genetic groups in their MAC-HCT patient set, while adverse genetic group patients had a significantly worse prognosis. Thus, HCT may reduce the prognostic impact of the ELN reporting system at AML diagnosis.

Since it has been recommended[1] to report outcomes for specific subsets within each ELN genetic group, we analyzed those with a sufficient number of patients available (Supplementary Figures S1–S3).

The favorable genetic group consists of patients with a core-binding factor AML (t(8;21)(q22;q22) and inv(16)(p13.1q22), or t(16;16)(p13.1;q22)), a mutation in the gene *CEBPA* or a *NPM1* mutation without *FLT3*-ITD (both with normal karyotype). In our cohort, no significant difference between the subsets was observed (Supplementary Figure S1). A significantly improved outcome within the favorable genetic group has been described for older patients with core-binding factor AML receiving a chemotherapy-based consolidation in first CR.[2,3] However, our set included only three patients with core-binding factor AML, preventing further analysis.

Similarly, no difference in outcome for the subsets was observed in the intermediate-I genetic group (Supplementary Figure S2). Interestingly, Mrózek *et al.*[3] and several other publications described a significantly worse outcome for older patients with *FLT3*-ITD who were consolidated with chemotherapy in first CR.[12,13] In our cohort of NMA-HCT treated patients, we found no significant impact of the presence of a *FLT3*-ITD on outcome within the intermediate-I genetic group, similar to a previous report analyzing the *FLT3*-ITD status in patients receiving HCT.[7,13] However, the number of patients in the intermediate-I group was low ($n = 35$), showing the need of further investigations of the impact of the presence of a *FLT3*-ITD in older NMA-HCT treated AML patients.

The adverse genetic group consists of several subsets, of which the complex karyotype is the largest. We found that the subsets within the adverse genetic group differed with regard to leukemia-free survival ($P = 0.03$) and overall survival ($P = 0.04$; Supplementary Figure S3). Patients with complex karyotype had the poorest prognosis within the adverse genetic group (data are shown in Supplementary Information). This is in concordance with data on older patients treated with chemotherapy in first CR, in which patients with complex karyotype also had the worst outcome within the adverse genetic group.[2–4,7] The second and third largest subsets in our cohort were patients with a monosomy 7, known to be the most frequent monosomy in AML, and loss of chromosome 5, respectively. Both aberrations have been described as negative prognosticators[14,15] in AML patients who for the most part did not receive HCT as consolidation in first CR. However, patients of both subsets treated with NMA-HCT showed an improved outcome compared to the complex karyotype within the adverse genetic group, which is similar to previous publications for older, transplanted patients.[7]

In conclusion, we observed outcome differences between the ELN genetic groups for AML patients receiving NMA-HCT, with patients within the favorable genetic group having the best prognosis. However, pairwise comparison revealed that only patients within the favorable genetic group performed better than patients in the intermediate-II or adverse genetic groups. The data presented here suggest that the ELN genetic grouping may have a reduced prognostic impact for patients receiving NMA-HCT as compared to those receiving a chemotherapy-based consolidation in first CR.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

MB and SS designed and performed the experiments. KS performed the experiments. MB, MJ and SS analyzed and interpreted the data. TL, MC, GB, VV, WP and G-NF provided administrative and technical support. MB and SS wrote, and all authors reviewed and approved the manuscript. DN and SS supervised the study.

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Supplementary Information accompanies this paper on Bone Marrow Transplantation website (http://www.nature.com/bmt)

2.3 Aberrant *BAALC*, *ERG*, and *MN1* expression at diagnosis as prognostic factors in older AML patients

BAALC, *ERG*, and *MN1* expression levels have been identified as prognostic markers in younger (< 60 years) CN-AML adults at diagnosis. However, most AML patients are diagnosed aged over 60 years, thus the prognostic impact of *BAALC*, *ERG*, and *MN1* expression levels in older patients required further investigation.

In the first paper integrated in this paragraph, diagnostic expression levels of *BAALC* and *ERG* in older AML patients were evaluated. Low *BAALC* and *ERG* expression levels were associated with better outcome in univariable and multivariable analyses. Expression levels of both *BAALC* and *ERG* were the only factors significantly associated with OS upon multivariable analysis. Furthermore, gene and miR expression signatures associated with *BAALC* and *ERG* expression were assessed.

In the second paper included in this paragraph, *MN1* expression levels prior to therapy were analyzed in older CN-AML patients. Low *MN1* expressers had higher CR rates and longer OS and event-free survival (EFS). *MN1* expresser-status-associated gene- and miR-expression signatures revealed underexpression of drug resistance and adverse outcome predictors, and overexpression of *HOX* genes and *HOX*-gene-embedded miRs in low *MN1* expressers.

Interestingly, similarities in the expression signatures in older AML patients in both papers compared to younger patients were revealed, suggesting that the historically evolved 60 years might not be the optimal age cut-off regarding biological differences in AML patients. In conclusion these two papers expanded the knowledge on the strong prognostic impact of *BAALC*, *ERG*, and *MN1* expression levels in older AML patients and elucidated the associated biology by demonstrating gene- and miR-expression signatures.

Manuscripts included in this paragraph:

- **Schwind S**, Marcucci G, Maharry K, Radmacher MD, Mrózek K, Holland KB, Margeson D, Becker H, Whitman SP, Wu Y, Metzeler KH, Powell BL, Kolitz JE, Carter TH, Moore JO, Baer MR, Carroll AJ, Caligiuri MA, Larson RA, Bloomfield CD: *BAALC* and *ERG* expression levels are associated with outcome and distinct gene- and microRNA-expression profiles in older patients with de novo cytogenetically normal acute myeloid leukemia: A Cancer and Leukemia Group B study. *Blood.* 2010; 116:5660-9.

- **Schwind S***, Marcucci G*, Kohlschmidt J, Radmacher MD, Mrózek K, Maharry K, Becker H, Metzeler KH, Whitman SP, Wu Y, Powell BL, Baer MR, Kolitz JE, Carroll AJ, Larson RA, Caligiuri MA, Bloomfield CD: Low expression of *MN1* associates with better treatment response in older patients with de novo cytogenetically normal acute myeloid leukemia. *Blood.* 2011; 118:188-98. *shared first-author

BAALC and *ERG* expression levels are associated with outcome and distinct gene and microRNA expression profiles in older patients with de novo cytogenetically normal acute myeloid leukemia: a Cancer and Leukemia Group B study

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***BAALC* and *ERG* expression levels are prognostic markers in younger (< 60 years) cytogenetically normal acute myeloid leukemia (CN-AML) adults; their prognostic impact in older (≥ 60 years) patients requires further investigation. We evaluated pretreatment expression of *BAALC* and *ERG* in 158 de novo patients treated on cytarabine/daunorubicin-based protocols. The patients were also characterized for other established molecular prognosticators. Low *BAALC* and *ERG* expression levels were associated with better outcome in univariable and multivariable analyses. Expression levels of both *BAALC* and *ERG* were the only factors significantly associated with overall survival upon multivariable analysis. To gain biological insights, we derived gene expression signatures associated with *BAALC* and *ERG* expression in older CN-AML patients. Furthermore, we derived the first microRNA expression signatures associated with the expression of these 2 genes. In low *BAALC* expressers, genes associated with undifferentiated hematopoietic precursors and unfavorable outcome predictors were down-regulated, whereas *HOX* genes and *HOX*-gene–embedded microRNAs were up-regulated. Low *ERG* expressers presented with down-regulation of genes involved in the DNA-methylation machinery, and up-regulation of *miR-148a*, which targets *DNMT3B*. We conclude that in older CN-AML patients, low *BAALC* and *ERG* expression associates with better outcome and distinct gene and microRNA expression signatures that could aid in identifying new targets and novel therapeutic strategies for older patients. (*Blood*. 2010; 116(25):5660-5669)**

Introduction

Acute myeloid leukemia (AML) is a cytogenetically and molecularly heterogeneous disease characterized by clonal proliferation of myeloid precursors and maturation arrest. Despite progress in our understanding of the biology of this disease and investigation of therapies targeting distinct clinical, cytogenetic, and/or molecular subsets, outcome remains poor for the majority of patients. This is especially true for patients aged 60 years or more, of whom only approximately 7%-15% achieve long-term survival.[1,2] The reasons for the poor outcome of this older patient population may not only relate to higher frequencies of secondary disease (ie, AML after antecedent hematologic disorders and/or therapy-related disease), high-risk cytogenetics, clinical comorbid conditions, and poor performance status, but also to the presence of specific molecular genetic alterations, including gene mutations and changes in gene expression.[3]

To date, the prognostic significance of molecular genetic alterations has been studied most extensively in younger (< 60 years) patients and found its maximum applicability in cytogenetically normal AML (CN-AML), which constitutes the largest AML subset.[4] In this cytogenetic subset, mutations in the *NPM1*[5] and *CEBPA* genes,[6,7] and lower expression levels of the *BAALC*[8,9] and *ERG*[10,11] genes have been associated with favorable outcome, whereas internal tandem duplication of the *FLT3* gene (*FLT3*-ITD)[12] and *WT1* mutations[13] have been shown to confer adverse prognosis. Furthermore, because these molecular alterations are not mutually exclusive, combinations of 2 or more of them have been used to refine prognostication of CN-AML patients and are recommended by best practice guidelines for cytogenetic/molecular risk stratification of AML patients.[14]

CN-AML is also the largest cytogenetic subset among patients aged 60 years or older.[1,2] But, despite the relatively large number of patients presenting with this feature, few studies have investigated the prognostic significance of molecular markers in this age group. Recently, we reported a study demonstrating that *NPM1* mutations are associated with a more favorable outcome in older CN-AML patients.[15] However, to our knowledge, studies testing the prognostic impact of *BAALC* and *ERG* gene expression levels in relatively large cohorts of older CN-AML patients have not been reported.

The *BAALC* (brain and acute leukemia, cytoplasmic) gene, located at chromosome band 8q22.3, was cloned in the course of

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research aimed at the identification of genes associated with a trisomy of chromosome 8 in AML.[16] High levels of *BAALC* expression were, indeed, found in AML patients with trisomy 8, but also in a subset of CN-AML patients.[8,16] The *ETS*-related gene, *ERG*, located at chromosome band 21q22.3, was first shown to be involved in leukemogenesis as a fusion partner with the *FUS* gene in the rare, but recurrent in AML, t(16;21)(p11;q22).[17] Moreover, *ERG* overexpression was demonstrated in AML patients with complex karyotypes with cryptic amplification of chromosome 21,[18] which was first discovered using spectral karyotyping,[19] and it was also found in a fraction of patients with CN-AML.[10] Our group was the first to report that high expression levels of the *BAALC* and *ERG* genes contribute to poor prognosis in younger CN-AML patients,[8,10] and these results have been corroborated by others.[20-24]

Herein, we sought to determine the prognostic impact of the expression of *BAALC* and *ERG* in older de novo CN-AML patients. We show, for the first time, that low levels of expression of these 2 genes are significantly associated with improved outcome in older CN-AML patients, even after adjustment for other prognostic clinical and molecular variables, including *NPM1* mutations. In addition, using genome-wide microarray profiling, we reveal changes in the expression of specific genes and microRNAs in low *BAALC* and *ERG* expressers that may contribute to the less aggressive disease in these patients. These biological features may potentially be exploited and lead to new treatment strategies.

Methods

Patients and treatment

A total of 158 patients aged 60 years or older with de novo CN-AML, who had pretreatment blood available, were analyzed for *BAALC* and *ERG* expression. The patients were treated with intensive cytarabine/daunorubicin-based regimens on Cancer and Leukemia Group B (CALGB) front-line clinical protocols 8525, 8923, 9420, 9720, or 10201 (see supplemental Methods for details, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Patients with antecedent hematologic disorders or therapy-related AML, and those transplanted in first complete remission (CR), were excluded. The Ohio State University Institutional Review Board–approved, written informed consent for participation in these studies was obtained from all patients in accordance with the Declaration of Helsinki.

Cytogenetics and additional mutation markers

Pretreatment cytogenetic analyses of bone marrow (BM) were performed by CALGB-approved institutional cytogenetic laboratories as part of CALGB 8461, a prospective cytogenetic companion study, and the results were reviewed centrally.[25] To be considered cytogenetically normal, at least 20 metaphase cells had to be analyzed and the karyotype found to be normal.[25]

The presence or absence of *FLT3*-ITD, *FLT3* tyrosine kinase domain mutations (*FLT3*-TKD), *MLL* partial tandem duplication (*MLL*-PTD), and mutations in the *NPM1*, *CEBPA*, *WT1*, *IDH1*, and *IDH2* genes was also determined centrally in blood or marrow pretreatment samples, as previously described.[5,7,12,13,26,27]

RNA extraction and real-time RT-PCR to measure *BAALC* and *ERG* expression levels

Preparation of pretreatment blood samples and analysis of *BAALC* and *ERG* expression were performed as previously described.[8-11] Briefly, total RNA was extracted using the Trizol method and complementary DNA was synthesized from total RNA. Quantitative real-time reverse-transcription–polymerase chain reaction (RT-PCR) amplification of *BAALC*, *ERG*, and *ABL1* was performed using standard curves. *BAALC* and *ERG* expression levels are reported as copy numbers normalized to *ABL1* copy numbers.

Gene and microRNA expression profiling

For gene and microRNA expression profiling, total RNA was extracted from pretreatment BM or blood mononuclear cells. The gene and micro RNA expression profiling was performed using the Affymetrix U133 plus 2.0 array (Affymetrix) and The Ohio State University custom microRNA array (OSU_CCC Version 4.0), respectively, as previously reported[15,28] and detailed in supplemental Methods. The microarray data are available on ArrayExpress under accession numbers E-TABM-1071 and E-TABM-1072.

Definition of clinical endpoints and statistical analysis

The main objective of this study was to evaluate the prognostic impact of *BAALC* and *ERG* expression on clinical outcome in older CN-AML patients. The median *BAALC*/*ABL1* or *ERG*/*ABL1* copy number values, respectively, were used to define low and high *BAALC* or *ERG* expressers. This cutoff was based on the trends for overall survival (OS) of patients divided into quartiles by expression values, where patients in quartile 1 had better outcome than patients in quartile 2, followed by patients in quartiles 3 and 4 for *BAALC* and *ERG* expression ($P < .001$ and $P < .001$, test for trend[29]).

Definitions of clinical endpoints (eg, CR, disease-free survival [DFS] and OS) and details of statistical analyses, including variable selection for statistical modeling, are provided in the supplemental material. Associations between patients with low or high expression of *BAALC* or *ERG* for baseline demographic, clinical, and molecular features were compared using the Fisher exact and Wilcoxon rank-sum tests for categorical and continuous variables, respectively. Estimated probabilities of DFS and OS were calculated using the Kaplan-Meier method, and the log-rank test evaluated differences between survival distributions. Multivariable logistic regression models were constructed to analyze factors related to the probability of achieving CR using a limited backward selection procedure. Multivariable proportional hazards models were constructed for DFS and OS to evaluate the impact of *BAALC* and *ERG* expression (low/high) by adjusting for other variables using a limited backward selection procedure. For achievement of CR, estimated odds ratios, and for survival endpoints, hazard ratios with their corresponding 95% confidence intervals were obtained for each significant prognostic factor.

For the gene and microRNA expression profiling, summary measures of gene and microRNA expression were computed, normalized, and filtered (see supplemental material). The profiles were derived by comparing gene and microRNA expression between low and high *BAALC* expressers and between low and high *ERG* expressers. Univariable significance levels of .001 for gene and .005 for microRNA expression profiling were used to determine, respectively, the probe sets and microRNA probes that comprised the signatures.

All analyses were performed by the CALGB Statistical Center.

Results

Associations of *BAALC* and *ERG* expression with clinical and molecular characteristics

At diagnosis, low *BAALC* expression was associated with mutated *NPM1* ($P < .001$), wild-type *CEBPA* ($P = .02$), and low *ERG* expression levels ($P < .001$; Table 1).

Low *ERG* expression was associated with lower white blood count (WBC; $P = .005$), lower percentages of blood ($P < .001$) and BM ($P = .001$) blasts, the absence of *FLT3*-ITD ($P < .001$), and low *BAALC* expression ($P < .001$; Table 1).

Prognostic value of *BAALC* and *ERG* expression

Low *BAALC* expressers had a higher CR rate (86% vs. 54%; $P < .001$) and longer DFS ($P = .01$; Figure 1A) and OS ($P < .001$; Figure 1B) than high expressers (Table 2). Similarly, patients with low *ERG* expression had a trend for better CR rates (76% vs. 65%;

Table 1. Clinical and molecular characteristics at diagnosis according to *BAALC* and *ERG* expression status in older CN-AML patients

Characteristic	Low *BAALC* (n = 79)	High *BAALC* (n = 79)	*P*	Low *ERG* (n = 79)	High *ERG* (n = 79)	*P*
Age, y			.17			.95
Median	67	69		68	69	
Range	60-79	60-83		60-81	60-83	
Sex, no. (%)			.52			.11
Male	46 (58)	41 (52)		38 (48)	49 (62)	
Female	33 (42)	38 (48)		41 (52)	30 (38)	
Race, no. (%)			.40			1.0
White	73 (94)	68 (89)		70 (92)	71 (91)	
Nonwhite	5 (6)	8 (11)		6 (8)	7 (9)	
Hemoglobin, g/dL			.43			.76
Median	9.6	9.5		9.5	9.5	
Range	5.4-15.0	6.8-14.5		5.4-13.6	6.0-15.0	
Platelet count, $\times 10^9$/L			.31			.51
Median	55	67		69	55	
Range	5-481	4-850		4-271	11-850	
WBC count, $\times 10^9$/L			.78			.005
Median	27.8	33.5		20.6	38.0	
Range	1.4-450.0	1.0-173.1		1.1-234.5	1.0-450.0	
Blood blasts, %			.36			< .001
Median	52	59		34	68	
Range	0-97	0-99		0-93	0-99	
BM blasts, %			.80			.001
Median	70	67		59	75	
Range	11-97	7-97		7-97	23-97	
Extramedullary involvement, no. (%)	16 (21)	19 (25)	.70	16 (21)	19 (25)	.56
***NPM1*, no. (%)**			< .001			.33
Mutated	64 (81)	33 (42)		45 (57)	52 (66)	
Wild-type	15 (19)	46 (58)		34 (43)	27 (34)	
***FLT3*-ITD, no. (%)**			.14			< .001
Present	24 (30)	34 (43)		12 (15)	46 (58)	
Absent	55 (70)	45 (57)		67 (85)	33 (42)	
***FLT3*-TKD, no. (%)**			.61			.61
Present	10 (13)	7 (9)		10 (13)	7 (9)	
Absent	69 (87)	72 (91)		69 (87)	72 (91)	
***WT1*, no. (%)**			.21			.21
Mutated	3 (4)	8 (10)		3 (4)	8 (10)	
Wild-type	76 (96)	71 (90)		76 (96)	71 (90)	
***CEBPA*, no. (%)**			.02			.65
Mutated	6 (8)	17 (22)		10 (13)	13 (16)	
Wild-type	73 (92)	62 (78)		69 (87)	66 (84)	
***IDH1*, no. (%)**			.09			.40
Mutated	4 (5)	10 (17)		5 (8)	9 (15)	
Wild-type	59 (94)	50 (83)		59 (92)	53 (85)	
***IDH2**, no. (%)**			.30			.10
R140 *IDH2*	18 (29)	7 (12)		17 (28)	8 (13)	
R172 *IDH2*	0 (0)	5 (8)		2 (3)	3 (5)	
Wild-type	45 (71)	48 (80)		42 (69)	51 (82)	
***MLL*-PTD, no. (%)**			.68			.42
Present	2 (4)	4 (7)		4 (7)	2 (3)	
Absent	55 (96)	55 (93)		51 (93)	59 (97)	
***ERG* expression†, no. (%)**			< .001			
Low	51 (65)	28 (35)				
High	28 (35)	51 (65)				
***BAALC* expression†, no. (%)**						< .001
Low				51 (65)	28 (35)	
High				28 (35)	51 (65)	

BM, bone marrow; *FLT3*-ITD, internal tandem duplication of the *FLT3* gene; *FLT3*-TKD, tyrosine kinase domain mutation of the *FLT3* gene; *MLL*-PTD, partial tandem duplication of the *MLL* gene; WBC, white blood count.

**P* value is for the comparison of *IDH2* mutated (R140 or R172) versus *IDH2* wild-type.

†The median expression value was used as a cut point.

$P = .12$) and longer DFS ($P = .03$; Figure 2A) and OS ($P = .003$; Figure 2B; Table 2). The median follow-up for patients alive was 3.8 years (range: 2.8-11.6 years).

In a multivariable model for CR attainment (see supplemental material for full model building description), *BAALC* expression remained a significant prognosticator ($P < .001$) after adjustment

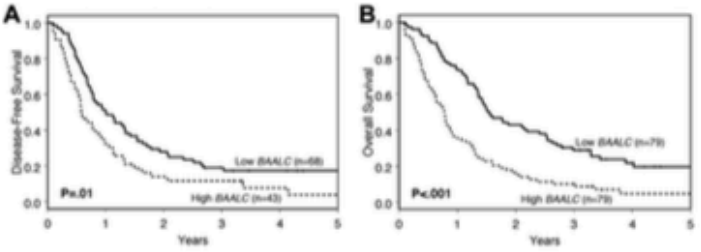

Figure 1. Outcome of cytogenetically normal older AML patients according to *BAALC* expression levels. (A) DFS. (B) OS.

for *NPM1* mutation status ($P = .04$) and WBC ($P = .02$; Table 3). The odds for achieving a CR were more than 4 times higher for the low *BAALC*-expressing group than for high *BAALC* expressers. *BAALC* expression, but not *ERG* expression, was also a significant factor for DFS ($P = .03$), after adjustment for the *FLT3*-ITD ($P < .001$) and age ($P = .05$; Table 3). The risk of relapse or death was reduced by 36% for low *BAALC* expressers. Furthermore, expression levels of both *BAALC* and *ERG* were the only factors significantly associated with OS upon multivariable analysis ($P < .001$ and $P = .03$, respectively; Table 3). The risk of death was reduced by approximately one-half in the low *BAALC*-expressing group and by approximately one-third in the low *ERG*-expressing group.

Because both expression markers were significantly associated with OS, we investigated the combination of the 2 markers. Patients who had lower expression of *BAALC* or *ERG*, or both, had a significantly longer DFS and OS ($P < .001$ for both DFS and OS; Figure 3) than those expressing both markers at high levels. At 3 years, 26% of the patients with low expression of *BAALC* or *ERG*, or both, were alive, compared with only 6% of patients who expressed *BAALC* and *ERG* at high levels. However, there were no significant differences in outcome between patients with low expression of both *BAALC* and *ERG* and those who had low expression of only 1 of these 2 genes.

We recently reported a strong prognostic impact of *NPM1* mutations on CR rates, DFS, and OS in older CN-AML patients.[15] However, in the set of patients investigated here, *NPM1* mutation status was retained only in our model for CR. Therefore, we compared different multivariable models for CR attainment, DFS, and OS using the Akaike information criterion (AIC; supplemental Table 1A-C). We found that the multivariable model for DFS that included *BAALC* expresser status, *FLT3*-ITD mutation status, and age, excluding *NPM1* mutation status, appeared to be better than other evaluated models that comprised *NPM1* mutation status (supplemental Table 1B). For OS, we found that a model including only the *BAALC* and *ERG* expresser status was better than 2 other evaluated models that comprised *NPM1* mutation status (supplemental Table 1C).

We have also recently reported a trend toward a lower CR rate and shorter OS in older CN-AML patients harboring *WT1* mutations.[13] This report suggested that the impact of *WT1* mutation status might be obscured by the generally poor outcome of older CN-AML patients. In the current study, we only had 11 patients who harbored *WT1* mutations, and we did not see a significant impact of *WT1* mutation status on outcome upon univariable analyses. Furthermore, only 3 of the 11 *WT1*-mutated cases were low *BAALC* or low *ERG* expressers. Therefore, we were not able to develop multivariable regression models for outcome that would have evaluated *WT1* mutations in the context of low *BAALC* or *ERG* expression status.

Genome-wide gene expression profiling

To gain insights into the biology of older CN-AML patients, we derived gene expression signatures associated with *BAALC* and *ERG* expresser status.

We observed that *BAALC* expression was associated with the differential expression of 482 probe sets, representing 292 annotated genes. Of these, 283 probe sets, which represented 165 annotated genes, were up-regulated, and 199 probe sets, representing 127 annotated genes, were down-regulated in low *BAALC* expressers (Figure 4A; supplemental Table 2). Probe sets representing *BAALC* were among the most down-regulated probe sets in low *BAALC* expressers, confirming our real-time RT-PCR results. The gene expression signature of *BAALC* derived in older CN-AML patients was very similar to that we reported in patients younger than 60 years.[9] Low *BAALC* expression was associated with up-regulation of genes of the *HOXA* and *HOXB* clusters, as well as the *HOX* cofactor, *MEIS1*, that are important for developmental processes and hematopoietic stem cell function.[30] We also observed a higher expression of *CTSG* and *AZU1*—genes that are expressed in mature neutrophils.[31] As in younger patients and consistent with the more favorable prognosis associated with low *BAALC* expression, we found that low *BAALC* expressers had down-regulation

Table 2. Outcomes according to *BAALC* and *ERG* expression in older CN-AML patients

Outcome	Low *BAALC* (n = 79)	High *BAALC* (n = 79)	*P*	Low *ERG* (n = 79)	High *ERG* (n = 79)	*P*
Complete remission rate, no. (%)	68 (86)	43 (54)	< .001	60 (76)	51 (65)	.12
Disease-free survival			.01			.03
Median, y	1.0	0.6		1.0	0.6	
Disease-free at 3 y, % (95% CI)	19 (11-29)	12 (4-23)		18 (10-29)	14 (6-25)	
Overall survival			< .001			.003
Median, y	1.5	0.8		1.4	0.8	
Alive at 3 y, % (95% CI)	29 (19-39)	10 (5-18)		24 (15-34)	15 (8-24)	

CI indicates confidence interval.

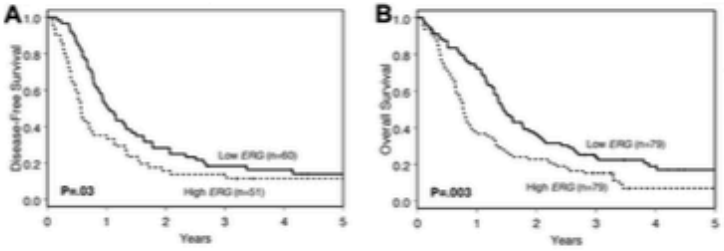

Figure 2. Outcome of cytogenetically normal older AML patients according to *ERG* expression levels. (A) DFS. (B) OS.

of genes previously associated with worse outcome in CN-AML, including the transcription coregulator, *MN1*,[32] the surface marker, *CD200*,[33] and the growth factor, *HGF*[34] (Figure 4A). Furthermore, we observed a down-regulation of genes found expressed in less differentiated precursors, such as *PROM1*, *CD34*, *JUP*, *C5orf23*, *FZD6*, *B4GALT6*, and *APP* (Figure 4A),[18,35,36] and *ABCB1* (*MDR1*), a gene encoding the multidrug resistance protein, whose high expression has been associated with worse outcome in older AML patients.[37]

The gene expression signature associated with *ERG* expression comprised 1554 differentially expressed probe sets, representing 1089 genes, of which 208 probe sets, representing 127 genes, were up-regulated, and 1346 probe sets, representing 962 genes, were down-regulated in low *ERG* expressers (Figure 5A; supplemental Table 3). Probe sets representing *ERG* were among the most down-regulated in low *ERG* expressers, again confirming our real-time RT-PCR results. We found previously identified putative *ERG* targets and binding partners, such as *ICAM2*, *SOCS2*, and *FLI1*,[38,39] down-regulated in low *ERG* expressers. Also down-regulated were *DNMT3A* and *DNMT3B*, genes associated with aberrant DNA hypermethylation in AML[40]; *SET*, which inhibits active DNA demethylation via chromatin modification and inhibits the activation of the tumor suppressor, PP2A phosphatase[41,42]; and the histone methyltransferases, *SMYD2* and *SMYD3*, that have a role in cell proliferation in cancer.[43,44] Among the overexpressed genes associated with low *ERG* levels were *TGFBR3*, which was shown to suppress breast cancer progression,[45] and *TOP1*, which has been associated with enhanced sensitivity to chemotherapy.[46] Furthermore, when we compared the gene expression signature of *ERG* derived in older CN-AML patients to the signature reported in patients younger than 60 years, we found them to be very similar.[10] We found that genes such as *BCL11A*, *DAPK1*, *GUCY1A3*, and *KLHDC1*,[10] were also down-regulated in low *ERG* expressers in the current study.

Genome-wide microRNA expression profiling

To further investigate the biology related to low *BAALC* and *ERG* expression, we derived microRNA expression signatures associated with *BAALC* and *ERG* expresser status. For the first time, we were able to derive a microRNA expression signature associated with *BAALC* expression (Figure 4B). We found 22 differentially expressed probes, representing 18 microRNAs, with 10 up-regulated and 8 down-regulated in low *BAALC*-expressing patients. Consistent with the higher expression of *HOX* genes in low *BAALC* expressers, we observed up-regulation of microRNAs embedded in the *HOX* cluster, namely, *miR-10a*, *miR-10b*, and *miR-9*. Underexpressed was *miR-126*, which we previously found correlated with *MN1* expression in younger patients,[32] as well as *miR-222*, which is known to target *KIT* and has been linked to hematologic lineage differentiation.[47]

We also report here the first microRNA expression signature associated with *ERG* expression in CN-AML. We observed 11 differentially expressed probes, representing 11 microRNAs, associated with *ERG* expression (Figure 5B), with 5 up-regulated and 6 down-regulated in low *ERG* expressers. Among the up-regulated microRNAs in low *ERG*-expressing patients was *miR-107*, known

Table 3. Multivariable logistic regression analysis for achievement of CR, DFS, and OS in older CN-AML patients

	CR*			DFS†			OS‡		
Group	OR	95% CI	*P*	HR	95% CI	*P*	HR	95% CI	*P*
BAALC, low vs high	4.32	1.83-10.20	< .001	0.64	0.42-0.97	.03	0.49‖	0.34-0.71	<.001
ERG, low vs high							0.69	0.48-0.97	.03
NPM1, mutated vs wild-type	2.34	1.03-5.35	.04						
FLT3-ITD, present vs absent				1.79§	1.11-2.87	< .001			
WBC, each 50-unit increase	0.68	0.49-0.93	.02						
Age, each 10-year increase				0.65	0.42-1.00	.05			

Odds ratios greater than (less than) 1.0 mean higher (lower) CR rate for the higher values of the continuous variables and the first category listed for the categorical variables. Hazard ratios greater than (less than) 1.0 indicate higher (lower) risk for relapse or death (DFS) or death (OS) for the higher values of the continuous variables and the first category listed for the categorical variables.

*Variables considered in the model based on univariable analyses were *BAALC* expression (low vs high; median cut), *ERG* expression (low vs high; median cut), *NPM1* (mutated vs wild-type), WBC (in 50-unit increments), and age (in 10-year increments).

†Variables considered in the model based on univariable analyses were *BAALC* expression (low vs high; median cut), *ERG* expression (low vs high; median cut), *FLT3*-ITD (positive vs negative), *FLT3*-TKD (positive vs negative), and age (in 10-year increments).

‡Variables considered in the model based on univariable analyses were *BAALC* expression (low vs high; median cut), *ERG* expression (low vs high; median cut), *NPM1* (mutated vs wild-type), *FLT3*-ITD (positive vs negative), and platelet count.

‖Does not meet the proportional hazards assumption. Low *BAALC* was associated with better OS until approximately 2 years, and after that, did not seem to impact OS. The hazard ratio for *BAALC*, low vs high, is reported at 1 year.

§Does not meet the proportional hazards assumption. *FLT3*-ITD was associated with worse DFS until approximately 1 year, and after that, its adverse impact for DFS declined. The hazard ratio for *FLT3*-ITD is reported at 9 months.

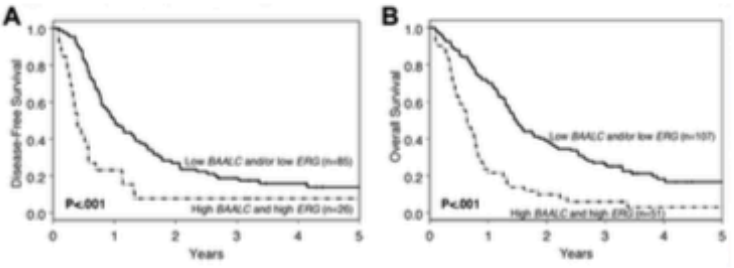

Figure 3. Outcome of cytogenetically normal older AML patients according to a combination of *BAALC* and *ERG* expression levels. Patients with low expression of *BAALC* or *ERG*, or both, were compared with those having high expression of both *BAALC* and *ERG*. (A) DFS. (B) OS.

to target *NFIX*, a gene involved in a regulatory feedback loop involving *miR-223* and *CEBPA* during granulocytic differentiation.[48] Interestingly, also up-regulated was *miR-148a*, which is shown to target *DNMT3B*,[49] and *miR-208*, which is predicted in silico to target *ERG* itself. Another noteworthy down-regulated microRNA was *miR-302d* that has been associated with early developmental stages and "stemness."[50]

Discussion

Although some studies have shown an association of mutation markers and outcome in older CN-AML patients,[13,15,26] relatively little is known with regard to the prognostic value of gene expression markers in older patients with CN-AML. The aim of this study was to elucidate the prognostic impact of *BAALC* and *ERG* expression, in the context of other well-established molecular markers, in older de novo CN-AML patients similarly treated with intensive chemotherapy.

First, we demonstrate that low expression levels of both *BAALC* and *ERG* are associated with better outcome in older patients with CN-AML. As in younger CN-AML patients,[9] low *BAALC* expression was associated with a higher CR rate and longer DFS and OS in older CN-AML patients investigated here. Low *ERG* expression was also associated with longer DFS and OS and a trend toward a higher CR rate in older patients, which is similar to our findings in younger CN-AML patients, where low *ERG* expressers had higher CR rates and longer event-free survival (EFS) than high *ERG* expressers.[11] In multivariable analyses, *BAALC*, but not *ERG*, expression levels were significant factors for CR attainment and DFS. Expression levels of both *BAALC* and *ERG* were the only factors associated with OS in our cohort of older CN-AML patients.

To our knowledge, only 3 studies that analyzed the prognostic significance of *BAALC* expression and one study analyzing *ERG* expression in CN-AML included patients age 60 years or older.[20,21,24] Two of these studies that comprised, respectively, 67 and 98 adults with CN-AML, including an unspecified number of patients age 60 years or older, showed that low *BAALC* expression was associated with better outcome, but did not evaluate older patients separately. The third study[20] also included older patients and showed that low *BAALC* expression was associated with higher probability of CR achievement and longer EFS and OS. In their subgroup of 101 patients age 60 years or older, low *BAALC* expression showed a trend toward a higher CR rate (66% vs. 50%; $P = .1$) and a longer EFS ($P = .03$; Klaus H. Metzeler, Ludwig-Maximilians-Universität München, e-mail, April 26, 2010). On the other hand, low *ERG* expression seemed to be a stronger factor for outcome in the study of Metzeler et al,[20] where it was significantly associated with a higher CR rate and longer EFS and OS in all patients analyzed. Subset analyses revealed a significant impact of low *ERG* expression on OS in both the younger patients and those aged 60 years or older, but neither of these age groups was evaluated separately in multivariable models considering other molecular markers.

Second, because we recently reported a strong prognostic impact of *NPM1* mutations on CR rates, DFS, and OS in older CN-AML patients,[15] it was of particular interest to us to investigate the relationship of *NPM1* mutation status with *BAALC* and *ERG* expresser status. In multivariable models, *NPM1* mutation status remained a strong, favorable prognostic marker for achievement of CR, but did not remain in the models for DFS and OS. Interestingly, in our previously reported gene expression signature associated with *NPM1* mutations in older patients with CN-AML, expression of both the *BAALC* and *ERG* genes was low.[15] We believe that measuring *BAALC* and *ERG* expression at diagnosis seems to provide more prognostic information with regard to survival than *NPM1* mutational status alone. Indeed, patients with high *BAALC* expression had worse outcome irrespective of their *NPM1* mutational status. This is supported by the comparison of different multivariable models in our dataset. Thus, once reliable methods to measure the pretreatment expression levels of *BAALC* and *ERG* in individual patients are available, these markers should be included in diagnostic testing for a more accurate risk stratification of older CN-AML patients.

Finally, to gain insights into the biology of the disease, we derived gene and, for the first time, microRNA expression signatures associated with *BAALC* and *ERG* expression. Many genes in the *BAALC* signature for older patients reported here have also been found in the gene expression signature in younger patients.[9] This indicates that low *BAALC* expression defines a distinct subset of biologically similar patients, regardless of age. The characteristic features of this signature included overexpression of the *HOXA* and *HOXB* gene clusters, underexpression of known adverse outcome predictors, and down-regulation of genes associated with an undifferentiated state. Furthermore, in the first microRNA expression signature associated with *BAALC* expression, we found down-regulation of microRNAs embedded in the *HOX* cluster and up-regulation of *miR-222*, which is known to target the tyrosine kinase, *KIT*. The lower expression of *ABCB1* (*MDR1*) observed in older CN-AML patients with low *BAALC* expression may account, at least in part, for the observed higher CR rate and better survival.

Interestingly, patient subgroups characterized by mutated *NPM1* or low *BAALC* expression shared some common features in their gene and microRNA expression profiles. The second most down-regulated gene in *NPM1* mutated patients was *BAALC*.[15] Both the gene and microRNA expression profiles associated with mutated *NPM1*[15] and those associated with low *BAALC* expression show down-regulation of *HOX* genes and cofactors such as *MEIS1* and

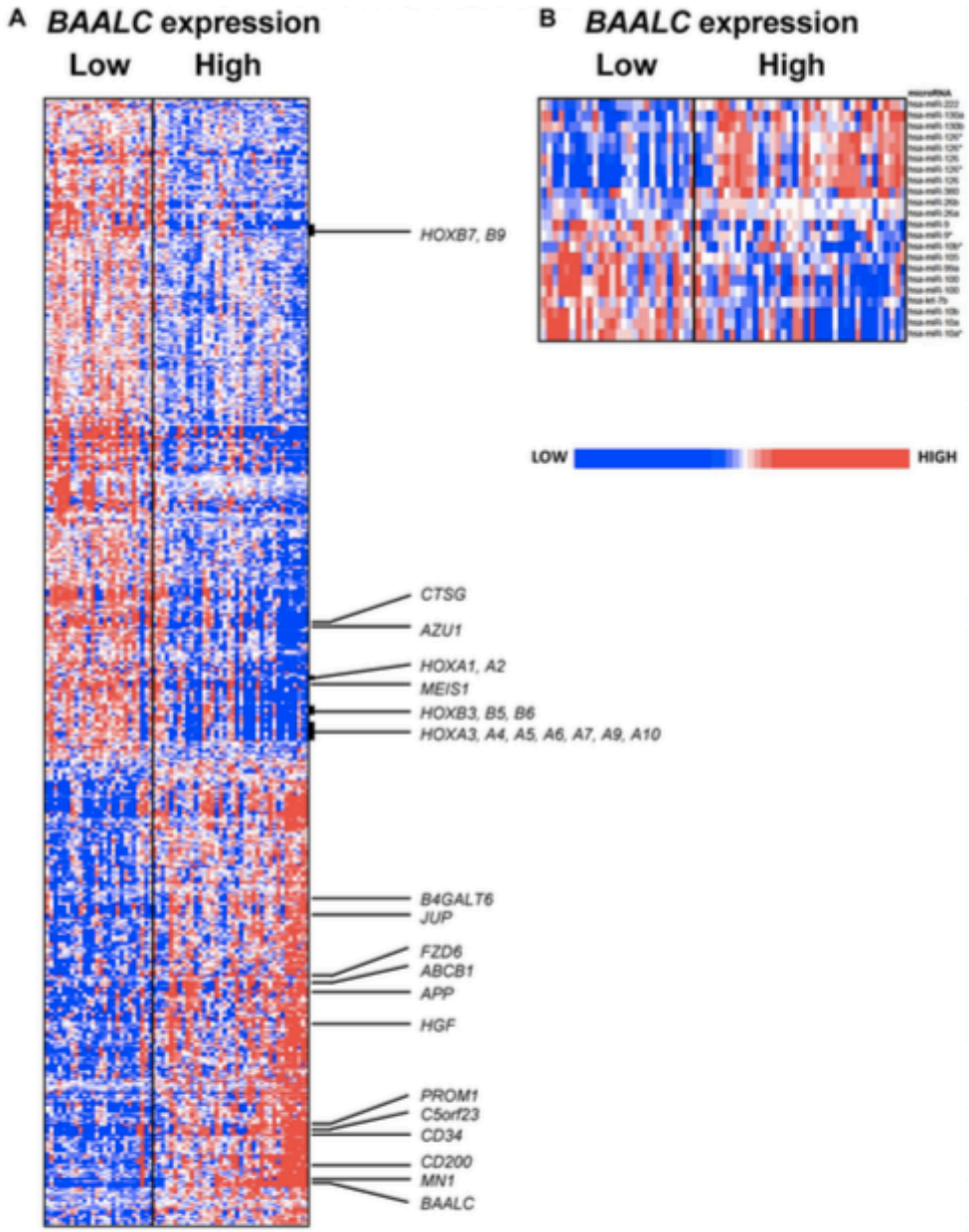

Figure 4. Heat maps of the *BAALC* signatures. Derived gene expression (A) and microRNA expression signatures (B) associated with *BAALC* expression in the group of CN-AML patients ≥ 60 years. Patients are ordered from left to right by increasing *BAALC* expression. Expression values of the gene probe sets (microRNA probes) are represented by color, with blue indicating expression less than and red indicating expression greater than the median value for the given gene probe set (microRNA probe). For the gene expression heat map, up- and down-regulated genes mentioned in the text are indicated.

up-regulation of *CTSG*, as well as overexpression of *miR-9*, *miR-10a*, *miR-10b*, and *miR-100*, and underexpression of *miR-126* and *miR-130a*. Thus, both low *BAALC* expression and mutated *NPM1* appear to share some biological features. To our knowledge, no molecular link between the 2 markers has been hitherto found. Further studies of possible biological links between those 2 markers are needed.

As with *BAALC*, the gene expression signature associated with *ERG* was similar to the signature we previously derived in younger patients.[10] Not surprisingly, we found lower expression of previously identified putative *ERG* targets and binding partners associated with low *ERG* expression. Up-regulation of topoisomerase 1 might, in part, explain outcome differences caused by enhanced sensitivity to chemotherapy of low *ERG* expressers. Furthermore,

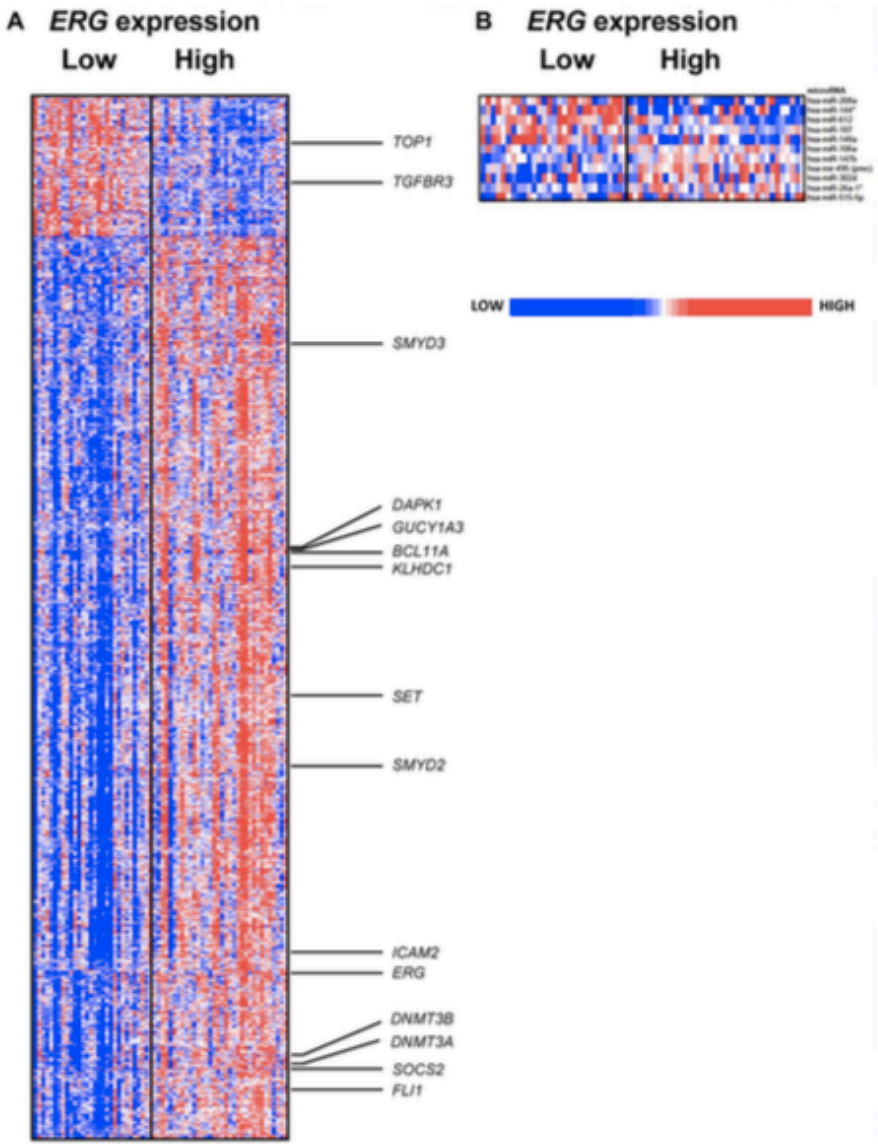

Figure 5. Heat maps of the *ERG* signatures. Derived gene expression (A) and microRNA expression signatures (B) associated with *ERG* expression in the group of CN-AML patients ≥ 60 years. Patients are ordered from left to right by increasing *ERG* expression. Expression values of the gene probe sets (microRNA probes) are represented by color, with blue indicating expression less than and red indicating expression greater than the median value for the given gene probe set (microRNA probe). For the gene expression heat map, up- and down-regulated genes mentioned in the text are indicated.

low *ERG* expressers also had down-regulation of DNA methyltransferases, histone methyltransferases, and an up-regulation of *miR-148a*, which targets *DNMT3B*.[49] We also observed a down-regulation of *SET*, which inhibits active DNA demethylation. This may indicate a higher activity of the DNA methylation machinery in high *ERG* expressers, which could contribute to their worse outcome. This should be further investigated and, if confirmed, might potentially lead to new treatment strategies in this subset of patients.

The similarity of the respective *BAALC* and *ERG* gene expression signatures between younger and older CN-AML patients, and the fact that these expression markers affect outcomes of both younger and older CN-AML patients, suggest that 60 years might not be an optimal age cutoff to separate younger and older patients' eligibility for treatment trials. Older patients with favorable molecular risk factors, such as low *BAALC* and/or *ERG* expression, if treated more intensively, might have outcomes comparable with those of younger patients with corresponding molecular features.

In conclusion, we show that low *BAALC* expression is an important factor for CR achievement and longer DFS; low *BAALC* and *ERG* expresser status are both significant factors associated with improved survival in older CN-AML patients. However, before the pretreatment expression of the 2 genes can be used for risk-stratification of older CN-AML patients, future studies should establish a standardized method of *BAALC* and *ERG* expression quantification and define absolute cutoff points. Once this obstacle is overcome, these important markers should be included in future risk-classification schemas. Furthermore, the derived gene and microRNA expression signatures shed light on the biology of this complex disease and identified new targets that might help in developing new therapeutic strategies for older CN-AML patients.

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Authorship

Contribution: S.S., G.M., K. Maharry, M.D.R., K. Mrózek, and C.D.B. contributed to the design and analysis of this study and the writing of the manuscript, and all authors agreed on the final version; S.S., H.B., S.P.W., Y.-Z.W., and K.H.M. carried out laboratory-based research; K. Maharry, M.D.R., K.B.H., and D.M. performed statistical analyses; and G.M., B.L.P., J.E.K., T.H.C., J.O.M., M.R.B., M.A.C., R.A.L., and C.D.B. were involved directly or indirectly in the care of patients and/or sample procurement.

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For a complete list of participating Cancer and Leukemia Group B institutions, principal investigators, and cytogeneticists, please see the supplemental Appendix.

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Low expression of *MN1* associates with better treatment response in older patients with de novo cytogenetically normal acute myeloid leukemia

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Low *MN1* expression bestows favorable prognosis in younger adults with cytogenetically normal acute myeloid leukemia (CN-AML), but its prognostic significance in older patients is unknown. We analyzed pretherapy *MN1* expression in 140 older (≥ 60 years) de novo CN-AML patients treated on cytarabine/daunorubicin-based protocols. Low *MN1* expressers had higher complete remission (CR) rates ($P = .001$), and longer overall survival ($P = .03$) and event-free survival (EFS; $P = .004$). In multivariable models, low *MN1* expression was associated with better CR rates and EFS. The impact of *MN1* expression on overall survival and EFS was predominantly in patients 70 years of age or older, with low *MN1* expressers with mutated *NPM1* having the best outcome. The impact of *MN1* expression was also observed in the Intermediate-I, but not the Favorable group of the European LeukemiaNet classification, where low *MN1* expressers had CR rates and EFS similar to those of Favorable group patients. *MN1* expresser-status-associated gene- and microRNA-expression signatures revealed underexpression of drug resistance and adverse outcome predictors, and overexpression of *HOX* genes and *HOX*-gene–embedded microRNAs in low *MN1* expressers. We conclude that low *MN1* expression confers better prognosis in older CN-AML patients and may refine the European LeukemiaNet classification. Biologic features associated with *MN1* expression may help identify new treatment targets. (*Blood*. 2011;118(15): 4188-4198)

Introduction

Over the past 3 decades, there has been relatively steady improvement of outcomes of patients with acute myeloid leukemia (AML) younger than 60 years. However, this has not occurred in older AML patients. Despite advances in our understanding of disease mechanisms and investigation of new therapies targeting distinct clinical, cytogenetic, and molecular subsets, the outcome of AML patients older than 60 years remains poor, with long-term survival rates of ~ 7%-15%.[1-3] The shorter survival of older AML patients compared with younger patients is probably related to clinical and biologic differences between them, including the failure to achieve a complete remission (CR) as a result of an increased intrinsic resistance of leukemic blasts to chemotherapy and the presence of specific cytogenetic and/or molecular alterations associated with worse outcome.[4]

As in younger patients, older patients with cytogenetically normal (CN) AML represent the largest AML subset.[5] This group is molecularly heterogeneous.[6,7] To date, however, the prognostic significance of molecular genetic alterations has been studied most extensively in younger (< 60 years) patients.[6-8] Recently, some, but not all, of these markers have also been shown to impact on outcome of older (≥ 60 years) CN-AML patients. For example, *NPM1* mutations,[9] and lower expression levels of the *BAALC* and *ERG*[10] genes have been associated with favorable outcome, whereas *FLT3* internal tandem duplication (*FLT3*-ITD)[11] and *WT1* mutations[12] have been shown to confer adverse prognosis in older patients, as they do in younger patients. However, to our knowledge, no study has investigated the prognostic impact of meningioma 1 (*MN1*) gene expression levels exclusively in CN-AML patients aged 60 years of age and older.

The *MN1* gene is localized at human chromosome band 22q12 and encodes a transcriptional coregulator.[13] *MN1* is involved in myeloid malignancies as a fusion partner of the *ETV6* gene in the recurrent translocation t(12;22)(p13;q11)[14] and has been shown to be overexpressed in subsets of AML.[15,16] We and others have shown that high *MN1* expression levels are prognosticators for poor outcome in younger CN-AML patients.[17,18]

With the hope to better predict the course of the disease, adjust therapeutic approaches, and improve outcome, we explored herein the prognostic significance of *MN1* expression in older de novo CN-AML patients. We have also analyzed genome-wide gene- and microRNA-expression profiles associated with *MN1* expression in these patients, to gain insights into *MN1*-associated disease.

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Methods

Patients and treatment

Pretreatment bone marrow (BM) samples of 140 adults 60 years of age or older with de novo CN-AML and material available were analyzed for *MN1* expression. The patients were enrolled on Cancer and Leukemia Group B (CALGB) front-line intensive cytarabine/daunorubicin-based treatment protocols (for protocol details see supplemental Methods, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Institutional Review Board–approved, written informed consent for participation in these studies was obtained from all patients in accordance with the Declaration of Helsinki.

Cytogenetics and additional molecular markers

Pretreatment cytogenetic analyses of BM were performed by CALGB-approved institutional cytogenetic laboratories as part of CALGB 8461, a prospective cytogenetic companion study, and the results reviewed centrally.[19,20] For a case to be considered CN, at least 20 metaphase cells had to be analyzed and the karyotype found to be normal.[20]

The presence or absence of *FLT3*-ITD mutations in the tyrosine kinase domain of the *FLT3* gene (*FLT3*-TKD) and mutations in the *CEBPA*, *IDH1*, *IDH2*, *NPM1*, *TET2*, and *WT1* genes was determined centrally in pretreatment samples as described previously.[9,11,12,21-28] The expression levels of the *BAALC* and *ERG* genes in peripheral blood were also assessed centrally in pretreatment samples as previously described.[10,29-32] *miR-181a* expression was evaluated as previously described.[33]

RNA extraction and real-time RT-PCR to measure *MN1* expression levels

Preparation of pretreatment BM samples and the analysis of *MN1* expression were performed as previously described.[18] Briefly, total RNA was extracted using Trizol reagent, and complementary DNA was synthesized from total RNA. Quantitative real-time RT-PCR amplifications of *MN1* and *ABL1* were performed using standard curves. *MN1* copy numbers were normalized to *ABL1* copy numbers.

Gene- and microRNA-expression profiling

For gene- and microRNA-expression profiling, total RNA was extracted from pretreatment BM or blood mononuclear cells. Gene- and microRNA-expression profiling was performed using the Affymetrix U133 plus Version 2.0 array (Affymetrix) and The Ohio State University custom microRNA array (OSU_CCC Version 4.0), respectively, as previously reported[9] and detailed in supplemental Methods.

Definition of clinical end points and statistical analysis

The main objective of this study was to evaluate the prognostic value of *MN1* expression on clinical outcome in older de novo CN-AML patients. For these patients, the median *MN1*/*ABL1* copy number value was chosen to define the low and high *MN1* expressers. This cut-off was based on the trend in overall survival (OS) of patients divided into quartiles by *MN1* level values; patients in the first 2 quartiles had a better outcome than patients in quartiles 3 and 4 ($P = .04$ test for trend).[34]

Definitions of clinical end points (ie, CR, disease-free survival [DFS], OS, and event-free survival [EFS]) are provided in supplemental Methods. Associations between patients with low or high expression of *MN1* for baseline demographic, clinical, and molecular features were compared using the Fisher exact and Wilcoxon rank-sum tests for categorical and continuous variables, respectively. Estimated probabilities of DFS, OS, and EFS were calculated using the Kaplan-Meier method, and the log-rank test evaluated differences between survival distributions. Multivariable analyses are detailed in supplemental Methods. Briefly, multivariable logistic regression models were constructed to analyze factors related to the probability of achieving CR using a limited backward selection procedure. Multivariable proportional hazards models were constructed for OS and

Table 1. Clinical and molecular characteristics according to *MN1* expression status in CN-AML patients 60 years of age or older

Characteristic	Low *MN1* (n = 70)	High *MN1* (n = 70)	*P*
Age, y			.57
Median	66	69	
Range	60-81	60-81	
Sex, no. (%) of males	40 (57)	32 (46)	.24
Race, no. (%)			.49
White	66 (96)	63 (93)	
Nonwhite	3 (4)	5 (7)	
Hemoglobin, g/dL			.16
Median	9.1	9.4	
Range	5.4-13.6	6.0-13.1	
Platelets, × 10^9/L			.43
Median	63	72	
Range	20-271	11-850	
WBC count, × 10^9/L			.15
Median	33.8	21.5	
Range	1.0-450.0	1.0-434.1	
Blood blasts, %			1.0
Median	45	49	
Range	0-96	0-99	
BM blasts, %			.32
Median	71	64	
Range	15-97	7-96	
Extramedullary involvement, no. (%)	19 (26)	15 (22)	.55
***FLT3*-ITD, no. (%)**			.59
Present	22 (31)	26 (37)	
Absent	48 (69)	44 (63)	
***FLT3*-TKD, no. (%)**			.08
Present	10 (14)	3 (4)	
Absent	60 (86)	67 (96)	
***CEBPA*, no. (%)**			.14
Mutated	6 (9)	13 (19)	
Single mutated	5	7	
Double mutated	1	6	
Wild-type	64 (91)	57 (81)	
***IDH1*, no. (%)**			.30
Mutated	10 (14)	6 (9)	
Wild-type	59 (86)	63 (91)	
***IDH2*, no. (%)**			.54
IDH2-mutated	13 (19)	17 (24)	
R140-*IDH2*-mutated	12	13	
R172-*IDH2*-mutated	1	4	
Wild-type	56 (81)	53 (76)	
***NPM1*, no. (%)**			< .001
Mutated	55 (79)	26 (37)	
Wild-type	15 (21)	44 (63)	
***TET2*, no. (%)**			.85
Mutated	19 (28)	18 (26)	
Wild-type	49 (72)	51 (74)	
***WT1*, no. (%)**			1.0
Mutated	3 (4)	3 (4)	
Wild-type	67 (96)	67 (96)	
***BAALC* expression,* no. (%)**			< .001
Low	48 (73)	24 (34)	
High	18 (27)	46 (66)	
***ERG* expression,* no. (%)**			.23
Low	36 (55)	30 (43)	
High	30 (45)	40 (57)	
***miR-181a* expression (continuous)**			.04
Median (log expression units)	11.89	12.23	
Range	9.06-15.43	8.81-14.66	
ELN risk group,† no. (%)			.03
Favorable	40 (57)	26 (37)	
Intermediate-I	30 (43)	44 (63)	

*The median expression value was used as a cutpoint.

†Favorable risk group consists of patients with *CEBPA* mutations or those who are *FLT3*-ITD-negative and harbor *NPM1* mutations. Intermediate-I genetic group is composed of patients who are not in the Favorable group (ie, those with wild-type *CEBPA* and wild-type *NPM1* with or without *FLT3*-ITD or mutated *NPM1* with *FLT3*-ITD).

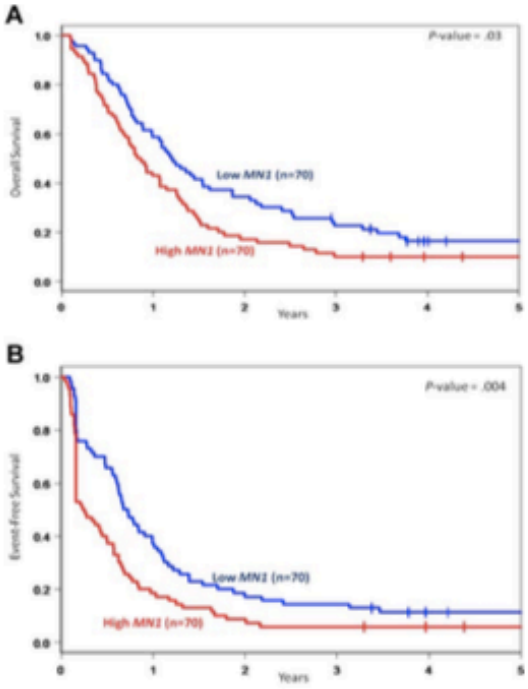

Figure 1. Outcome of CN-AML patients 60 years of age or older with respect to *MN1* expression. (A) OS. (B) EFS.

EFS to evaluate the impact of low or high expression of *MN1* by adjusting for other variables using a limited backward selection procedure. For achievement of CR, estimated odds ratios, and for survival end points, hazard ratios with their corresponding 95% confidence intervals (CIs) were examined.

For the gene- and microRNA-expression profiling, summary measures of gene and microRNA expression were computed, normalized, and filtered (supplemental Methods). *MN1*-associated signatures were derived by comparing gene and microRNA expression between low and high *MN1* expressers. Univariable significance levels of .001 for gene and .005 for microRNA expression profiling were used to determine, respectively, the probe sets and microRNA probes that constituted the signatures.

All analyses were performed by the CALGB Statistical Center.

Results

Associations of *MN1* expression with clinical and molecular characteristics and clinical outcome in older CN-AML patients

At diagnosis, the low and high *MN1* expresser groups did not differ significantly with regard to any of the clinical pretreatment characteristics. However, low *MN1* expression was associated with mutated *NPM1* ($P < .001$), lower *BAALC* expression levels ($P < .001$), and lower *miR-181a* expression levels ($P = .04$), as well as a trend for the presence of *FLT3*-TKD ($P = .08$; Table 1).

With a median follow-up for living patients of 4.0 years (range, 3-11.6 years) and for those who did not have an event of 4.2 years (range, 3.3-11.6 years), low *MN1* expressers had a higher CR rate (80% vs 53%, $P = .001$) and longer OS ($P = .03$; Figure 1A) and EFS ($P = .004$; Figure 1B) than high *MN1* expressers (Table 2). We did not observe a significant difference in DFS between high and low *MN1* expressers ($P = .29$; Table 2)

In a multivariable model for CR, *MN1* expression was a strong prognostic factor ($P = .01$), when controlling for *BAALC* expression ($P < .001$) and WBC ($P = .01$; Table 3). In a multivariable analysis for EFS, *MN1* expression remained prognostic ($P = .03$), after adjustment for *BAALC* expression ($P = .002$), WBC ($P < .001$), and platelets ($P = .002$; Table 3). The risk of having an event (ie, induction failure, relapse, or death) for low *MN1* expressers was half that for high expressers (hazard ratio [HR] = 0.54; 95% CI, 0.34-0.86). However, *MN1* expression did not remain an important predictor in a multivariable model for OS.

Prognostic impact of *MN1* expression by 60 to 69 years of age and 70 years of age or older subgroups

We recently reported that the prognostic significance of *FLT3*-ITD and *NPM1* mutations in older adults differed between patients 60-69 years of age and those 70 years of age or older, with the adverse impact of *FLT3*-ITD being found mostly in the former[11] and the favorable impact of *NPM1* mutations in the latter.[9] Therefore, we analyzed the prognostic impact of *MN1* expression in these 2 age subgroups (Table 2). Low *MN1* expression was associated with higher CR rates both in patients 60-69 years of age (80% vs 54%, $P = .03$) and in those 70 years of age or older (81% vs 51%, $P = .03$). In contrast, a significantly longer OS ($P = .006$; 3-year OS rates, 31% vs 9%) and EFS ($P = .007$; 3-year EFS rates, 27% vs 6%) and a trend toward longer DFS ($P = .09$; 3-year DFS rates, 33% vs 11%) were observed only in patients 70 years of age and older (Table 2).

MN1 expression status remained independently associated with probability of achieving a CR for both age subgroups (60-69 years of age, $P = .02$, data not shown; 70 years of age or older, $P = .02$, Table 3), with no other variable remaining in the final model. Concerning patients 70 years of age or older (Table 3), low *MN1* expressers had almost 4 times greater odds of attaining a CR (odds ratio [OR] = 3.97; 95% CI, 1.22-12.90; Table 3). When we considered OS and EFS in this age group, we found an interaction between *MN1* expression and *NPM1* mutation status. The favorable impact of low *MN1* expression on OS and EFS appeared to be limited to patients who simultaneously carried an *NPM1* mutation ($P = .04$ and $P = .02$, respectively; Table 3), whereas there was no significant difference in OS or EFS between low and high *MN1* expressers with wild-type *NPM1* ($P = .58$ and $P = .87$, respectively; Table 3).

Taking into account the aforementioned OS and EFS interaction and the fact that we previously reported that the impact of *NPM1* mutations on outcome was much stronger in the 70 years of age or older subgroup,[9] we examined the relationship between *NPM1* mutation status and *MN1* expression status within this patient subgroup more closely. Among CN-AML patients 70 years of age or older, those with *NPM1* mutations who had low *MN1* expression had a trend for better CR rates ($P = .15$) and significantly longer DFS ($P = .003$), OS ($P = .002$; Figure 2A), and EFS ($P = .002$; Figure 2B) compared with the 3 other molecular subsets combined (ie, low *MN1* expressers with wild-type *NPM1*, high *MN1* expressers with mutated *NPM1*, and high *MN1* expressers with wild-type *NPM1*).

Prognostic impact of *MN1* expression within the ELN classification

Recently, the European LeukemiaNet (ELN) guidelines classified CN-AML patients into Favorable or Intermediate-I genetic groups based on the mutational status of the *CEBPA*, *NPM1*, and *FLT3*

Table 2. Outcomes according to *MN1* expression status in all CN-AML patients 60 years of age or older and, separately, in those 60-69 years of age and 70 years of age or older

Outcome	Low *MN1*	High *MN1*	*P**	OR/HR (95% CI)
All patients	n = 70	n = 70		
CR rate, no. (%)	56 (80)	37 (53)	.001	3.57 (1.68, 7.56)
DFS			.29	0.79 (0.51, 1.23)
Median, y	0.9	0.6		
Disease-free at 3 y, % (95% CI)	18 (9-29)	11 (3-23)		
OS			.03	0.68 (0.48, 0.96)
Median, y	1.2	0.8		
Alive at 3 y, % (95% CI)	23 (14-33)	10 (4-18)		
EFS			.004	0.50† (0.33, 0.76)
Median, y	0.7	0.2		
Event-free at 3 y, % (95% CI)	14 (7-23)	6 (2-13)		
Patients 60-69 y	n = 44	n = 35		
CR rate, no. (%)	35 (80)	19 (54)	.03	3.28 (1.22, 8.81)
DFS			.82	0.94 (0.52, 1.69)
Median, y	0.7	0.5		
Disease-free at 3 y, % (95% CI)	9 (2-21)	11 (2-28)		
OS			.44	0.83 (0.52, 1.33)
Median, y	1.1	0.8		
Alive at 3 y, % (95% CI)	18 (9-31)	11 (4-24)		
EFS			.11	0.69 (0.44, 1.10)
Median, y	0.6	0.2		
Event-free at 3 y, % (95% CI)	7 (2-17)	6 (1-17)		
Patients 70 y or older	n = 26	n = 35		
CR rate, no. (%)	21 (81)	18 (51)	.03	3.97 (1.22, 12.90)
DFS			.09	0.54 (0.27, 1.10)
Median, y	1.3	0.7		
Disease-free at 3 y, % (95% CI)	33 (15-53)	11 (2-30)		
OS			.006	0.46 (0.26, 0.81)
Median, y	2.0	0.9		
Alive at 3 y, % (95% CI)	31 (15-49)	9 (2-21)		
EFS			.007	0.48 (0.27, 0.84)
Median, y	1.0	0.3		
Event-free at 3 y, % (95% CI)	27 (12-44)	6 (1-17)		

OR indicates the odds of achieving a CR for low *MN1* vs high *MN1* expressers; HR, the hazard of having an event for low *MN1* vs high *MN1* expressers; and CI, confidence interval.

**P* values for categorical variables are from Fisher exact test. *P* values for time-to-event variables are from the log-rank test (OS, DFS, and EFS).

†Does not meet the proportional hazards assumption, HR reported at 3 months.

genes.[5] The ELN Favorable genetic group is composed of CN-AML patients with *CEBPA* mutation and/or *NPM1* mutation without *FLT3*-ITD, whereas the Intermediate-I genetic group encompasses all other CN-AML patients (ie, CN-AML patients with wild-type *CEBPA* and either *NPM1* mutation with *FLT3*-ITD or wild-type *NPM1* with or without *FLT3*-ITD). We thus investigated the prognostic impact of *MN1* expression within these ELN genetic groups. Among the 140 patients, 66 were in the Favorable genetic group and 74 in the Intermediate-I genetic group. Lower *MN1* expression levels were found more frequently in the Favorable than Intermediate-I group patients (57% vs 43%, $P = .03$; Table 1).

Within the ELN Favorable group, we observed no significant differences in CR rates ($P = .24$), DFS ($P = .84$), OS ($P = .81$), or EFS ($P = .69$) between low and high *MN1* expressers (Table 4). In contrast, within the Intermediate-I genetic group, CN-AML patients with low *MN1* expression had better CR rates (77% vs 43%, $P = .008$), a trend toward longer DFS ($P = .15$; 3-year DFS rates, 13% vs 0%), and significantly longer OS ($P = .05$; 3-year OS rates, 10% vs 2%) and EFS ($P = .003$; 3-year EFS rates, 10% vs 0%; Table 4). The CR rate of 77% in patients with low *MN1* expression in the Intermediate-I genetic group was comparable to CR rates of patients with both low and high *MN1* expression in the ELN Favorable group (83% and 69%, respectively). Likewise, the EFS of low *MN1* expressers in the Intermediate-I genetic group was not significantly different from the EFS of patients in the ELN Favorable genetic group (Table 4; Figure 3B).

Because each of the ELN genetic groups is composed of specific molecular subsets, the ELN guidelines recommend reporting outcome measures also by these specific subsets. There was no impact of *MN1* expression on either of the 2 CN-AML molecular subsets within the ELN Favorable genetic group (data not shown). The situation was different when we analyzed the impact of *MN1* expression in the 3 molecular subsets composing the Intermediate-I genetic group. As seen in supplemental Table 1, all patients in the subset characterized by wild-type *NPM1* genes and the presence of *FLT3*-ITD had high *MN1* expression, thus precluding assessment of the prognostic significance of *MN1* expression in this subset. Of the remaining 2 subsets, significant differences in CR rates, OS, and EFS between the low and high *MN1* expressers were observed only among patients with mutated *NPM1* who harbored *FLT3*-ITD, whereas these outcome measures did not differ significantly in the subset encompassing patients with wild-type *NPM1* and no *LT3*-ITD (supplemental Table 1). However, because the numbers of patients in each subset composing the ELN Intermediate-I genetic group were relatively small (29, 11, and 34 patients, respectively), our analyses

Table 3. Multivariable regression analysis for outcome according to the *MN1* expression status in all older patients with de novo CN-AML, and in those 70 years of age or older

End point	Variables in final models	OR/HR	95% CI	*P*
All patients				
CR*	*MN1*, low vs high	3.16	1.29, 7.70	.01
	BAALC, low vs high	4.39	1.85, 10.44	< .001
	WBC, each 2-fold increase	0.76	0.62, 0.93	.01
EFS†	*MN1*, low vs high	0.54	0.34, 0.86	.03¶
	BAALC, low vs high	0.43	0.27, 0.68	.002¶
	WBC, each 2-fold increase	1.22	1.09, 1.35	< .001¶
	Platelets, each 50-unit increase	1.15	1.05, 1.25	.002
Patients 70 y or older				
CR‡	*MN1*, low vs high	3.97	1.22, 12.90	.02
OS§	Interaction of *MN1* and *NPM1*			.29
	Mutated *NPM1*:	0.41	0.17, 0.97	.04
	MN1, low vs high			
	Wild-type *NPM1*:			.58
	MN1, low vs high			
EFS‖	Interaction of *MN1* and *NPM1*			.14
	Mutated *NPM1*:	0.37	0.15, 0.88	.02
	MN1, low vs high			
	Wild-type *NPM1*:			.87
	MN1, low vs high			

OR > 1 (< 1) indicates higher (lower) CR rate for the higher values of the continuous variables and the first category listed for the categorical variables. HR > 1 (< 1) indicates higher (lower) risk for an event for the higher values of the continuous variables and the first category listed for the categorical variables.

*Variables considered in the model based on univariable analyses were *MN1* expression (high vs low; median cut), *BAALC* expression (high vs low; median cut), *FLT3*-ITD (positive vs negative), *IDH2* (mutated vs wild-type), *NPM1* (mutated vs wild-type), WBC (continuous, log base 2), and platelets (continuous, 50-unit increase).

†Variables considered in the model based on univariable analyses were *MN1* expression (high vs low; median cut), *BAALC* expression (high vs low; median cut), *ERG* expression (high vs low; median cut), *FLT3*-ITD (positive vs negative), *IDH2* (mutated vs wild-type), *NPM1* (mutated vs wild-type), *WT1* (mutated vs wild-type), WBC (continuous, log base 2), and platelets (continuous, 50-unit increase).

‡Variables considered in the model based on univariable analyses were *MN1* expression (high vs low; median cut), *BAALC* expression (high vs low; median cut), platelets (continuous, 50-unit increase), and *NPM1* (mutated vs wild-type).

§Variables considered in the model based on univariable analyses were *MN1* expression (high vs low; median cut), *BAALC* expression (high vs low; median cut), *IDH1* (mutated vs wild-type), *NPM1* (mutated vs wild-type), and platelets.

‖Variables considered in the model based on univariable analyses were *MN1* expression (high vs low; median cut), *BAALC* expression (high vs low; median cut), *IDH2* (mutated vs wild-type), *NPM1* (mutated vs wild-type), and platelets.

¶Does not meet the proportional hazards assumption. For EFS, the HR for *BAALC*, high vs low (median cut), *MN1*, high vs low (median cut), and WBC are reported at 3 months.

should be considered preliminary and of a descriptive nature, and the results have to be confirmed by larger studies.

Genome-wide gene-expression profiling

To gain insights into the biology of older CN-AML patients differentially expressing *MN1*, we derived a genome-wide gene expression signature. The *MN1*-associated gene expression signature consisted of 507 probe sets, representing 323 annotated genes (Figure 4).

In low *MN1* expressers, 258 probe sets, representing 158 genes, were found underexpressed, and 249 probe sets, representing 164 genes, overexpressed compared with high *MN1* expressers. The probe set representing *MN1* was among the most underexpressed probe sets in the low *MN1*-expressing patients, corroborating the quantification of *MN1* expression obtained by real-time RT-PCR (Figure 4). All microarray gene expression data are available on ArrayExpress under accession number E-TABM-1189.

Consistent with our observation in younger patients,[18] patients with low *MN1* expression had lower expression of genes previously associated with worse outcome in AML, such as *BAALC*,[19,29,30] the surface marker *CD200*,[35] the growth factor *HGF*,[36] and *CD34*, as well as the adhesion molecule *CD44*, a key regulator of AML leukemic stem cells necessary for the stem cells to interact with their microenvironment (Figure 4).[37,38] We also observed lower expression of *ABCB1* (*MDR1*), a gene encoding the multidrug resistance protein, whose high expression also has been associated with worse outcome in older AML patients.[39] Furthermore, patients with low *MN1* expression had lower expression of *AKT3*, a member of the *AKT* kinase family, which has a central role in cell proliferation, survival, and drug resistance in AML,[40] and of the transcription factor *STAT5B*. Indeed, Heuser et al[41] previously showed that STAT5 signaling is critical for leukemia stem cell self-renewal in an *MN1* and *HOXA9*-expressing leukemia model.

Highly expressed in low *MN1*-expressing patients were the *HOXA* and *HOXB* cluster genes, as well as the *HOX* cofactor *MEIS1*, which are important for developmental processes and hematopoietic stem cell function (Figure 4).[42] We also observed higher expression of the tumor suppressor *TP53BP2*, which is known to interact with and inhibit the antiapoptotic protein BCL2.[43]

Genome-wide microRNA expression profiling

To further elucidate the biologic features associated with low *MN1* expression, we derived a microRNA expression signature. The *MN1*-associated microRNA expression signature was composed of 20 probes (Figure 5), 13 of which, representing 9 microRNAs, were underexpressed, and 7, representing 7 microRNAs, overexpressed in low *MN1* expressers compared with high *MN1* expressers. All microRNA data are available on ArrayExpress under accession number E-TABM-1190.

In the low *MN1*-expressing patients, we found *miR-126* and its passenger strand *miR-126** among the most underexpressed microRNAs, which is consistent with our previous findings in

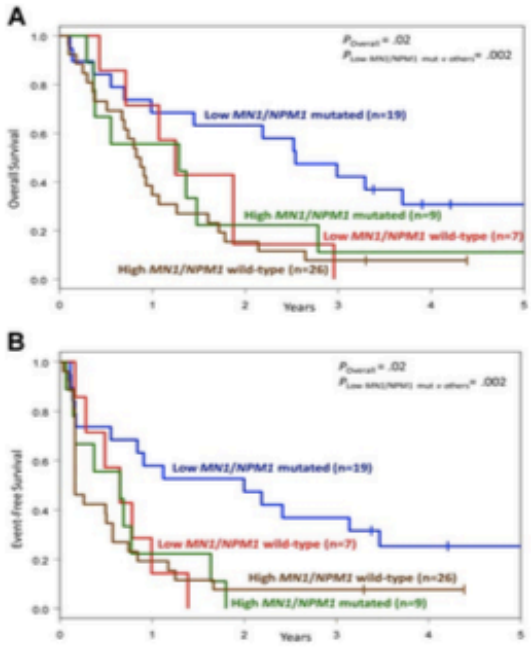

Figure 2. Outcome of CN-AML patients 70 years of age or older with respect to *MN1* expression and *NPM1* mutation status. (A) OS. (B) EFS.

younger patients.[18] In addition, underexpressed were *miR-146a*, low expression of which has been associated with the 5q− syndrome,[44] and *miR-146b*. Furthermore, we observed a lower expression of *miR-30b*, whose amplification and overexpression have been linked to medulloblastoma.[45]

Consistent with the higher expression of *HOX* genes in low *MN1* expressers, we observed higher expression of the *HOX*-gene embedded microRNAs *miR-10a* and *miR-10b*. We also observed higher expression of *let-7b*, a member of a known tumor-suppressor microRNA family, which has been found down-regulated in AML with favorable cytogenetics [ie, t(8;21), inv(16) and t(15;17)].[46] We observed higher expression of *miR-449a*, shown to target *HDAC1* and induce growth arrest in prostate cancer.[47]

Discussion

The majority of patients with AML are older than 60 years at diagnosis. Although our knowledge of molecular prognostic markers is most extensive in younger CN-AML patients,[7] recently there has been progress in our understanding of the role molecular alterations play in prognostication of older patients.[9-12,27,28] The main objective of this study was to elucidate the prognostic impact of *MN1* expression in older CN-AML patients, and to determine whether this knowledge can be integrated into the landscape of other established molecular markers.

We demonstrate here that CN-AML patients 60 years of age or older with low *MN1* expression have higher CR rates and that their OS and EFS are longer than those of patients with high *MN1* expression. However, we did not observe a significant difference in DFS, which is somewhat different from our findings in younger patients, where low *MN1* expression associated with higher CR rates and longer DFS, OS, and EFS.[18] This discrepancy might be related to the differences in the intensity of treatment regimens administered to the younger and older patients. As in younger CN-AML patients, we observed an association of low *MN1* expression with mutated *NPM1* and lower *BAALC* expression. In multivariable analyses, *MN1* expresser status remained a significant prognosticator for CR attainment, even in the context of other molecular markers, including *NPM1* mutation and *BAALC* expresser status. Indeed, the expresser status of *MN1* and *BAALC* were the only molecular markers associated with CR achievement

Table 4. Outcomes according to *MN1* expression in older CN-AML within the ELN genetic groups

End point	All	Low *MN1*	High *MN1*	*P**	OR/HR (95% CI)
ELN Favorable group	n = 66	n = 40	n = 26		
CR, no. (%)	51 (77)	33 (83)	18 (69)	.24	2.10 (0.65, 6.72)
DFS				.84	
Median, y	0.9	1.0	0.7		1.07 (0.56, 2.03)
Disease-free at 3 y, % (95% CI)	22 (12-34)	21 (9-36)	22 (7-43)		
OS				.81	0.94 (0.54, 1.61)
Median, y	1.5	1.5	1.4		
Alive at 3 y, % (95% CI)	29 (18-40)	32 (19-47)	23 (9-40)		
EFS				.69	0.90 (0.53, 1.53)
Median, y	0.8	0.9	0.6		
Event-free at 3 y, % (95% CI)	17 (9-27)	18 (8-31)	15 (5-31)		
ELN Intermediate-I group	n = 74	n = 30	n = 44		
CR, no. (%)	42 (57)	23 (77)	19 (43)	.008	4.32 (1.54, 12.17)
DFS				.15	0.63 (0.33, 1.20)
Median, y	0.5	0.6	0.5		
Disease-free at 3 y, % (95% CI)	7(2-17)	13 (3-30)	0 (NA)		
OS				.05	0.61 (0.38, 1.00)
Median, y	0.7	0.9	0.7		
Alive at 3 y, % (95% CI)	5 (2-12)	10 (3-24)	2 (.1-10)		
EFS				.003	0.49 (0.30, 0.80)
Median, y	0.3	0.6	0.2		
Event-free at 3 y, % (95% CI)	4 (1-10)	10 (3-24)	0 (NA)		

OR indicates the odds of achieving a CR for low *MN1* vs high *MN1* expressers; HR, the hazard of having an event for low *MN1* vs high *MN1* expressers; CI, confidence interval; and NA, not applicable (CI could not be attained).

**P* values for categorical variables are from Fisher exact test. *P* values for time-to-event variables are from the log-rank test (OS, DFS, and EFS).

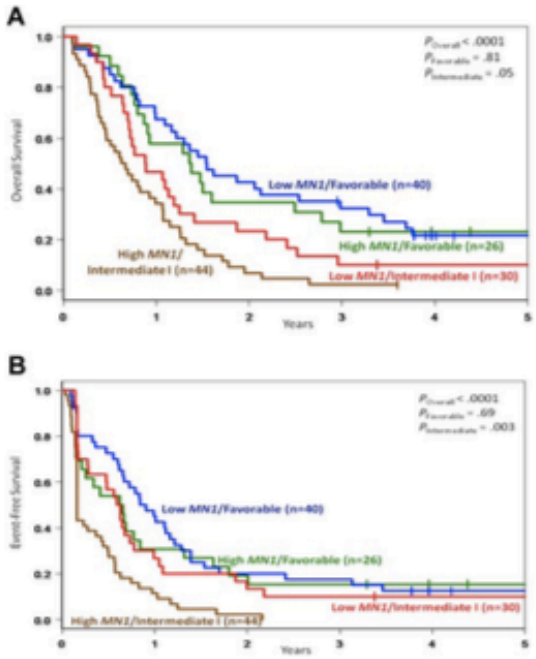

Figure 3. Outcome of CN-AML patients 60 years of age or older with respect to *MN1* expression within the ELN Favorable and ELN Intermediate-I genetic groups. (A) OS. (B) EFS.

in the patients investigated in the current study. We did not find an independent impact of *MN1* expression status on OS in the entire cohort of older patients that we analyzed, in contrast to younger patients.[18] This might be accounted for by differences in disease biology but might also be related to the lower intensity of postremission treatment administered to older AML patients compared with younger patients.

To our knowledge, only one recent study of the prognostic significance of *MN1* expression in CN-AML included patients 60 years of age or older among those analyzed.[48] This study showed that high *MN1* expression was associated with lower probability of CR achievement and shorter relapse-free survival, OS, and EFS.[48] However, in contrast to our findings, *MN1* expression was not an independent prognostic factor in the entire cohort of 210 patients analyzed by Metzeler et al,[48] and the outcome data were not reported separately for a subgroup of 101 patients 60 years of age or older.[48]

Our group recently reported age-related differences with respect to the impact on outcome of 2 molecular markers in older CN-AML patients. We found a stronger impact of *NPM1* mutations in patients 70 years of age or older as opposed to those 60-69 years of age,[9] and a stronger impact of *FLT3*-ITD in patients 60-69 years of age as opposed to those 70 years of age and older.[11] The current study provides evidence that the prognostic impact of *MN1* expression is also influenced by the patients' age. Although low *MN1* expressers in both age subgroups had higher probability of achieving a CR, *MN1* expression was prognostic with respect to OS and EFS only in the subgroup 70 years of age or older. Moreover, our data suggest that our finding of better outcome associated with *NPM1* mutations in patients 70 years of age or older does not pertain to all such patients but mostly to those who, in addition to *NPM1* mutation, have low *MN1* expression. Consequently, if our findings are confirmed and a standardized method of *MN1* expression quantification is established, testing for both *NPM1* mutations and *MN1* expression could be recommended to achieve the best prognostic stratification of CN-AML patients 60 years of age or older. The reasons for the age-related differences in the impact of *MN1* expression, *NPM1* mutations, or *FLT3*-ITD on outcome of older patients remain unknown.

Recently, the ELN expert panel proposed a novel risk classification for AML based on cytogenetics and molecular markers.[8] Within this classification, CN-AML patients are assigned to Favorable or Intermediate-I genetic groups based on the mutational status of the *CEBPA*, *NPM1*, and *FLT3* genes.[8] To evaluate whether determination of *MN1* expression levels can improve this classification, we analyzed the prognostic significance of *MN1* expression status separately within the ELN Favorable and the Intermediate-I genetic groups of CN-AML. In our patient cohort, *MN1* expression did not impact on outcome of the Favorable group. However, we observed a strong impact of *MN1* expression status on patients belonging to the Intermediate-I genetic group. Within this group, patients with low expression of *MN1* had better outcome than those with high *MN1* expression, and their CR rates and EFS were not significantly different from those in the ELN Favorable group. However, DFS and OS of low *MN1* expressers in the Intermediate-I genetic group were better than high *MN1* expressers, but not comparable with those in the ELN Favorable group. If our findings are confirmed, *MN1* expression status might become a molecular marker that will help refine the ELN classification. Furthermore, our analysis of molecular subsets within the Intermediate-I genetic group suggests that patients who benefit most from having low *MN1* expression are those who harbor both *NPM1* mutation and *FLT3*-ITD. This finding requires corroboration in a larger set of patients.

The molecular mechanisms by which *MN1* contributes to leukemia remain elusive. To gain deeper insights into the biology of the disease, we derived gene- and microRNA-expression signatures associated with *MN1* expression. The genome-wide microarray profiling supports the prognostic significance of low *MN1* expression levels by demonstrating concurrent underexpression of genes and microRNAs associated with biologic features of aggressive phenotypes. Not surprisingly, we found the gene expression signature associated with *MN1* expression derived in older CN-AML patients to be similar to the one we reported in patients younger than 60 years.[18] It is known that *MN1* expression not only negatively impacts on cell differentiation but also affects chemotherapy response,[49] which is in line with our finding of the importance of low *MN1* expression for CR achievement. Heuser et al[50] previously showed that genes that are associated with undifferentiated hematologic precursor cells also associate with a poor response to induction therapy. Consistently, in both younger and older patients, low *MN1* expression was associated with higher CR rates and lower expression of known adverse outcome predictors and of genes involved in chemotherapy resistance, such as *ABCB1*. These findings may, at least in part, explain the observed, independent association with better treatment response of low *MN1* expressers in older CN-AML. We also found overlapping microRNA expression features in younger and older patients with low *MN1* expression, including down-regulation of *miR-126* and *miR-130b*, and this may indicate that these microRNAs play an important role in modifying patients' response to therapy.

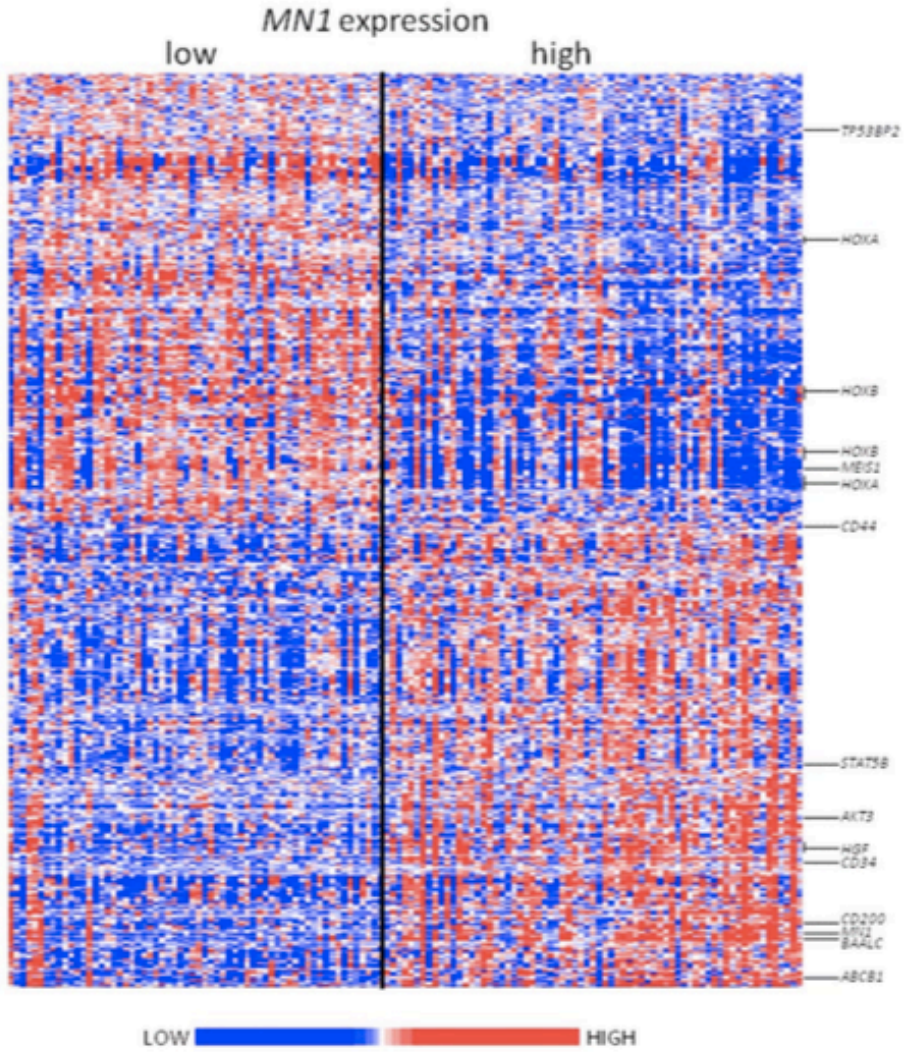

Figure 4. Heat map of the derived gene expression signature associated with *MN1* expression in the group of CN-AML patients 60 years of age or older. The patients are ordered from left to right by increasing expression of *MN1*. Expression values of the probe sets are represented by color: blue represents expression less than the median value for the given probe set; and red, expression greater than the median value for the given probe set. Up- and down-regulated genes that are mentioned in the text are indicated along the side.

In conclusion, we show that *MN1* expression is an important predictor of treatment response in older de novo CN-AML patients. Prognostic impact of *MN1* expression is especially strong in patients 70 years of age or older, and a combination of low *MN1* expression and mutated *NPM1* identifies a subset of these patients with a particularly good outcome. Furthermore, the gene- and microRNA-expression profiles we derived may help to shed light on the complex biology of *MN1*-associated disease. Once a standardized method of expression quantification is established (eg, by digital mRNA quantification technologies) and absolute cutpoints are defined, measurements of pretreatment *MN1* expression may be included in diagnostic panels and used to improve risk stratification of older CN-AML patients and to guide treatment decisions in clinical trials testing new agents targeting genes, such as *ABCB1* or even *MN1* itself.

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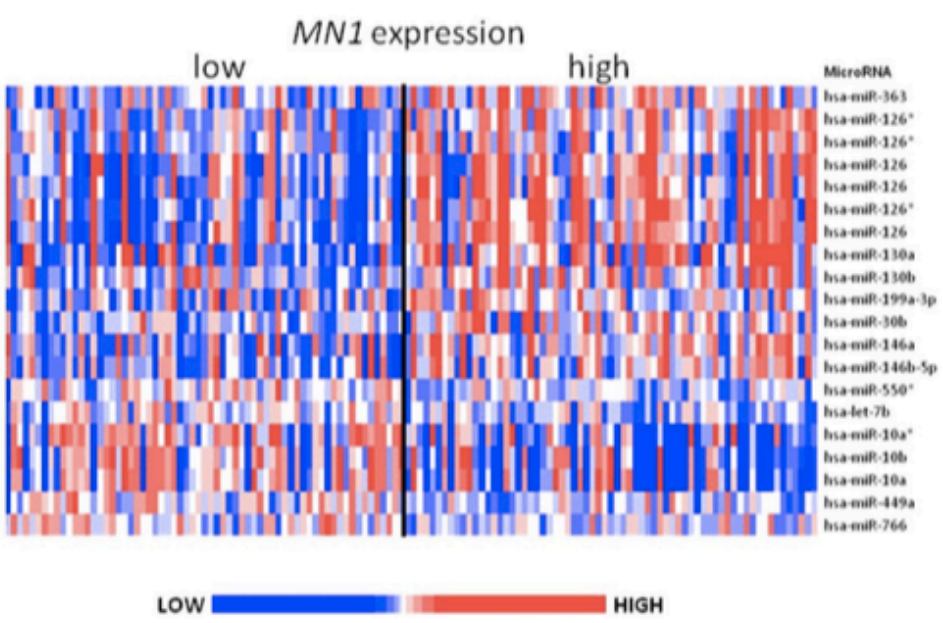
A



B

	Underexpressed in low *MN1* patients	
microRNA	Fold-change	
miR-30b	0.72	
miR-126	0.43	
*miR-126**	0.47	
miR-130a	0.48	
miR-130b	0.77	
miR-146a	0.67	
miR-146b	0.63	
miR-199a	0.76	
miR-363	0.68	

	Overexpressed in low *MN1* patients	
microRNA	Fold-change	
let-7b	1.24	
miR-10a	2.8	
*miR-10a**	2.8	
miR-10b	2.7	
miR-449a	1.22	
*miR-550**	1.24	
miR-766	1.2	

Figure 5. Heat map and table of the derived microRNA expression signature associated with *MN1* expression in the group of CN-AML patients 60 years of age or older. (A) Heat map of the derived microRNA expression signature associated with *MN1* expression in the group of CN-AML patients 60 years of age or older. The patients are ordered from left to right by increasing expression of *MN1*. Expression values of the probe sets are represented by color: blue represents expression less than the median value for the given probe; and red, expression greater than the median value for the given probe. Up- and down-regulated genes and microRNAs are indicated along the side. (B) Tables showing fold changes of the microRNAs that form the signature with corresponding fold changes.

Authorship

Contribution: S.S., G.M., J.K., M.D.R., K. Mrózek, K. Maharry, M.A.C., and C.D.B. contributed to the design and analysis of this study and the writing of this manuscript; S.S., H.B., K.H.M., S.P.W., and Y.-Z.W. carried out laboratory-based research; J.E.K., M.D.R., and K. Maharry performed statistical analyses; G.M., B.L.P., M.R.B., J.E.K., A.J.C., R.A.L., M.A.C., and C.D.B. were involved directly or indirectly in care of patients and/or sample procurement; and all authors agreed on the final version.

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2.4 High *BAALC* expression as residual disease marker prior to allogeneic stem cell transplantation

Over the last years, assessment of MRD for risk stratification during AML disease course became of growing interest. MRD allows the detection of a small residual disease population despite morphologic remission. Thus, it has the potential to add valuable prognostic information for AML patients and will likely guide treatment decisions in the future.

The paper included in this paragraph evaluates the feasability of *BAALC* copy number assessment for MRD detection by the novel digital droplet PCR technique, prior to allogeneic HSCT in CR. It shows that a *BAALC* copy number in peripheral blood prior to an allogeneic HSCT that is higher than the copy number expressed in a healthy control cohort associates with a higher CIR and shorter OS in AML patients. These findings are most likely mediated by residual disease cells in patients with high *BAALC* copy numbers. The paper is the first to analyze *BAALC* as a MRD marker in a patient cohort receiving allogeneic HSCT and shows the feasability of MRD assessment independent of a disease-specific molecular alteration.

Manuscripts included in this paragraph:

- Jentzsch M, Bill M, Grimm J, Schulz J, Schubert K, Beinicke S, Häntschel J, Pönisch W, Franke GN, Vucinic V, Behre G, Lange T, Niederwieser D, **Schwind S**. High *BAALC* copy numbers in peripheral blood prior to allogeneic transplantation predict early relapse in acute myeloid leukemia patients. *Oncotarget*. 2017. doi: 10.18632/oncotarget.21322

High *BAALC* copy numbers in peripheral blood prior to allogeneic transplantation predict early relapse in acute myeloid leukemia patients

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ABSTRACT

High *BAALC* expression levels at acute myeloid leukemia diagnosis have been linked to adverse outcomes. Recent data indicate that high *BAALC* expression levels may also be used as marker for residual disease following acute myeloid leukemia treatment. Allogeneic hematopoietic stem cell transplantation (HSCT) offers a curative treatment for acute myeloid leukemia patients. However, disease recurrence remains a major clinical challenge and identification of high-risk patients prior to HSCT is crucial to improve outcomes. We performed absolute quantification of *BAALC* copy numbers in peripheral blood prior (median 7 days) to HSCT in complete remission (CR) or CR with incomplete peripheral recovery in 82 acute myeloid leukemia patients using digital droplet PCR (ddPCR) technology. An optimal cut-off of 0.14 *BAALC/ABL1* copy numbers was determined and applied to define patients with high or low *BAALC/ABL1* copy numbers. High pre-HSCT *BAALC/ABL1* copy numbers significantly associated with higher cumulative incidence of relapse and shorter overall survival in univariable and multivariable models. Patients with high pre-HSCT *BAALC/ABL1* copy numbers were more likely to experience relapse within 100 days after HSCT. Evaluation of pre-HSCT *BAALC/ABL1* copy numbers in peripheral blood by ddPCR represents a feasible and rapid way to identify acute myeloid leukemia patients at high risk of early relapse after HSCT. The prognostic impact was also observed independently of other known clinical, genetic, and molecular prognosticators. In the future, prospective studies should evaluate whether acute myeloid leukemia patients with high pre-HSCT *BAALC/ABL1* copy numbers benefit from additional treatment before or early intervention after HSCT.

INTRODUCTION

The identification of cytogenetic, molecular, and clinical factors impacting on outcome at acute myeloid leukemia (AML) diagnosis improved risk stratification [1, 2]. But pre-treatment AML characterization may not capture all parameters important for outcome, e.g. response or resistance to therapy [3]. Early detection of measurable residual disease (MRD) through multiparameter flow cytometric (MFC) or quantitative real time PCR (qRT-PCR) assays may allow treatment intervention before overt relapse occurs [3–5]. MFC enables MRD assessment through detection of aberrant surface antigen expression in complete remission (CR) [Wormann *et al*, ASH 1991,6,7]. However, heterogenic outcomes were observed in MFC-MRD studies [8] and

reproducibility of MFC-MRD assessment is limited by the need of specialized laboratories [3, 4]. Sensitive qRT-PCR enabled MRD detection in AML cases with common fusion genes and in *NPM1* mutated AML [3, 9, 10]. Thus qRT-PCR MRD monitoring is widely restricted to patients carrying specific molecular alterations [11] with the exception of Wilms' tumor gene 1 (*WT1*) expression [9, 12]. Because clonal evolution can occur at disease progression and might complicate early disease detection at relapse [13], it seems reasonable to track several MRD markers per patient.

The gene brain and acute leukemia, cytoplasmic (*BAALC*) has been suggested as a suitable MRD marker as it is expressed at low levels in peripheral blood and bone marrow of healthy individuals [14, 15], but upregulated in AML patients [15]. High *BAALC* expression levels at AML diagnosis have been shown to associate with adverse outcomes [16–19]. Recently, high *BAALC* levels have also been linked to worse outcome if measured by qRT-PCR after achievement of CR [15], completion of induction therapy [11, 20] or after allogeneic stem cell transplantation (HSCT) [21]. However, qRT-PCR has the disadvantage of the need of calibration curves and poor inter-laboratory comparability. In chronic myeloid leukemia (CML) this led to complex harmonization efforts for *BCR-ABL1* detection [22], which are not yet clinical practice for MRD markers in AML. Here we adopted digital droplet PCR (ddPCR), a new technique which allows an absolute quantification without the need of standard curves [23].

Allogeneic HSCT is a potential curative treatment option for AML patients and offers the highest chance of sustained remissions [2]. Non-myeloablative conditioning regimens (NMA), in which the therapeutic success is mainly based on graft-versus-leukemia (GvL) effects, enabled allogeneic HSCT in comorbid or older individuals [24]. Disease recurrence after HSCT remains a major clinical problem with short patient survival [25]. Until today, no study evaluated the feasibility of *BAALC* expression levels for risk stratification in AML patients prior to allogeneic HSCT in CR or CR with incomplete peripheral recovery (CRi), which was the main objective of our study. Early identification of AML patients at high risk of relapse may result in adjustment of treatment strategies prior to morphologic relapse and subsequently improve outcomes. With the goal of a robust, rapid, and reproducible approach, we used peripheral blood to assess the feasibility of ddPCR for absolute quantification of *BAALC/ABL1* copy numbers.

RESULTS

BAALC/ABL1 copy numbers in AML patients prior to HSCT and in healthy individuals

Within the patient cohort in CR or CRi prior to HSCT, we observed a median pre-HSCT *BAALC/ABL1* copy number of 0.03 (range 0.00-2.58, Figure 1). In the healthy control cohort, median *BAALC/ABL1* copy numbers were 0.04 (range 0.03-0.10). Overall, there was no significant difference in the *BAALC/ABL1* copy

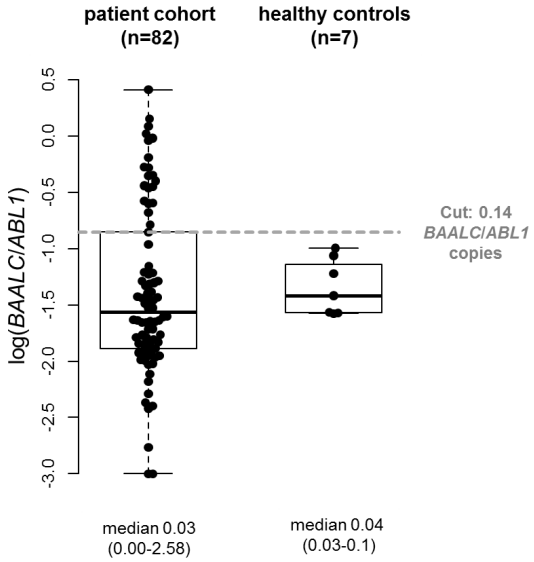

Figure 1: Comparison of absolute *BAALC/ABL1* copy numbers in AML patients pre-HSCT (n=82) and healthy controls (n=7).

numbers between both groups (*P*=.34). The patient cohort and the healthy control cohort were evenly matched in age (*P*=1) and sex (*P*=1, Supplementary Table 2).

Associations of high pre-HSCT *BAALC/ABL1* copy numbers with clinical and biological characteristics

Patients with high and low pre-HSCT *BAALC/ABL1* copy numbers did not differ significantly in the evaluated characteristics at diagnosis (Table 1, Supplementary Table 1). However, there was a trend for a lower incidence of *CEBPA* mutations in patients with high pre-HSCT *BAALC/ABL1* copy numbers (*P*=.09). Patients with high and low pre-HSCT *BAALC/ABL1* copy numbers also did not differ significantly in pre-HSCT characteristics; specifically, no significant differences were found regarding the remission status at HSCT, white blood count at time of blood sampling for *BAALC/ABL1* copy number evaluation or time from blood sampling to HSCT (Supplementary Table 1).

Prognostic significance of pre-HSCT *BAALC/ABL1* copy numbers

Patients with high pre-HSCT *BAALC/ABL1* copy numbers had a significantly higher cumulative incidence of relapse (CIR, *P*=.02, Figure 2A) and shorter overall survival (OS, *P*=.03, Figure 2B) which was reproduced when we restricted our analysis to patients with a normal karyotype (n=38, *P*=.007 and *P*=.11, respectively, Figures 2C and 2D). Subgroup analyses for patients harboring *de novo* disease (n=52, Supplementary Figure 2), patients transplanted in CR (n=68, Supplementary Figure 3), CD34-positive AML (n=31, Supplementary Figure 4), patients surviving longer than 100 days after HSCT (n=71, Supplementary Figure 5), as well as patients with diagnostic *BAALC/ABL1* copy number information available (n=51, Supplementary Figure 6) are shown in the Supplementary Material.

One year after HSCT, 52% of patients with high pre-HSCT *BAALC/ABL1* copy numbers relapsed compared to 25% of patients with low pre-HSCT *BAALC/ABL1* copy numbers. Furthermore, 38% of patients with high pre-HSCT *BAALC/ABL1* copy numbers were alive compared to 68% of patients with low pre-HSCT *BAALC/ABL1* copy numbers. Patients with high pre-HSCT *BAALC/ABL1* copy numbers suffering relapse had a trend for shorter time to relapse after HSCT (median 78, range 19-244 days) compared to patients with low pre-HSCT *BAALC/ABL1* copy numbers (median 116, range 27-543 days, *P*=.07). Furthermore, for patients without non-relapse mortality after 100 days and six months after HSCT, those with high pre-HSCT *BAALC/ABL1* copy numbers more often relapsed compared to patients with low pre-HSCT *BAALC/ABL1* copy numbers (37% *vs*. 11%, *P*=.02 [Figure 3], and 73% *vs*. 27%, *P*=.002, respectively). In multivariable analysis, high pre-HSCT *BAALC/ABL1* copy numbers significantly associated with higher CIR (Hazard Ratio [HR] 2.6, Confidence Interval [CI] 1.2-5.7, *P*=.01) after adjustment for disease status at HSCT (*P*=.003) and disease origin (*P*=.009) and shorter OS (HR 2.1, CI 1.1-4.1, *P*=.03, Table 2).

Detailed comparisons between the four groups of patients experiencing relapse or remaining in remission with high or low pre-HSCT *BAALC/ABL1* copy numbers are shown in the Supplementary Material.

DISCUSSION

Assessment of residual disease provides a powerful tool to measure treatment responses and to identify patients at high risk of relapse [4]. Although we still lack data of prospective MRD-guided trials in non-APL (acute promyelocyte leukemia) AML, MRD assessment may allow preemptive therapy to delay or even prevent relapse and improve outcomes [5, 26, 27]. However, about 40% of AML patients do not harbor the today commonly used molecular MRD targets [3, 4], reflecting a need for new MRD markers.

While the prognostic impact of high *BAALC* expression levels at diagnosis has been widely evaluated [3, 16–19], only a few studies with limited patient numbers evaluated *BAALC* expression levels during disease course using qRT-PCR [11, 15, 20, 21]. For MRD evaluation in AML in general, it remains unclear whether peripheral blood or bone marrow should be analyzed [7, 28]. For *BAALC*, high correlations of expression levels in peripheral blood and bone marrow in both newly diagnosed AML patients and healthy individuals have been shown [15, 16]. While some authors only used bone marrow [21], others used *BAALC* expression levels of either blood or bone marrow for survival analysis at diagnosis and during disease course [11, 20]. Peripheral blood is derived faster, with lower risk of complications and a higher convenience for the patient than bone marrow aspiration and results in comparable *BAALC* expression data [15, 16]. Therefore, we decided to restrict our analysis to peripheral blood samples to examine the prognostic impact of absolute pre-HSCT *BAALC/ABL1* copy numbers in patients receiving NMA-HSCT.

None of the aforementioned studies focusing on *BAALC* expression levels during disease course reported significant diagnostic clinical or genetic associations with different *BAALC* expression levels at a defined point in time in CR. In our study, we also did not detect any significant association of high pre-HSCT *BAALC/ABL1* copy numbers with tested pre-treatment or pre-HSCT parameters. This may indicate that the observed higher CIR and subsequent shorter OS is indeed driven by residual disease detected by high pre-HSCT *BAALC/ABL1* copy numbers rather than other commonly tested prognostic parameters. The first

Table 1: Clinical characteristics of 82 AML patients treated with HSCT according to absolute pre-HSCT *BAALC/ABL1* copy numbers (high *vs.* low, 0.14 cut)

Characteristic	All patients (n=82)	Low pre-HSCT *BAALC/ABL1* copy numbers (n=61)	High pre-HSCT *BAALC/ABL1* copy numbers (n=21)	*P*
Pre-HSCT *BAALC/ABL1* copy numbers				<.001
Median	0.03	0.02	0.44	
Range	0.00-2.58	0.00-0.11	0.14-2.58	
Age at HSCT, years				.79
Median	63.9	64.9	63.9	
Range	50.8-76.2	51.5-76.2	50.8-74.9	
Sex, n (%)				.80
Male	37	27 (44)	10 (48)	
Female	45	34 (56)	11 (52)	
Hemoglobin at diagnosis, g/dL				.54
Median	8.7	9.0	8.5	
Range	4.5-14.4	5.5-14.4	4.5-11.3	
Platelet count at diagnosis, x 10^9/L				.76
Median	65	71	63	
Range	3-224	3-167	13-224	
WBC count at diagnosis, x 10^9/L				.13
Median	7.2	4.6	22.4	
Range	0.7-385	0.8-324	0.7-385	
Blood blasts at diagnosis, %				.48
Median	22	21	28	
Range	0-97	0-97	2-97	
BM blasts at diagnosis, %				.87
Median	50	52	43	
Range	3-95	3-95	10-95	
Karyotype, n (%)				.45
Abnormal	41	32 (55)	9 (43)	
Normal	38	26 (45)	12 (57)	
ELN 2010 Genetic Group, n (%)[36]				.86
Favorable	17	12 (22)	5 (26)	
Intermediate-I	19	13 (24)	6 (32)	
Intermediate-II	19	15 (27)	4 (21)	
Adverse	19	15 (27)	4 (21)	
Disease origin, n (%)				.60
De novo	52	40 (66)	12 (57)	
Secondary	30	21 (34)	9 (43)	

(*Continued*)

Characteristic	All patients (n=82)	Low pre-HSCT *BAALC/ABL1* copy numbers (n=61)	High pre-HSCT *BAALC/ABL1* copy numbers (n=21)	*P*
NPM1 at diagnosis, n (%)				.76
Wild-type	51	36 (77)	15 (71)	
Mutated	17	11 (23)	6 (29)	
FLT3-ITD at diagnosis, n (%)				1
Absent	54	38 (79)	16 (80)	
Present	14	10 (21)	4 (20)	
CEBPA at diagnosis, n (%)				.09
Wild-type	51	34 (83)	17 (100)	
Mutated	7	7 (17)	0 (0)	

ABL1, Abelson murine leukemia viral oncogene homolog 1 gene; *BAALC*, brain and acute leukemia, cytoplasmatic gene; BM, bone marrow; *CEBPA*, CCAAT/enhancer-binding protein alpha gene; ELN, European LeukemiaNet classification 2010; *FLT3*-ITD, internal tandem duplication of the fms like tyrosine kinase 3 gene; HSCT, hematopoietic stem cell transplantation; *NPM1*, nucleophosmin 1 gene; WBC, white blood cell.

study to propose *BAALC* as a potential MRD marker analyzed 45 patients with *de novo* acute leukemia, but also included six patients with APL and 11 patients with lymphoid leukemia in their analysis [15]. The authors were able to show a superior disease-free survival in patients with lower *BAALC/GAPDH* expression levels in bone marrow after CR achievement. Another small study focused on 45 patients harboring core-binding factor (CBF) AML that received an allogeneic or autologous HSCT and evaluated *BAALC*/*ABL1* expression levels in bone marrow at diagnosis, as well as in CR after the first induction cycle, pre-HSCT, and at day 60 post-HSCT. While the authors showed significantly shorter OS, event-free survival (EFS) and higher CIR in patients with high *BAALC*/*ABL1* expression levels at diagnosis and post-HSCT, there was no significant impact on outcome after first induction cycle or pre-HSCT [21]. In contrast, we observed a strong prognostic impact of high pre-HSCT *BAALC*/*ABL1* copy numbers on CIR and OS in univariable and multivariable models. These differences might be explained by a lower patient number (n=45) and/or the restriction to CBF AML in the study of Yoon *et al.* [21] In 27 CN-AML patients with high initial *BAALC*/*ABL1* expression levels, Weber *et al.* [11] observed shorter EFS for individuals with sustained high *BAALC*/*ABL1* expression levels in peripheral blood or bone marrow after two induction cycles. Later, this data was extended to 46 and 33 patients after completion of two induction cycles and 3-6 months after completion of two induction cycles, respectively [11]. Again, patients with high *BAALC*/*ABL1* expression levels at either of both time points had shorter EFS. Despite this promising data, possible limitations of *BAALC* as MRD marker still have to be determined. While most studies showed a prognostic impact without a prior assessment of the CD34 expression status [11, 20, 21], Najima *et al.* [15] postulated *BAALC* as MRD marker limited to CD34-positive AML as *BAALC* is upregulated in CD34-positive AML [14]. Restricting our analysis to patients with CD34-positive AML, we also observed a trend for higher CIR in patients with high pre-HSCT *BAALC*/*ABL1* copy numbers despite low patient numbers (*P*=.06, n=31, Supplementary Material). Limited numbers of patients with CD34-negative AML prevented a separate analysis for this subset. However, we observed no difference in CD34-positivity or CD34 expression at diagnosis between patients with high or low pre-HSCT *BAALC*/*ABL1* copy numbers (Supplementary Table 1). Further studies are needed to evaluate whether there are specific AML subgroups for which *BAALC* represents a more suitable MRD marker than for others.

To our knowledge until today all studies used qRT-PCR for *BAALC* evaluation but different approaches to define a cut-off for high or low *BAALC* expression levels during follow-up. Najima *et al* [15]. used the two-fold standard deviation over the median of a healthy cohort, while Yoon *et al.* [21] focused on the relative *BAALC*/*ABL1* expression of the tested patients and used a ROC (receiver operation characteristic) curve to define the optimal cut for each point in time individually. The

latter resembles our approach and – despite different methodology - the evaluated cut-off in our cohort was also slightly higher than the two-fold standard deviation over the median of healthy *BAALC*/*ABL1* copy numbers in peripheral blood (0.14 vs. 0.10, respectively). Finally, Weber *et al.* used the median *BAALC*/*ABL1* expression at diagnosis of the initial cohort [11] to define high or low expression during disease course but restricted their analysis to patients with initially high *BAALC*/*ABL1* expression levels [11, 20]. In our study, for a subset of the analyzed patients (n=51) diagnostic material for *BAALC*/*ABL1* copy number assessment was available. For patients' characteristic, as well as clinical and biological associations linked with high *BAALC*/*ABL1* copy numbers at diagnosis see the Supplementary Material. When we restricted our outcome analyses to patients with low or high *BAALC*/*ABL1* copy numbers at diagnosis - despite the limited number of patients - we observed a trend for higher CIR and shorter OS for patients with high pre-HSCT *BAALC*/*ABL1* copy numbers in patients irrespective of the diagnostic *BAALC*/*ABL1* copy number (Supplementary Figure 6). In fact five of the patients with low diagnostic *BAALC*/*ABL1* copy numbers had high pre-HSCT *BAALC*/*ABL1* copy numbers, of which three subsequently relapsed (see Supplementary Material for details). Thus, despite the limited number of patients, our data indicate that pre-HSCT *BAALC/ABL1* copy

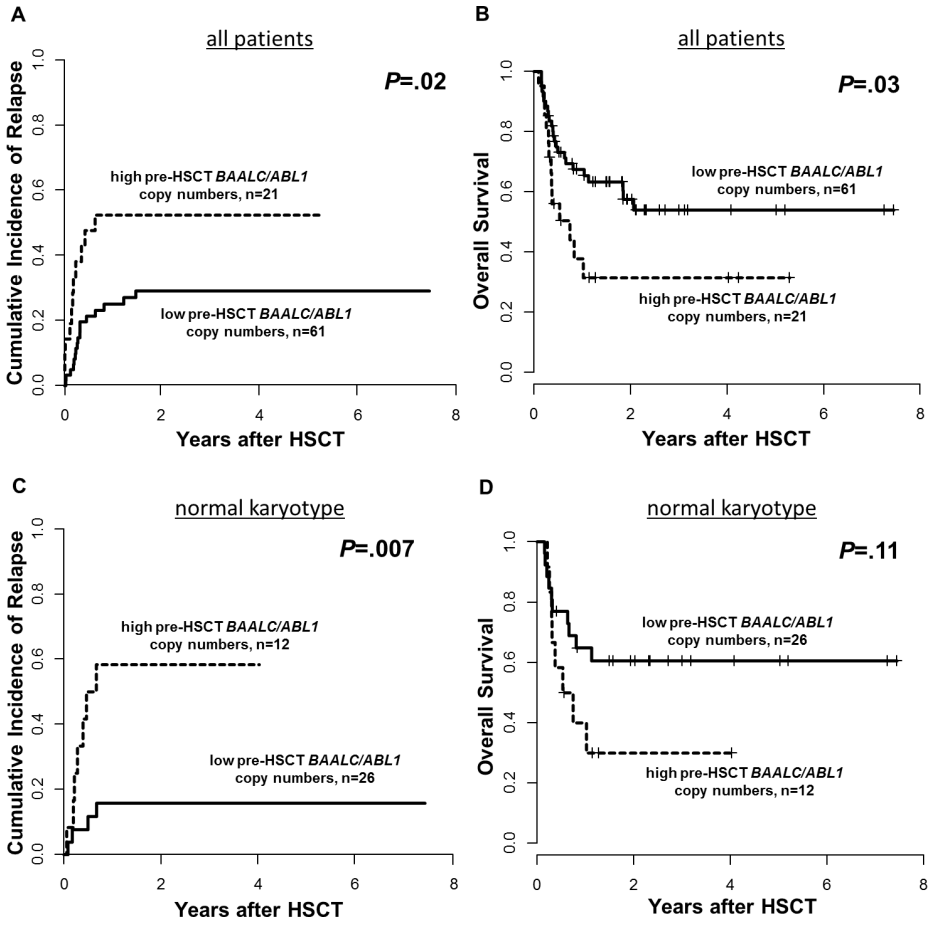

Figure 2: Outcome of patients according to pre-HSCT *BAALC*/*ABL1* copy numbers, high *vs* low, 0. 14 cut, **(A)** Cumulative Incidence of Relapse and **(B)** Overall Survival for the entire set (n=82) and **(C)** Cumulative Incidence of relapse and **(D)** Overall Survival in patients with a normal karyotype (n=38).

number determination can provide valuable clinical information also in patients with low diagnostic *BAALC*/*ABL1* copy numbers.

Considering the small number of studies focusing on *BAALC* expression as a MRD marker, the optimal cut-off needs validation. However, assessment of *BCR-ABL1* as MRD marker in CML showed us the technical difficulties of standard curves and in achieving an inter-laboratory comparability to ensure consistent analyses [22]. ddPCR has already been shown to provide comparable sensitivity to qRT-PCR but seems to have an improved day-to-day reproducibility and greater precision [23, 29, Huang et al, ASH 2015]. Therefore, ddPCR may represent a promising new method for gene expression analyses for MRD monitoring in the future.

Our here presented study is the first to demonstrate that ddPCR is a feasible method for evaluation of absolute *BAALC*/*ABL1* copy numbers prior to allogeneic HSCT. We were able to show that patients with high pre-HSCT *BAALC*/*ABL1* copy numbers had a significant higher CIR and shorter OS (P=.02 and P=.03, respectively, Figure 2). Patients with high pre-HSCT *BAALC*/*ABL1* copy numbers had an over 2.5-fold higher risk of relapse and an over 2-fold higher risk of death after HSCT compared to patients with low pre-HSCT *BAALC*/*ABL1* copy numbers (Table 2). Patients with high pre-HSCT *BAALC*/*ABL1* copy numbers more often suffered relapse within the first 100 days after HSCT (37% *vs.* 11%, P=.02) and the time from HSCT to relapse was shorter in patients with high pre-HSCT *BAALC*/*ABL1* copy numbers by trend (P=.07, Figure 3). To our knowledge, no other study reported on early relapses detected by high *BAALC* expression levels. We postulate that high pre-HSCT *BAALC*/*ABL1* copy numbers might indicate a residual disease burden in AML patients that subsequently may lead to early relapse during follow-up. Noteworthy, for all patients, peripheral blood was used in the analyses facilitating repetitive MRD assessment. We and others [11, 15, 20, 21] were able to show that *BAALC* has the potential to allow further risk stratification during disease course and subsequently may improve MRD assessment in addition to other established MRD markers such as *PML-RARA*, *CBFB-MYH11, RUNX1-RUNX1T1* or *NPM1* mutations. Furthermore, since *BAALC* is expressed at different amounts in all AML patients, it might allow molecular MRD detection in patients lacking molecular alterations commonly used for MRD assessment.

Restrictions of our study are the retrospective nature and the limited patient numbers. Future prospective clinical trials are needed to validate the here-established cut-off value and the resulting outcome findings in larger patient populations.

Even with a variety of possible treatment options such as reduction of immunosuppression, donor lymphocyte infusions or treatment with hypomethylating agents, patients suffering from morphologic relapse after HSCT have a very poor prognosis [25, 30, 31]. Pre-HSCT *BAALC*/*ABL1* copy number evaluation allows early identification of patients at higher risk of relapse and subsequently closer monitoring for relapse in the post-transplant period. In the future pre-HSCT *BAALC*/*ABL1* evaluation might guide preemptive treatment to improve the poor prognosis of AML patients with a risk for morphologic relapse. Furthermore, prospective studies will be required to evaluate whether AML patients with high pre-HSCT *BAALC*/*ABL1* copy numbers might benefit from additional treatment or intensification of the conditioning regimen prior to allogeneic HSCT.

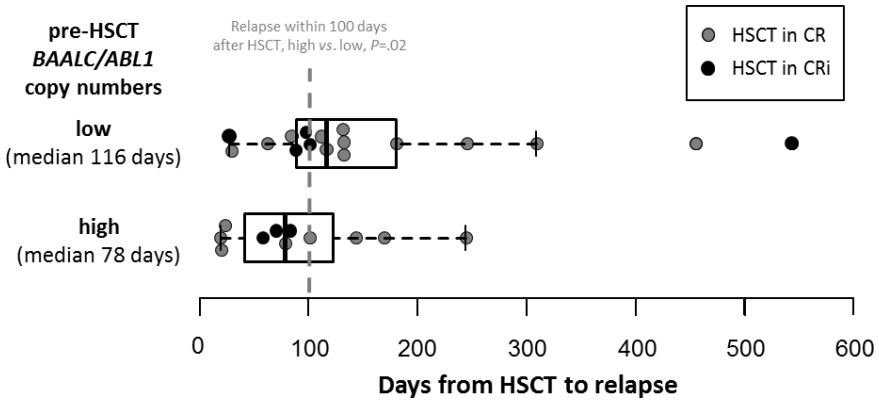

Figure 3: Time from HSCT to relapse according to high (median 78, range 19-244 days) or low (median 116, range 27-543 days) absolute pre-HSCT *BAALC*/*ABL1* copy numbers in relapsed patients (n=28).

Table 2: Multivariable outcome analyses of 82 AML patients treated with HSCT

Variable	Cumulative Incidence of Relapse		Overall survival	
	HR[a] (95% CI)	*P*	HR[a] (95% CI)	*P*
pre-HSCT *BAALC*/*ABL1* copy numbers (high *vs.* low, 0.14 cut)	2.6 (1.2-5.7)	.012	2.1 (1.1-4.1)	.03
Disease origin (*de novo vs.* secondary)	0.4 (0.2-0.8)	.009	-	-
Disease status at HSCT (CR *vs.* CRi)	0.3 (0.1-0.7)	.003	-	-

ABL1, Abelson murine leukemia viral oncogene homolog 1 gene; *BAALC*, brain and acute leukemia, cytoplasmatic gene; CI, confidence interval; CR, complete remission; CRi, CR with incomplete peripheral recovery; HSCT, hematopoietic cell transplantation; HR, hazard ratio.

[a] HR, hazard ratio, <1 (>1) indicate lower (higher) risk for an event for the first category listed for the dichotomous variables.

Variables considered in the models were those significant at α=0.20 in univariable analyses. For OS endpoint, variables considered were hemoglobin count at diagnosis, white blood cell count at diagnosis, pre-HSCT *BAALC/ABL1* copy numbers (high vs. low) and HLA match (antigen match vs mismatch) while for CIR endpoint, variables considered were disease origin (de novo vs. secondary), *BAALC/ABL1* copy numbers (high vs. low), disease status at HSCT (CR vs. CRi) and ELN 2010 Genetic Group.

MATERIALS AND METHODS

Patients and treatment

A total of 82 adult AML patients who received allogeneic HSCT at the University of Leipzig between September 2002 and December 2015 were retrospectively included in this analysis. All patients had peripheral blood samples up to 14 days prior to HSCT (median 7, range 0-14 days) for *BAALC*/*ABL1* copy number assessment available. White blood count (WBC) was assessed at time of blood sampling for analysis. Additionally, for 51 of these patients diagnostic peripheral blood or bone marrow samples were available for *BAALC*/*ABL1* copy number analysis. For details see Supplementary Material and Supplementary Table 3.

All patients received age-dependent cytarabine based chemotherapy protocols (under or over 60 years) and were consolidated with HSCT in first (60%) or second CR (23%) or CRi (17%). For details please see Supplementary Material. Median age at HSCT was 63.9 (range 50.8-76.2) years. Written informed consent for participation in these studies was obtained in accordance with the Declaration of Helsinki.

All patients received NMA conditioning with fludarabine 30 mg/m^2 for three days followed by 2 Gy total body irradiation [32, 33] and infusion of granulocyte colony stimulating factor (G-CSF)-mobilized peripheral blood stem cells on day 0. Reasons for choosing a NMA protocol were age over 50 years for patients receiving unrelated HSCT (n=71) or age over 55 years for patients receiving related HSCT (n=11). Patients' characteristics are shown in Table 1 and Supplementary Table 1. For Information regarding prevention and incidence of acute and chronic graft-versus-host disease see Supplementary Material. Median follow-up after HSCT for patients alive was 1.8 years.

Healthy control cohort

In a control cohort of 7 healthy volunteers (median age of 62.7, range 39.6-82.0 years), absolute *BAALC*/*ABL1* copy numbers in peripheral blood were evaluated. Written informed consent was obtained for all healthy individuals; their characteristics are shown in Supplementary Table 2.

Cytogenetic, molecular genetic, and flow cytometric analyses

At diagnosis, cytogenetic analyses, the presence of internal tandem duplication in the *FLT3* gene (*FLT3*-ITD) as well as mutations in the *FLT3* tyrosine kinase domain (*FLT3*-TKD), *NPM1* and *CEBPA* genes were determined as previously described [34]. For details see Supplementary Material. For patients with material available, the CD34 and CD38 expression on mononuclear cells in bone marrow at diagnosis was determined as previously described [35].

ddPCR assessment of *BAALC/ABL1* copy numbers

Absolute *BAALC* copy numbers were assessed using a specific ddPCR assay (BioRad, Hercules,

California, USA; Assay ID: dHsaCPE5025566) according to manufacturer's specifications. Primers and probe sequences for *ABL1* copy number assessment (Biomers, Ulm, Germany) are shown in the Supplementary Material. ddPCR was performed on a QX100 platform (BioRad) and QuantaSoft software (Biorad) was used for raw data processing. With the droplet generator, each sample was divided into approximately 10,000 - 20,000 partitions (droplets). After PCR amplification (for details see Supplementary Material) the samples were placed into the droplet reader, where each droplet was read as positive or negative for the gene expression by issuing specific fluorescence signals (FAM and HEX). Redistribution according to the Poisson's algorithm determined the target copy number in the original sample. Two examples of the ddPCR droplet reader output are given in Supplementary Figure 1.

BAALC/*ABL1* cut-off point definition

Using the R package 'OptimalCutpoints' a cut-off point of 0.1397 absolute pre-HSCT *BAALC*/*ABL1* copies was determined and used to define patients with high (n=21, 26%) and low (n=61, 74%) pre-HSCT *BAALC*/*ABL1* copy numbers in peripheral blood. For details see Supplementary Material.

End points and statistical analyses

For definition of clinical endpoints and statistical analyses for associations and survival (univariable and multivariable) see Supplementary Material.

Author contributions

MJ and SS contributed to the design and analysis of this study and the writing of the manuscript, and all authors agreed on the final version. MJ, MB, KG, JS, JG, JH and SB carried out the laboratory-based research; MJ and SS performed statistical analyses; and MJ, WP, GNF, VV, GB, TL, DN and SS were involved directly or indirectly in the care of patients and/or sample procurement.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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2.5 Aberrant microRNA expression as prognostic markers in AML and their clinical and therapeutic implications

In recent years it became clearer that differential miR expression levels may not only be used for risk stratification but that exploring their biology may provide important insights into AML pathogenesis. Furthermore, identifiying ways to manipulate their expression levels may present new therapeutic avenues for AML treatment.

The first part of this paragraph focuses on *miR-181a*. The first presented paper evaluated the prognostic significance of *miR-181a* expression levels in the context of established molecular markers in CN-AML, and aided to gain insight into the leukemogenic role of *miR-181a*. This paper was the first to provide evidence that the expression of a single miR is associated with clinical outcome of patients with CN-AML, as higher *miR-181a* expression associated with longer diseas-free survival (DFS) and OS.

The second paper included in this paragraph demonstrates that high *miR-181a* expression levels lead to a less aggressive AML phenotype by directly downregulating KRAS, NRAS, and MAPK1 and decrease AML growth. Furthermore, the paper shows that *miR-181a* expression levels not only represent a strong prognostic marker in AML, but are also involved in AML biology by targeting the RAS-MAPK-pathway, and that *miR-181a* mimics represent a novel promising therapeutic approach for AML and possibly for other RAS-driven cancers.

In the second part of this paragraph the role of *miR-29b* in AML is characterized and potential new treatment strategies are discussed. High expression levels of *miR-29b* expression associated with clinical response to the hypomethylating agent decitabine in older AML patients. Bortezomib-induced *miR-29b* up-regulation resulted in loss of transcriptional activation for several genes relevant to AML leukemogenesis. In the third paper a phase 1 trial is presented that demonstrates the feasibility and preliminary clinical activity of bortezomib plus decitabine in AML, and identified FLT3 as a novel pharmacodynamic end point for future trials.

In the fourth paper it is demonstrated that the potent histone deacetylase inhibitor AR-42 increased *miR-29b* levels and led to downregulation of known *miR-29b* targets (i.e. *SP1*, *DNMT1*, *DNMT3A* and *DNMT3B*). It also shows that the sequential administration of AR-42

followed by decitabine resulted in a strong anti-leukemic activity. These preclinical results with AR-42 priming before decitabine administration represent a promising, novel treatment approach and a paradigm shift with regard to the combination of epigenetic-targeting compounds in AML, where decitabine has been traditionally given before histone deacetylase inhibitors.

In the final paper included in this paragraph it is demonstrated that treatment with a transferrin-conjugated nanoparticle loaded with synthetic *miR-29b* (Tf-NP-*miR-29b*) significantly downregulated *DNMT*s, *CDK6*, *SP1*, *KIT*, and *FLT3*, decreased AML cell growth and impaired colony formation. Mice engrafted with AML cells and then treated with Tf-NP-*miR-29b* had significantly longer survival. Furthermore, priming AML cell with Tf-NP-*miR-29b* before treatment with decitabine resulted in marked decrease in cell viability and showed improved antileukemic activity compared with decitabine alone *in vivo*.

Manuscripts included in this paragraph:

- **Schwind S**, Maharry K, Radmacher MD, Mrózek K, Holland KB, Margeson D, Whitman SP, Hickey C, Becker H, Metzeler KH, Paschka P, Baldus CD, Liu S, Garzon R, Powell BL, Kolitz JE, Carroll AJ, Caligiuri MA, Larson RA, Marcucci G, Bloomfield CD: Prognostic Significance of Expression of a Single microRNA, *miR-181a*, in Cytogenetically Normal Acute Myeloid Leukemia: A Cancer and Leukemia Group B Study. *J Clin Oncol.* 2010; 28:5257-64.

- Huang X*, **Schwind S***, Santhanam R, Eisfeld AK, Chiang C, Yu B, Hoellerbauer P, Dorrance A, Jin Y, Tarighat SS, Khalife J, Walker A, Chan KK, Caligiuri M, Perrotti D, Muthusamy N, Bloomfield CD, Garzon R, Lee RJ, Lee JL, Marcucci G. Targeting the RAS/MAPK pathway with *miR-181a* in Acute Myeloid Leukemia. *Oncotarget.* 2016; 7:59273-86. *shared first-author

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ORIGINAL REPORT

Prognostic Significance of Expression of a Single MicroRNA, *miR-181a*, in Cytogenetically Normal Acute Myeloid Leukemia: A Cancer and Leukemia Group B Study

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ABSTRACT

Purpose
To evaluate the prognostic significance of expression levels of a single microRNA, *miR-181a*, in the context of established molecular markers in cytogenetically normal acute myeloid leukemia (CN-AML), and to gain insight into the leukemogenic role of *miR-181a*.

Patients and Methods
miR-181a expression was measured in pretreatment marrow using Ohio State University Comprehensive Cancer Center version 3.0 arrays in 187 younger (< 60 years) adults with CN-AML. Presence of other molecular prognosticators was assessed centrally. A gene-expression profile associated with *miR-181a* expression was derived using microarrays and evaluated by Gene-Ontology analysis.

Results
Higher *miR-181a* expression associated with a higher complete remission (CR) rate ($P = .04$), longer overall survival (OS; $P = .01$) and a trend for longer disease-free survival (DFS; $P = .09$). The impact of *miR-181a* was most striking in poor molecular risk patients with *FLT3*-internal tandem duplication (*FLT3*-ITD) and/or *NPM1* wild-type, where higher *miR-181a* expression associated with a higher CR rate ($P = .009$), and longer DFS ($P < .001$) and OS ($P < .001$). In multivariable analyses, higher *miR-181a* expression was significantly associated with better outcome, both in the whole patient cohort and in patients with *FLT3*-ITD and/or *NPM1* wild-type. These results were also validated in an independent set of older (≥ 60 years) patients with CN-AML. A *miR-181a*-associated gene-expression profile was characterized by enrichment of genes usually involved in innate immunity.

Conclusion
To our knowledge, we provide the first evidence that the expression of a single microRNA, *miR-181a*, is associated with clinical outcome of patients with CN-AML and may refine their molecular risk classification. Targeted treatments that increase endogenous levels of *miR-181a* might represent novel therapeutic strategies.

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INTRODUCTION

Several recent studies have revealed that microRNAs, short noncoding RNAs that hybridize to their target mRNAs and repress the expression of the encoded proteins,[1] are not only involved in such biologic processes as cellular differentiation, proliferation, and survival, but also play an essential role in the development of solid tumors and acute myeloid leukemia (AML).[2-6] In AML, genome-wide microRNA-expression profiling has revealed distinctive microRNA-expression signatures capable of differentiating among specific cytogenetic subtypes, such as core-binding factor (CBF) -AML with t(8;21), CBF-AML with inv(16) or t(16;16), and acute promyelocytic leukemia with t(15;17), and setting them apart from other AML subtypes.[7-9] Moreover, microRNA expression signatures have been associated with mutations of *NPM1*,[7,10] *FLT3*,[7,10,11] and *CEBPA*,[7,12] which are genetic alterations known to affect clinical outcome of patients belonging to the largest subset of AML—cytogenetically normal AML (CN-AML).[13,14]

Furthermore, we have recently demonstrated that deregulated microRNA expression may also be associated with outcome in CN-AML.[5,11] Using

microRNA-expression profiling in patients with CN-AML with unfavorable molecular features—*FLT3*-ITD and/or *NPM1* wild-type (*NPM1*wt)—we discovered a prognostic microRNA signature consisting of 12 microRNA probes, five of which corresponded to members of the *miR-181* family.[5] Although these data provided initial support for the usefulness of microRNAs for assessment of molecular risk in AML, microRNAs have been linked to prognosis in AML mainly in the context of genome-wide profiling. This approach, however, is based on population analysis, and therefore, is relatively difficult to implement for prospectively assessing the molecular risk of individual patients. Thus new strategies are needed to increase the clinical applicability of microRNA expression–based prognostication in AML.

To our knowledge, the independent prognostic impact of expression levels of individual microRNAs, which are relatively easy to measure for molecular risk assessment of individual patients at diagnosis, has not been demonstrated in CN-AML outside of microRNA expression profiles. Thus, we sought evidence here that the expression levels of a single microRNA, *miR-181a*, could provide prognostic information in patients with CN-AML independently from a comprehensive panel of other established clinical and molecular predictors, and therefore, be readily applicable as a risk-stratification tool. We show that expression of *miR-181a* is strongly associated with outcome, which suggests that *miR-181a* expression could be used for individual patients' molecular risk assessment and perhaps as a potential therapeutic target.

PATIENTS AND METHODS

Patients, Treatment, and Cytogenetic Analysis

A total of 187 adult patients younger than 60 years (range, 18 to 59 years) with untreated, primary CN-AML and material available for analysis were included. Patients were treated similarly with intensive induction chemotherapy and consolidation with autologous peripheral blood stem-cell transplantation on Cancer and Leukemia Group B (CALGB) protocols 9621 (n = 89) and 19808 (n = 98).[15,16] Of those who achieved a complete remission (CR), 82% received an autologous transplant. Cytogenetic analyses of pretreatment bone marrow (BM) samples were performed by CALGB-approved institutional cytogenetic laboratories as part of CALGB 8461, a prospective cytogenetic companion study, and centrally reviewed.[17,18] All patients gave informed consent for the research use of their specimens, in accordance with the Declaration of Helsinki. No patient received allogeneic stem-cell transplantation in first CR.

A cohort of 122 CN-AML patients age 60 years or older, treated on first-line CALGB protocols (Appendix, online only), constituted an independent validation set for outcome analyses.

Molecular Analyses

The presence or absence of additional molecular markers such as *FLT3*-ITD, *FLT3* tyrosine kinase domain mutations (*FLT3*-TKD), mutations in the *NPM1*, *CEBPA*, *WT1*, *IDH1*, and *IDH2* genes, *MLL* partial tandem duplication (*MLL*-PTD), and *BAALC* and *ERG* expression levels were assessed centrally, as previously reported.[12,19-29]

miR-181a *Expression Analyses*

For microRNA expression, total RNA was extracted from pretreatment BM or blood mononuclear cells, and biotinylated first-strand complementary DNA was synthesized and hybridized to microRNA microarray chips.[5] Images of the microRNA microarray chips were acquired, and calculation, normalization, and filtering of signal intensity for each microarray spot and batch-effect adjustment were performed.[5] *miR-181a* expression was measured using Ohio State University Comprehensive Cancer Center version 3.0 arrays. Log intensities for *miR-181a* probes were averaged and used as a continuous variable for analyses. To validate measurements of *miR-181a* expression made using the microRNA microarrays, quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR) was performed in a subgroup of younger patients (Appendix).

Gene Expression Profiling

To gain further insight into the biologic processes associated with *miR-181a* in CN-AML, we performed gene-expression profiling using the AffymetrixU133 plus 2.0 array (Affymetrix, Santa Clara, CA), and Gene Ontology analysis as reported previously,[30] and described in the Appendix.

Definition of Clinical End Points and Statistical Analysis

The main objective of our study was to evaluate the impact of *miR-181a* expression on outcome (for definition of clinical end points, see Appendix).

The associations of *miR-181a* expression, considered as a continuous variable, with baseline clinical, demographic, and molecular features were analyzed using one-way analysis of variance. Univariable logistic regression models were constructed to evaluate *miR-181a* expression for achievement of CR, and univariable Cox proportional hazards models were used to evaluate the associations of *miR-181a* expression with disease-free survival (DFS) and overall survival (OS). Multivariable logistic regression models were constructed to analyze factors related to the probability of achieving CR, and multivariable Cox proportional hazards models were constructed to analyze factors important for DFS and OS (multivariable analyses are detailed in the Appendix).

RESULTS

Associations of miR-181a *Expression With Clinical and Molecular Characteristics in Patients With CN-AML*

At diagnosis, higher expression of *miR-181a*, analyzed here as a continuous variable, was significantly associated with higher hemoglobin ($P = .05$) and percentage of circulating blasts ($P < .001$), French-American-British M1 and M2 subtypes ($P < .001$) and the absence of extramedullary disease, especially skin and gum involvement ($P = .04$; Table 1). Higher *miR-181a* expression was also significantly associated with higher frequency of wild-type *NPM1* ($P = .003$), *CEBPA* mutations ($P < .001$), *IDH1* mutations ($P = .007$), and lower *ERG* ($P = .02$) and higher *BAALC* ($P = .05$) expresser status (Table 1).

Prognostic Value of miR-181a *Expression in CN-AML*

Patients with higher *miR-181a* expression had a higher CR rate (odds ratio [OR], 1.38; $P = .04$). With a median follow-up time for patients alive at the last follow-up visit of 6.5 years (range, 3.1 to 11.0 years), higher *miR-181a* expressers had a trend for longer DFS ($P = .09$) and had longer OS (hazard ratio [HR], 0.82; $P = .01$; Table 2). The prognostic impact of *miR-181a* expression levels measured using microRNA microarrays was technically validated by outcome analyses in a subgroup of 30 patients for whom *miR-181a* expression was also determined using real-time RT-PCR (Appendix).

In multivariable analyses (Table 3), higher *miR-181a* expression levels were associated with an increased rate of CR (OR, 2.36; $P = .02$), after adjusting for *ERG* ($P = .008$) and *BAALC* expression status ($P = .01$) and age ($P = .01$). Higher *miR-181a* expression was also associated with longer DFS (HR, 0.8; $P = .02$), after adjusting for *CEBPA* ($P = .005$), *NPM1* ($P < .001$), *WT1* ($P = .003$), *FLT3*-ITD ($P < .001$) and *FLT3*-TKD ($P = .02$) mutational status, and with longer OS (HR, 0.81; $P = .01$), after adjusting for *CEBPA* ($P < .001$),

Table 1. Relationship of Clinical and Molecular Characteristics With *miR-181a* Expression in the Whole Group of 187 Younger Patients With Cytogenetically Normal Acute Myeloid Leukemia at Diagnosis

Characteristic	No.	%	P*
Median age, years	45		.08 ↓
Range	18-59		
Sex			.39
Female	98	52	
Male	89	48	
Race			.91
White	163	88	
Nonwhite	23	12	
Median hemoglobin, g/L	9.3		.05 ↑
Range	4.6-13.6		
Median platelet count, $\times 10^9$/L	58		.29
Range	7-466		
Median WBC, $\times 10^9$/L	27.9		.13 ↓
Range	0.9-295.0		
Median blood blasts, %	62		< .001 ↑
Range	0-97		
Median bone marrow blasts, %	67		.58
Range	21-95		
FAB			< .001
M1/M2	**92**	**59**	
M4/M5	56	36	
Extramedullary involvement†			.04
No	**129**	**70**	
Yes	56	30	
FLT3-ITD			.94
Negative	117	63	
Positive	70	37	
FLT3-TKD			.06
Negative	**167**	**90**	
Positive	18	10	
NPM1			.003
Wild type	**67**	**36**	
Mutated	120	64	
CEBPA			< .001
Wild type	152	83	
Mutated	**32**	**17**	
WT1			.16
Wild type	**161**	**88**	
Mutated	22	12	
MLL-PTD			.59
Negative	175	94	
Positive	12	6	
IDH1			.007
Wild type	124	87	
Mutated	**19**	**13**	
IDH2			.88
Wild type	126	88	
Mutated	17	12	
ERG expression			.02
Low	**83**	**62**	
High	50	38	
BAALC expression			.05
Low	70	50	
High	**70**	**50**	

Abbreviations: FAB, French-American-British classification; *FLT3*-ITD, internal tandem duplication of the *FLT3* gene; *FLT3*-TKD, tyrosine kinase domain mutation of the *FLT3* gene; *MLL*-PTD, partial tandem duplication of the *MLL* gene.

*P values are from the one-way analysis of variance overall F-test, evaluating the presence of any linear relationship between *miR-181a* expression and the variable tested. For tests with a P value < .20, ↑ indicates that higher values of the continuous variable associate with higher *miR-181a* expression and ↓ indicates that lower values of the continuous variable associate with higher *miR-181a* expression; for the categorical variables, those associated with higher *miR-181a* expression are indicated using bold type.

†Primarily extramedullary skin and gum involvement.

Table 2. Relationship Between *miR-181a* Expression and Outcome of Younger Patients With Cytogenetically Normal Acute Myeloid Leukemia

End Point	OR/HR	95% CI	P
Analyses in all CN-AML patients			
Complete remission	1.38	1.01 to 1.88	.04
Disease-free survival	—	—	.09
Overall survival	0.82	0.71 to 0.96	.01
Analyses in *FLT3*-ITD and/or *NPM1*wt patients			
Complete remission	1.64	1.12 to 2.42	.009
Disease-free survival	0.66	0.53 to 0.84	< .001
Overall survival	0.71	0.60 to 0.84	< .001

NOTE: An OR greater than 1.0 means a higher complete remission rate for higher values of *miR-181a* expression. An HR lower than 1.0 means longer survival for higher values of *miR-181a* expression. The sample size for the entire set was n = 187 for complete remission and overall survival and n = 154 for disease-free survival. The sample size for *FLT3*-ITD and/or *NPM1*wt patients was n = 122 for complete remission and overall survival and n = 96 for disease-free survival.

Abbreviations: HR, hazard ratio; OR, odds ratio.

NPM1 ($P < .001$), *WT1* ($P < .001$), and *FLT3*-ITD ($P = .003$) mutational status, and WBC ($P = .005$).

Association of miR-181a *Expression Levels With Outcome in Distinct CN-AML Molecular Groups*

The presence or absence of *FLT3*-ITD and *NPM1* mutations has been reported to stratify patients with CN-AML into prognostically distinct categories. Patients with *NPM1* mutations, but no *FLT3*-ITD had a more favorable outcome, whereas those with *FLT3*-ITD and/or *NPM1*wt had worse prognosis.[23] Thus, to better understand the prognostic significance of higher *miR-181a* expression levels in CN-AML, we analyzed their impact on the aforementioned prognostic subsets. While there was no prognostic impact of *miR-181a* expression on patients with *NPM1* mutations and no *FLT3*-ITD (n = 65; CR rate, $P = .58$; DFS, $P = .76$; and OS, $P = .66$), we observed that higher *miR-181a* expression levels were associated with a significantly higher CR rate (OR, 1.64; $P = .009$), and longer DFS (HR, 0.66; $P < .001$) and OS (HR, 0.71; $P < .001$) in patients with *FLT3*-ITD and/or *NPM1*wt (n = 122; Table 2).

In multivariable analysis restricted to patients with *FLT3*-ITD and/or *NPM1*wt (Table 3), higher *miR-181a* expression levels were associated with higher odds of achieving a CR (OR, 1.61; $P = .02$), after adjusting for age ($P = .009$), with longer DFS (HR = 0.74; $P = .02$), after adjusting for *CEBPA* ($P < .001$), *NPM1* ($P = .007$), and *FLT3*-ITD ($P = .02$) mutational status, and hemoglobin levels ($P = .04$), and with longer OS (HR, 0.74; $P = .002$), after adjusting for *CEBPA* ($P < .001$), *NPM1* ($P = .007$), and *WT1* ($P = .01$) mutational status, WBC ($P < .001$), and extramedullary involvement ($P = .01$).

In the aforementioned analyses, we used *miR-181a* expression values as a continuous variable. To graphically display the relationship between *miR-181a* expression and achievement of CR, we compared *miR-181a* expression in patients achieving CR with that of patients experiencing failure with induction therapy within the subgroup of patients with *FLT3*-ITD and/or *NPM1*wt (Fig 1A). Furthermore, to graphically display the relationship between *miR-181a* expression and DFS and OS, we dichotomized *miR-181a* expression values at the median, and present survival curves for the high and low *miR-181a* expressers within the subgroup of patients with *FLT3*-ITD and/or *NPM1*wt (Fig 1B and 1C).

Table 3. Multivariable Analyses Evaluating *miR-181a* Expression for Clinical Outcome in Younger Patients With CN-AML

Variables in Final Models	OR/HR	95% CI	*P*
Multivariable analyses in all patients with CN-AML			
CR[a]			
miR-181a expression	2.36	1.17 to 4.78	.02
ERG expression; low *v* high	5.86	1.60 to 21.52	.008
BAALC expression; low *v* high	6.69	1.56 to 28.74	.01
Age	0.36	0.17 to 0.78	.01
DFS[b]			
miR-181a expression	0.80	0.66 to 0.97	.02
CEBPA; mutated *v* wild type	0.38	0.19 to 0.75	.005
NPM1; mutated *v* wild type	0.42	0.24 to 0.75	< .001[c]
WT1; mutated *v* wild type	2.54	1.39 to 4.65	.003
FLT3-ITD; positive *v* negative	2.68	1.65 to 4.36	< .001[c]
FLT3-TKD; positive *v* negative	2.19	1.14 to 4.19	.02
OS[d]			
miR-181a expression	0.81	0.69 to 0.95	.01
CEBPA; mutated *v* wild type	0.32	0.16 to 0.62	< .001
NPM1; mutated *v* wild type	0.47	0.28 to 0.79	< .001[c]
WT1; mutated *v* wild type	2.65	1.54 to 4.57	< .001
FLT3-ITD; positive *v* negative	2.39	1.46 to 3.93	.003[c]
WBC	1.37	1.13 to 1.67	.005[c]
Multivariable analyses in patients with *FLT3*-ITD and/or *NPM1*wt			
CR[e]			
miR-181a expression	1.61	1.07 to 2.42	.02
Age	0.53	0.33 to 0.85	.009
DFS[f]			
miR-181a expression	0.74	0.57 to 0.96	.02
CEBPA; mutated *v* wild type	0.27	0.13 to 0.58	< .001
NPM1; mutated *v* wild type	0.33	0.14 to 0.79	.007[g]
FLT3-ITD; positive *v* negative	3.05	1.30 to 7.14	.02[g]
Hemoglobin	0.75	0.57 to 0.99	.04
OS[h]			
miR-181a expression	0.74	0.61 to 0.90	.002
CEBPA; mutated *v* wild type	0.29	0.14 to 0.59	< .001
NPM1; mutated *v* wild type	0.41	0.22 to 0.78	.007[g]
WT1; mutated *v* wild type	2.23	1.18 to 4.23	.01
WBC	1.40	1.15 to 1.71	< .001
Extramedullary involvement; absent *v* present	2.45	1.27 to 4.71	.01[g]

NOTE. Further details of the multivariable analyses are found in the Appendix (online only). ORs greater than 1.0 mean higher and those less than 1.0 mean lower CR rate for the higher values of the continuous variables and the first category listed for the categorical variables. HRs greater than 1.0 indicate higher and those less than 1.0 indicate lower risk for relapse or death (DFS) or death (OS) for the higher values of the continuous variables and the first category listed for the categorical variables.

Abbreviations: CN-AML, cytogenetically normal acute myeloid leukemia; CR, complete remission; DFS, disease-free survival; *FLT3*-ITD, internal tandem duplication of the *FLT3* gene; *FLT3*-TKD, tyrosine kinase domain of the *FLT3* gene; HR, hazard ratio; OS, overall survival; OR, odds ratio.

[a]Variables considered in the model based on univariable analyses were *miR-181a* expression, *ERG* expression (low *v* high), *FLT3*-ITD (positive *v* negative), *BAALC* expression (low *v* high), age (in 10-year increments), hemoglobin (in 2-unit increments), and WBC (in 50-unit increments).

[b]Variables considered in the model based on univariable analyses were *miR-181a* expression, *CEBPA* (mutated *v* wild type), *ERG* expression (low *v* high), *WT1* (mutated *v* wild type), *BAALC* expression (low *v* high), *FLT3*-ITD (positive *v* negative), *FLT3*-TKD (positive *v* negative), *MLL*-PTD (mutated *v* wild type), *NPM1* (mutated *v* wild type), WBC (in 50-unit increments), extramedullary involvement, and race.

[c]Does not meet the proportional hazards assumption. For DFS, the HR for *FLT3*-ITD and *NPM1* are reported at 9 months; for OS, the HR for *NPM1*, *FLT3*-ITD, and WBC are reported at 9 months.

[d]Variables considered in the model based on univariable analyses were *miR-181a* expression, *CEBPA* (mutated *v* wild type), *ERG* expression (low *v* high), *FLT3*-ITD (positive *v* negative), *WT1* (mutated *v* wild type), *BAALC* expression (low *v* high), *NPM1* (mutated *v* wild type), WBC (in 50-unit increments), age (in 10-year increments), hemoglobin (in 2-unit increments), platelet count, percentage of blood blasts, and extramedullary involvement.

[e]Variables considered in the model based on univariable analyses were *miR-181a* expression, age (in 10-year increments), hemoglobin (in 2-unit increments), and WBC (in 50-unit increments).

[f]Variables considered in the model based on univariable analyses were *miR-181a* expression, *CEBPA* (mutated *v* wild type), *ERG* expression (low *v* high), *WT1* (mutated *v* wild type), *FLT3*-ITD (positive *v* negative), *FLT3*-TKD (positive *v* negative), *NPM1* (mutated *v* wild type), hemoglobin (in 2-unit increments), WBC (in 50-unit increments), and race.

[g]Does not meet the proportional hazards assumption. For DFS, the HR for *FLT3*-ITD is reported at 1 year, *NPM1* is reported at 9 months; for OS, the HR for *NPM1* is reported at 1.5 years, extramedullary involvement is reported at 1 year.

[h]Variables considered in the model based on univariable analyses were *miR-181a* expression, *CEBPA* (mutated *v* wild type), *ERG* expression (low *v* high), *WT1* (mutated *v* wild type), *FLT3*-ITD (positive *v* negative), *NPM1* (mutated *v* wild type), hemoglobin (in 2-unit increments), WBC (in 50-unit increments), and extramedullary involvement.

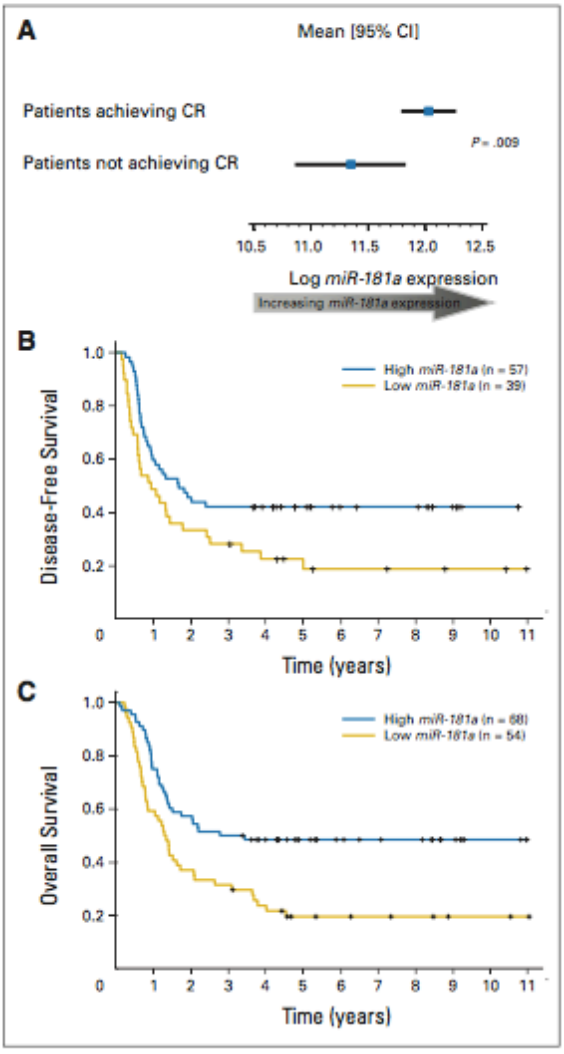

Fig 1. Favorable outcome of patients with *FLT3*-ITD and/or *NPM1*wt and higher *miR-181a* expression levels. (A) *miR-181a* expression in patients who achieved a complete response (CR) versus patients who did not achieve a CR; (B) disease-free and (C) overall survival according to *miR-181a* expression levels in patients with CN-AML dichotomized into high (above the median *miR-181a* expression value) or low (at or below the median *miR-181a* expression value) expression groups.

Importantly, an independent set of older patients with CN-AML with *FLT3*-ITD and/or *NPM1*wt (n = 122) was analyzed by microRNA microarray assays to validate the prognostic impact of *miR-181a* found in younger patients (Appendix). In this validation set, higher expression of *miR-181a*, used as a continuous variable, did not impact on the CR rate ($P = .52$), but was associated with longer DFS ($P = .04$) and with a trend for longer OS ($P = .08$). In multivariable models for this validation set, *miR-181a* was independently associated with longer DFS ($P = .04$) and OS ($P = .05$), even after adjusting for other clinical and molecular variables (Appendix Table A1, online only).

Biologic Insights

In order to gain insights into the functional contribution of *miR-181a* expression levels to the poor molecular risk CN-AML subset, we first derived a gene-expression signature associated with *miR-181a* expression in patients with *FLT3*-ITD and/or *NPM1*wt. We observed that the expression of 1,174 probe sets significantly correlated ($P < .001$) with that of *miR-181a*; 1,002 probe sets correlated negatively and 172 probe sets positively (Fig 2). Among other genes, we observed a negative correlation of *miR-181a* expression with the expression of the *HOXA* and *HOXB* clusters, as well as the *HOX* cofactor *MEIS1*. These genes are important for developmental processes and have also been linked to leukemogenesis and the self-renewal of leukemic stem cells.[31,32] We also observed a negative correlation of *miR-181a* expression with the expression of the

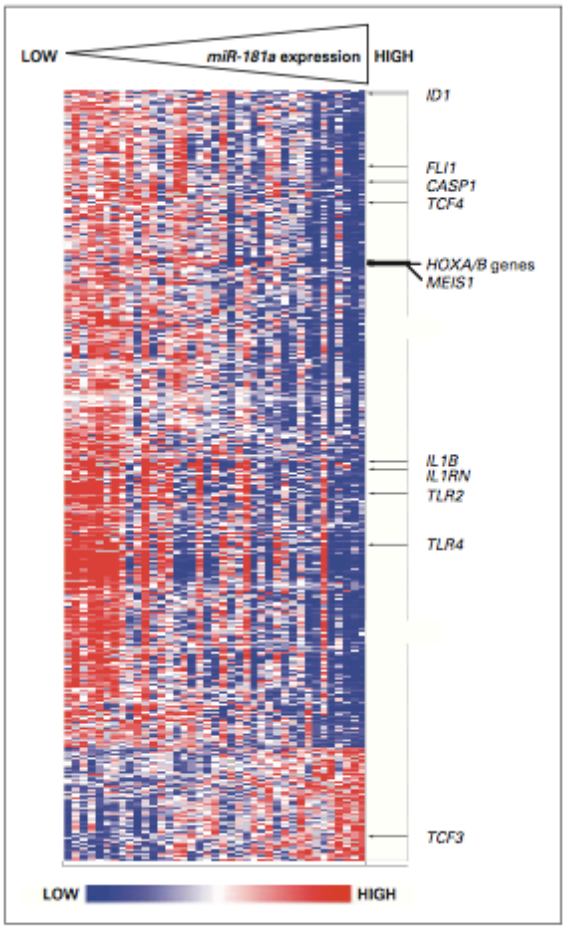

Fig 2. Heat map of the derived gene-expression signature correlated with *miR-181a* expression. Rows represent probe sets and columns represent patients. Probe sets are ordered by hierarchical cluster analysis. Patients are ordered from left to right by increasing *miR-181a* expression. Expression values of the probe sets are represented by color, with blue indicating expression less than and red indicating expression greater than the median value for the given probe set. Arrows indicate genes that are discussed in the text.

transcription coregulator *ID1*, which is able to prevent hematopoietic differentiation and has recently been associated with adverse outcome in AML[33]; the *FLI1* gene, a known suppressor of erythroid differentiation[34]; and the transcription factor *TCF4*, which contributes to neoplastic transformation as a downstream target of the WNT-pathway.[35] In contrast, we observed a positive correlation of *miR-181a* expression with the expression of *TCF3*, a gene encoding a transcription factor that has been shown to regulate the homeostasis of the hematopoietic stem cell pool and promote differentiation of hematopoietic progenitors.[36,37]

To further understand the potential functional role of *miR-181a* expression in CN-AML, we performed a Gene Ontology analysis. Biologic processes that relate to cytokine and native immunity-mediated processes, including those involving toll-like receptors (eg, *TLR4* and *TLR2*) and the interleukin pathways (eg, *IL1B, IL1RN*, and *CASP1*), were over-represented in the *miR-181a*-associated gene-expression signature (Table 4).

Table 4. GO Terms of Biological Processes Significantly Overrepresented in the *miR-181a*-Expression Profile

GO ID	GO Terms	Percentage of Members of the GO Term Present in the *miR-181a* Profile	*P*
50715	Positive regulation of cytokine secretion	83.33	< .001
50706	Regulation of interleukin-1 beta secretion	80	< .001
50716	Positive regulation of interleukin-1 secretion	80	< .001
50704	Regulation of interleukin-1 secretion	80	< .001
50718	Positive regulation of interleukin-1 beta secretion	80	< .001
50707	Regulation of cytokine secretion	77.78	< .001
45123	Cellular extravasation	66.67	< .001
50701	Interleukin-1 secretion	66.67	.001
50702	Interleukin-1 beta secretion	66.67	.001
7159	Leukocyte adhesion	66.67	.002
50663	Cytokine secretion	66.67	< .001
9595	Detection of biotic stimulus	62.5	< .001
50709	Negative regulation of protein secretion	60	.003
30593	Neutrophil chemotaxis	60	< .001
45408	Regulation of interleukin-6 biosynthetic process	57.14	.002
45576	Mast cell activation	57.14	.004
30149	Sphingolipid catabolic process	55.56	< .001
42226	Interleukin-6 biosynthetic process	50	.003
32635	Interleukin-6 production	50	.003
50714	Positive regulation of protein secretion	50	< .001
46466	Membrane lipid catabolic process	50	< .001

NOTE. Shown are significantly overrepresented GO terms with ≥ 50% of their assigned members represented in the gene expression signature associated with higher *miR-181a* expression. Gray shading identifies terms associated with genes encoding proteins in the interleukin-1β and toll-like receptor pathways (eg, *IL1B, IL1BRN, CASP1, TLR2, TLR4*, etc).
Abbreviation: GO, Gene Ontology.

DISCUSSION

We report here that expression levels of *miR-181a* constitute a strong prognostic factor in younger patients with CN-AML enrolled on similar CALGB first-line treatment protocols. We show that higher levels of *miR-181a* expression directly correlate with higher odds of achieving a CR and lower risk of experiencing relapse and/or death in patients with CN-AML. This study is the first to demonstrate that a single noncoding RNA associates with clinical outcome in CN-AML, even in the context of other well-established molecular markers including *CEBPA* and *NPM1* mutations, that were recently recognized by the WHO classification as defining markers for novel provisional AML entities,[38] and *FLT3*-ITD. Furthermore, we technically validated these results by using quantitative RT-PCR.

The prognostic impact was most striking in patients with *FLT3*-ITD and/or *NPM1*wt, which are associated with adverse outcome. These patients constitute approximately 65% of all CN-AML and one third of all AML patients younger than 60 years.[13] Notably, in this group, when other molecular prognostic markers were considered in multivariable models, higher expression of *miR-181a* was the only molecular marker that independently associated with higher odds of achieving CR, thereby suggesting a potential impact of this microRNA on mechanisms of resistance to chemotherapy-induced apoptosis. Higher expression of *miR-181a* was also associated with longer DFS after adjusting for the impact of *NPM1, CEBPA*, and *FLT3*-ITD mutational status and hemoglobin levels, and OS after adjusting for the impact of *NPM1, CEBPA*, and *WT1* mutational status, extramedullary involvement, and WBC. These results were validated by demonstrating the positive prognostic impact of higher *miR-181a* expression in an independent validation set of older patients with CN-AML.

Recently, a modified prognostic classification of CN-AML has been recommended by an international expert panel on behalf of the European LeukemiaNet, in which the intermediate I prognostic category also includes patients with *FLT3*-ITD and/or *NPM1*wt, but only those who lack *CEBPA* mutations; patients with *FLT3*-ITD and/or *NPM1*wt and *CEBPA* mutations are classified in the favorable category.[39] When we analyzed the prognostic significance of *miR-181a* expression in this European LeukemiaNet intermediate I prognostic category (n = 92), higher *miR-181a* expression levels were still associated with a significantly higher CR rate (OR, 1.56; $P = .04$), and longer DFS (HR, 0.72; $P = .03$) and OS (HR, 0.77; $P = .01$). Altogether, these data support a pivotal role of *miR-181a* expression levels for the response to treatment of patients with CN-AML, and suggest that since *miR-181a* expression provides additional prognostic information it can be used to further refine this newly devised molecular-risk classification of CN-AML.[39] Moreover, the identification of low levels of *miR-181a* as an adverse prognostic factor provides opportunity for potential therapeutic intervention with agents capable of increasing

low endogenous levels of *miR-181a* and/or with synthetic *miR-181a* compounds.

But how do changes of *miR-181a* expression levels in myeloid blasts affect the aggressiveness of the disease in patients with CN-AML? The biologic role of microRNAs may vary according to their expression in distinct cell populations of normal or neoplastic tissues. *miR-181a* has been described as a tumor suppressor in gliomas,[40] but also has been found elevated in hepatocellular carcinoma cells with features of hepatic cancer stem cells.[41] Currently, relatively little is known about the function of *miR-181a* in normal or malignant hematopoiesis. Previous studies reported that *miR-181* regulated B-cell development and influenced T-cell sensitivity to antigens by modulating T-cell receptor signaling strength.[42,43] Furthermore, *miR-181a* may also play a regulatory role in earlier steps of hematopoiesis.[44] Recently, it was shown that higher levels of *miR-181* are expressed during early erythroid differentiation.[45] In line with these findings, in this study, we observed a positive correlation between *miR-181a* expression and hemoglobin levels, and a negative correlation between *miR-181a* expression and expression of *FLI1*, a known suppressor of erythroid differentiation.[35] Furthermore, we found a negative correlation of *miR-181a* expression with the expression of *ID1*, an inhibitor of hematopoietic differentiation, and *TCF4*, a transcription factor promoting neoplastic transformation.[35] We also observed a negative correlation of *miR-181a* expression with the expression of the *HOXA* and *HOXB* clusters, as previously reported.[45] In contrast, we observed a positive correlation between *miR-181a* expression and *TCF3*, a transcription factor that seemingly promotes development of hematopoietic progenitors and contributes to regulating hematopoietic cell differentiation.[37]

In an effort to further understand how changes in *miR-181a* expression affect the aggressiveness of the disease, response to treatment, and outcome of patients with CN-AML, we used a Gene Ontology analysis. We show an over-representation of cytokine and native immunity-mediated processes in the *miR-181a*-associated gene-expression signature. The expression of the *TLR4*, *TLR2*, *IL1B*, *IL1RN*, and *CASP1* genes was negatively correlated with *miR-181a* expression, and we find some of these genes, namely *TLR4* and *IL1B* and *CASP1* to be predicted to be direct targets of *miR-181a*. Of these genes, *TLR4* and *IL1B* have previously been implicated in human cancer.[47-50] *TLR4* has been shown to promote tumor growth and interfere with response to chemotherapy in ovarian cancer,[46] and to contribute to the development of cytopenias in myelodysplastic syndromes.[47] In addition, TLR4 signaling has also been linked to blocking myeloid differentiation of hematopoietic stem and progenitor cells in severe sepsis.[48] IL-1β has been previously shown to be produced in an autocrine fashion and to stimulate the proliferation of AML blasts.[49,50] It is, therefore, tempting to speculate that high expression of *miR-181a* associates with a less aggressive disease by downregulating genes like *TLR4* and *IL1B*, that modulate the innate immune response to microbial pathogens in the normal host, but also when upregulated may support survival and proliferation of malignant blasts in AML patients.[47-50] However, the mechanisms through which the changes in levels of *miR-181a* expression contribute to different degrees of disease aggressiveness in patients with CN-AML and why *miR-181a* expression differs among individual patients remain to be elucidated.

In summary, we report here for the first time that the expression of a single microRNA, *miR-181a*, associates with clinical outcome in CN-AML. Moreover, it does so independently from other validated clinical and genetic variables, thus adding information useful for a better risk-stratification of patients with CN-AML. High *miR-181a* expression levels identify those patients with CN-AML who despite having molecular features associated with adverse outcome, such as *NPM1*wt and/or *FLT3*-ITD, might not need intensive treatment, such as allogeneic stem-cell transplantation. Moreover, for those patients with low *miR-181a* expression levels, it is hoped that the development of reliable methods of delivery of this microRNA directly to the leukemia cells and/or identification of agents capable of increasing endogenous levels of *miR-181a* may provide new therapeutic options. Further prospective studies should be done to confirm our findings. Establishment of standardized methods of microRNA quantification will allow prospective classification of patients according to their *miR-181a* levels. Finally, the combination of *miR-181a*-associated gene-expression profiling and Gene Ontology analyses provide insights into the leukemogenic role of genes that are either direct or indirect targets of *miR-181a*, and therefore should also be investigated as potential therapeutic targets in patients with CN-AML with low *miR-181a* expression.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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Manuscript writing: All authors
Final approval of manuscript: All authors

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Research Paper

Targeting the RAS/MAPK pathway with *miR-181a* in acute myeloid leukemia

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ABSTRACT

Deregulation of microRNAs' expression frequently occurs in acute myeloid leukemia (AML). Lower *miR-181a* expression is associated with worse outcomes, but the exact mechanisms by which *miR-181a* mediates this effect remain elusive. Aberrant activation of the RAS pathway contributes to myeloid leukemogenesis. Here, we report that *miR-181a* directly binds to 3'-untranslated regions (UTRs); downregulates KRAS, NRAS and MAPK1; and decreases AML growth. The delivery of *miR-181a* mimics to target AML cells using transferrin-targeting lipopolyplex nanoparticles (NP) increased mature *miR-181a*; downregulated KRAS, NRAS and MAPK1; and resulted in decreased phosphorylation of the downstream RAS effectors. NP-mediated upregulation of *miR-181a* led to reduced proliferation, impaired colony formation and increased sensitivity to chemotherapy. Ectopic expression of KRAS, NRAS and MAPK1 attenuated the anti-leukemic activity of *miR-181a* mimics, thereby validating the relevance of the deregulated *miR-181a*-RAS network in AML. Finally, treatment with *miR-181a*-NP in a murine AML model resulted in longer survival compared to mice treated with scramble-NP control. These data support that targeting the RAS-MAPK-pathway by *miR-181a* mimics represents a novel promising therapeutic approach for AML and possibly for other RAS-driven cancers.

INTRODUCTION

Acute myeloid leukemia (AML) is a complex neoplastic disease of the hematopoietic system resulting in maturation arrest and aberrant proliferation of leukemic cells. Despite the use of cytogenetic and molecular risk stratification for treatment guidance, the majority of AML patients still do not achieve long-term survival. A better knowledge of the disease biology and novel targeted therapeutic approaches may improve cure rates.

Recently, we and others reported that the deregulated expression of microRNAs (miRs) – small non-coding RNA molecules regulating post-transcription protein expression – is associated with AML [1, 2]. Assessing the

expression levels of some miRs refines patients' molecular risk classification and helps selecting treatment regimens [1–11]. These results are being translated into the clinic, and early clinical trials targeting miRs have been initiated. [12–16].

The *miR-181* family comprises four mature miRs (*miR-181a*, *miR-181b*, *miR-181c*, *miR-181d*) and has been associated with the regulation of inflammatory mechanisms [17, 18]. Physiologically, *miR-181* may accelerate the megakaryocyte differentiation of CD34-positive hematopoietic cells [19]. Furthermore, these miRs have been found to be deregulated in several types of human cancers, including leukemias [2, 9, 20–26]. In solid tumors the role of *miR-181* seems to be organ-specific. High expression of *miR-181* has been associated with poor clinical outcomes in patients with colorectal cancer [20] and lymph node metastasis in oral squamous cell carcinoma [21]. However, in glioma high expression of *miR-181* seems to have tumor suppressor activity [22]. In hematologic malignancies higher expression of *miR-181* is associated with better outcomes [2, 9, 26–28]. Indeed, we recently reported the favorable impact of higher *miR-181a* expression in both AML cytogenetically normal (CN) or abnormal (CA) patients [2, 9, 28]. To date, however the molecular basis for the attenuation of disease aggressiveness by *miR-181a* remains to be fully elucidated.

RAS proto-oncogenes encode small GTPase proteins, that is, KRAS, NRAS and HRAS, that are involved in homeostatic mechanisms of proliferation, differentiation and apoptosis of normal cells [29]. Whereas *KRAS* and *NRAS* are frequently mutated and activated in AML, *HRAS* mutations are rare, and *HRAS* wild-type expression is the lowest with respect to the other RAS isoforms in the hematopoietic system [29]. Aberrant activation of RAS signal transduction is often found in human neoplasia [30–43]. In hematopoietic malignancies, including AML, activating oncogenic RAS mutations contribute to malignant phenotypes by phosphorylating and activating downstream effectors such as the mitogen-activated protein kinase kinase (MAPKK, also known as MEK), mitogen-activated protein kinase (MAPK), and the PI3K-AKT downstream effectors, thereby promoting aberrant cell proliferation and survival [29]. However, to date, an effective therapeutic approach targeting RAS directly remains to be developed.

Recently, *KRAS* was shown to be a direct *miR-181a* target in oral squamous cell carcinoma [44]. Additionally, *NRAS* and the RAS-downstream effector *MAPK1* are *in silico* predicted to be putative *miR-181a* targets. We hypothesized that higher *miR-181a* levels attenuate AML aggressiveness by targeting RAS and/or its downstream effectors in myeloid blasts, thereby reducing proliferation and decreasing the apoptotic threshold. Therefore, we reasoned that the delivery of synthetic *miR-181a* mimics may increase the low endogenous levels of *miR-181a* in AML blasts and lead to anti-leukemic activity.

RESULTS

Anti-leukemic activity of *miR-181a*

We previously reported that chemotherapy-treated patients with AML with higher *miR-181a* expression achieved complete remission (CR) more frequently and had longer survival compared to lower *miR-181a* expressing patients [2, 9]. In line with these clinical observations, we and others showed that *miR-181a* expression is associated with a higher sensitivity to cytarabine in AML cell lines [45, 46].

These findings led us to postulate a tumor suppressor activity of *miR-181a* that we first tested by overexpressing or knocking-down *miR-181a* in the FLT3-ITD positive MV4-11 AML cell line by lentiviral infection (Figure 1A). Overexpression of *miR-181a* (lenti-*181a*) inhibited cell growth (Figure 1B; lenti-*181a* vs. lenti-sc: $P = 0.009$), whereas downregulation of *miR-181a* (lenti-*anti-181a*) enhanced cell proliferation compared to cells transfected with a vector carrying a scramble sequence (lenti-sc) (Figure 1B; lenti-sc vs. lenti-*anti-181a*: $P = 0.028$). We next engrafted 5×10^6 virally transduced MV4-11 cells into NOD/SCID mice subcutaneously ($n = 3$ in each group). On day 11, the average tumor weights for animals engrafted with the lenti-*anti-181a* or lenti-sc transduced cells were 1.642 ± 0.65 g and 0.076 ± 0.022 g, respectively (Figure 1C). No tumor growth was evident in animals engrafted with lenti-*181a* transduced cells. On day 23, the average tumor weights for the lenti-sc and the lenti-*181a* transduced cell-engrafted groups were 0.65 ± 0.49 g and 0.037 ± 0.025 g, respectively (Figure 1C).

To further support the putative tumor suppressor activity of *miR-181a*, we engrafted NSG mice with virally transduced MV4-11 cells through a tail vein. The median survival for the animals engrafted with the lenti-*miR-181a*, lenti-sc and lenti-*anti-181a* transduced cells were 43, 33.5 and 28.5 days, respectively (Figure 1D). Compared to the control group (lenti-sc), the lenti-*anti-181a* mice lived significantly shorter ($P = 0.002$, log-rank test) and lenti-*miR-181a* mice significantly longer ($P = 0.02$). Though the mice in three groups showed survival time differences, they all died from AML-like disease (Supplementary Figure S1).

We concluded that higher *miR-181a* expression leads to a less aggressive AML phenotype, thereby functionally validating the previously reported prognostic results [2, 9, 28].

KRAS, NRAS and MAPK1 are direct targets of *miR-181a*

The RAS-MAPK1 and RAS-AKT-pathways are often aberrantly activated in AML and are known to contribute to myeloid leukemogenesis [29–43]. *KRAS* has been shown to be a direct *miR-181a* target in oral squamous cell carcinoma [44]. Here, we first

tested whether *KRAS* and other genes involved in these pathways, including *NRAS* and its downstream effectors (i.e., *MAPK1*), were *miR-181a* targets in AML. Utilizing *in silico* tools (targetscan.org, http://diana.imis.athena-innovation.gr/ and microrna.org) we first identified putative *miR-181a*-binding sites in the 3′-untranslated regions (3′-UTRs) of *KRAS*, *NRAS* and *MAPK1*. In contrast, we could not identify putative *miR-181a* binding sites in the 3′-UTR of *HRAS*, which is rarely mutated in AML. We then tested whether *miR-181a* was able to reduce the expression of these genes in AML cells. *miR181a* overexpression by a lenti-*181a* vector reduced KRAS, NRAS, and MAPK1 protein levels 5.2, 2.1, and 6.5-fold, respectively, compared to scramble expressing controls in MV4-11 cells (Figure 2A). Consistent with these results, knock-down of *miR-181a* by a lenti-*anti-181a* increased KRAS, NRAS and MAPK1 1.5, 1.5 and 1.8-fold compared to scramble controls (Figure 2A).

Next we showed that the modulation of KRAS, NRAS and MAPK1 expression by *miR-181a* was caused by direct binding to the respective 3′-UTRs. We first validated *KRAS* as a direct *miR-181a* target. We identified two *miR-181a*-binding sites in the *KRAS* 3′-UTR and observed a 28 ± 4% ($P = 0.003$) and a 25 ± 1% ($P = 0.007$) downregulation of luciferase activity on site 1 and site 2 after co-transfecting 293T cells with *miR-181a* compared with scramble expressing controls. Mutations in the seed sequences of the *KRAS* 3′-UTRs rescued the *miR-181a*-induced downregulation (Figure 2B). Next, to demonstrate that *NRAS* is also a direct *miR-181a* target, we cloned the predicted *miR-181a*-binding-site in the *NRAS* 3′-UTR into a luciferase reporter, and we observed a 26 ± 6% ($P < 0.0001$) downregulation of luciferase activity. An introduced mutation in the seed sequence rescued the *miR-181a*-induced downregulation (Figure 2C). We also identified two putative *miR-181a* binding sites in the *MAPK1* 3′-UTR. Because of the short distance between the two binding sites (149 base pairs), we cloned the two binding sites into the same luciferase reporter construct. We observed a 33 ± 2% ($P = 0.0002$) downregulation of luciferase activity with *miR-181a* treatment compared to cells with scramble control treatment. When we mutated the two sites separately, we observed a 13 ± 3% (site 1;

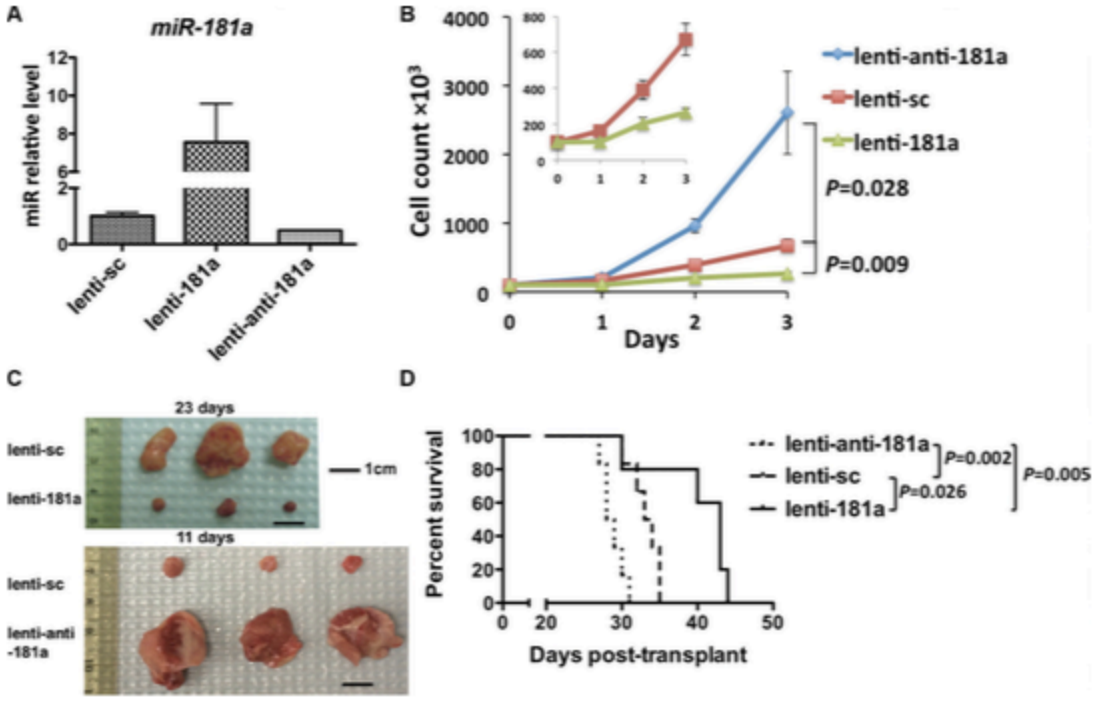

Figure 1: Higher levels of *miR-181a* are associated with a less aggressive phenotype in AML cells and longer survival in a murine AML model. (**A**) *miR-181a* expression in MV4-11 cells after lentiviral infection. (**B**) Growth curve of MV4-11 cells transduced with lentiviral constructs either overexpressing *miR-181a* (lenti-181a), expressing a scramble sequence (lenti-sc; = control) or a knock-down construct of *miR-181a* (lenti-anti-181a). (**C**) Five million lentiviral transduced cells were engrafted subcutaneously in NOD/SCID mice. At day 11, tumors from lenti-anti-181a and lenti-sc group ($n = 3$ in each group) were isolated and weighed (no tumor in lenti-181a group). At day 23, tumors from lenti-181a and lenti-sc group ($n = 3$ in each group) were isolated and weighed. (**D**) 1.5 million lentiviral transduced MV4-11 cells were engrafted into NSG mice. Survival curves of the mice in the three groups.

$P = 0.004$) and a 15 ± 3% (site 2; $P = 0.006$) *miR-181a*-induced downregulation of the luciferase activity. However, mutations on both sites of *MAPK1* could completely rescue the *miR-181a*-induced downregulation (Figure 2D). Collectively, these results support that KRAS, NRAS and MAPK1 are direct *miR-181a* targets.

Delivery of synthetic *miR-181a* mimic by transferrin (Tf)-conjugated nanoparticles (NP) enhanced *miR-181a* levels and inhibited RAS-dependent signaling pathways in AML

Because higher *miR-181a* levels are associated with improved outcomes in AML [2, 9, 26–28], and because *miR-181a* downregulation contributed to leukemia growth (Figure 1) and directly targeted KRAS, NRAS and MAPK1, we reasoned that increasing *miR-181a* may have therapeutic value in AML. We have previously demonstrated the successful delivery of miR mimics to AML blasts via transferrin (Tf)-targeted anionic lipid-based lipopolyplex nanoparticles (NP) [47]. Here, we used a similar approach to deliver synthetic *miR-181a* mimics. We chose KG1a, MV4-11 and OCI-AML cells as models because of the relatively low *miR-181a* levels and activated RAS pathways (Supplementary Figure S2). Following treatment with Tf-NPs encapsulating *miR-181a* double-stranded mimic molecules (Tf-NP-*miR-181a*; 10 nM) or scramble control molecules (Tf-NP-sc; 10 nM), levels of mature *miR-181a* were measured by qRT-PCR. After 24 hours exposure, mature *miR-181a* levels increased 211 ± 31, 880 ± 10 and 142 ± 10-fold in KG1a, OCI-AML3 and MV4-11 cells, respectively, whereas levels of *miR-181b* and unrelated *miR-140* remained unchanged (Figure 3A).

Having shown that the Tf-NP-delivery-system was able to deliver *miR-181a* to AML blasts, we next tested the impact of Tf-NP-*miR-181a* on RAS activity. First, we found that the delivered synthetic *miR-181a* was functional, as it downregulated KRAS, NRAS and MAPK1 proteins (KG1a: 4.3, 4.4 and 5.5-fold; OCI-AML3: 3.2, 3.9 and 2.2-fold; MV4-11: 1.5, 4.4 and 4.6-fold, respectively) compared to Tf-NP-sc treatment (Figure 3B). Compared to Tf-NP-sc, Tf-NP-*miR-181a* decreased p-MEK protein by 6.8, 2.2 and 4.5-fold and p-AKT

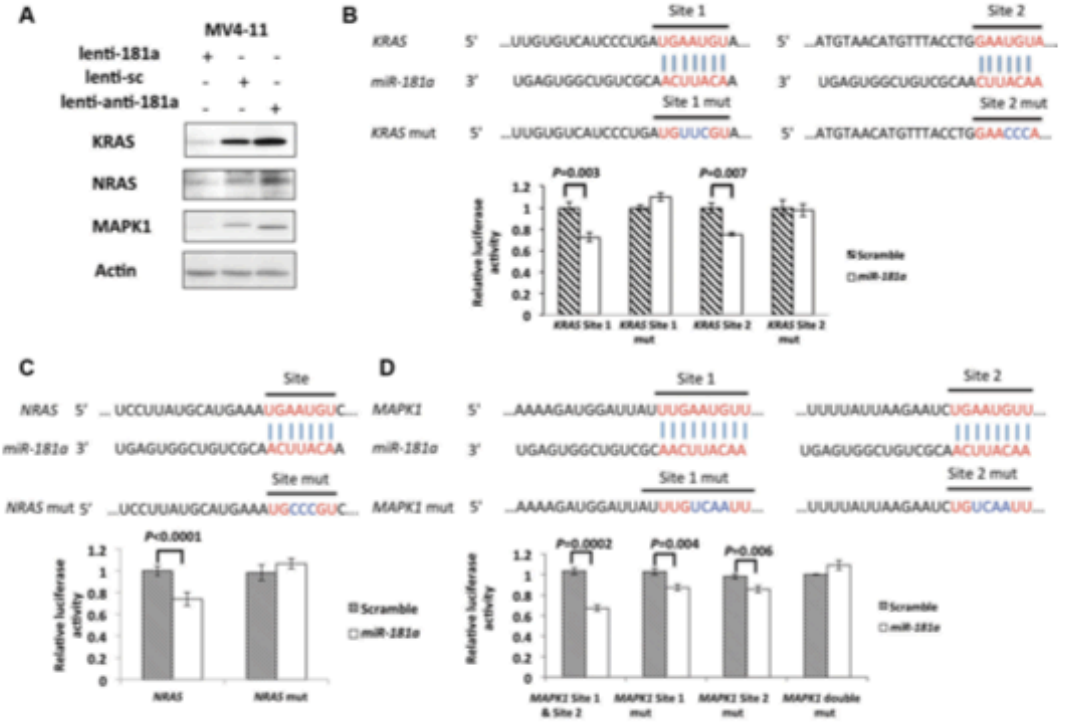

Figure 2: NRAS, KRAS and MAPK1 are direct targets of *miR-181a*. (**A**) KRAS, NRAS and MAPK1 protein expression in infected MV4-11 and OCI-AML3 cells with lenti-181a, lenti-sc or lenti-anti-181a. Dual luciferase assays of HEK293T cells co-transfected with firefly luciferase constructs containing the *KRAS* (**B**), *NRAS* (**C**) and *MAPK1* (**D**) wild-type or mutated 3′-UTRs and *miR-181a* mimics or scramble mimics (as controls). The firefly luciferase activity was normalized to Renilla luciferase activity. The data are shown as relative luciferase activity of *miR-181a* mimic transfected cells with respect to the scramble control of nine data points from three independent transfections. Error bars represent the standard deviation (SD).

protein by 2.0, 2.5 and 5.7-fold in KG1a, OCI-AML3 and MV4-11 cells, respectively (Figure 3B). Finally, we assessed the expression of the oncogenic transcription factor MYC, whose protein stability is enhanced by the RAS-MAPK1 phosphorylation pathway [48]. There was a 4.8, 4.3 and 7.8-fold reduction of MYC protein in KG1a, OCI-AML3 and MV4-11 treated with Tf-NP-*miR-181a* compared to those treated with Tf-NP-sc control (Figure 3B). Consistent with these results, anti-*miR-181a* treatment resulted in upregulation of the KRAS, NRAS and MAPK1 proteins in HL60 cells that present with higher levels of endogenous *miR-181a* (Supplementary Figures S2A and S3).

To validate these results, we treated primary AML blasts having activated RAS from three AML patients (Patient No 1-3; Supplementary Table S2) (Supplementary Figure S2) with Tf-NP-*miR-181a* and again observed an increase in *miR-181a* (Figure 3C). After 24 hours, mature *miR-181a* levels increased 45 ± 4, 35 ± 0.1 and 125 ± 16-fold, respectively, in the three patient blasts samples treated with Tf-NP-*miR-181a* compared to the Tf-NP-sc treated controls, whereas levels of *miR-181b* and *miR-140* remained unchanged (Figure 3C). Increased levels of *miR-181a* resulted in decreased protein levels of KRAS, NRAS and MAPK1 by 6.3, 6.8 and 5.6-fold in patient 1; 6.4, 1.6 and 19.7-fold in patient 2; and 2.3, 2.4 and 3.4-fold in patient 3, respectively (Figure 3D). Downregulation of RAS and MAPK1 resulted in RAS-MAPK1 inhibition, decreased MEK and AKT phosphorylation and decreased MYC levels. We observed a 1.4, 3.5 and 2.0-fold decrease of p-MEK, 1.8, 9.3 and 2.0-fold decrease of p-AKT, as well as a 5.3, 7.6 and 2.8-fold decrease of MYC normalized in the patient blasts treated with Tf-NP-*miR-181a* compared to Tf-NP-sc treatment (Figure 3D).

In summary, we showed the effective delivery of *miR-181a* via Tf-conjugated nanoparticles and in turn downregulation of KRAS, NRAS and MAPK1 and inhibition of the RAS-MAPK1 and RAS-AKT-kinase signaling cascade.

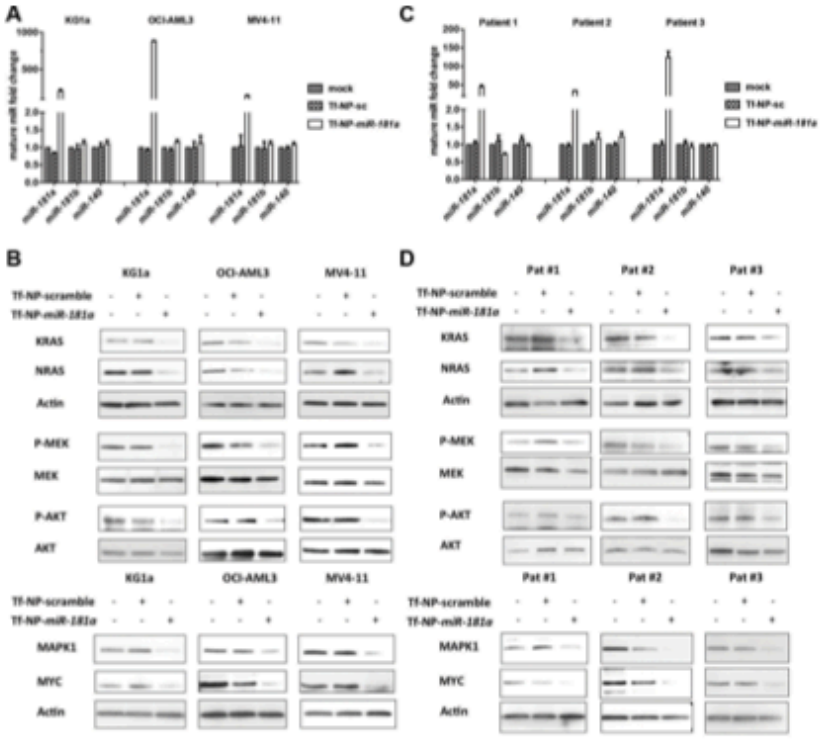

Figure 3: Treatment with Tf-NP-*miR-181a* increased mature *miR-181a* levels; downregulated KRAS, NRAS, and MAPK1; and inhibited the RAS-MAPK1 signaling pathway. Mature *miR-181a*, *miR-181b* and *miR-140* expression levels in KG1a, OCI-AML3 and MV4-11 cells (**A**) and primary patient blasts ($n = 3$) (**C**). NRAS, KRAS, p-MEK, MEK, p-AKT, AKT, MAPK1, and MYC expression in KG1a, OCI-AML3 and MV4-11 cells (**B**) and primary patient blasts ($n = 3$) (**D**) treated with mock, Tf-NP-sc and Tf-NP-*miR-181a*.

Tf-NP-*miR-181a* treatment in AML cells

Next, we demonstrated the anti-leukemic activity of the Tf-NP-*miR-181a*, which led to reduced proliferation of KG1a cells by 40% ($P = 0.015$), OCI-AML3 cells by 25% ($P = 0.023$) and MV4-11 cells by 32% ($P < 0.0001$) after 72 hours compared to Tf-NP-sc control (Figure 4A). To validate the RAS-MAPK1 and RAS-AKT-kinase-pathways as relevant anti-leukemic *miR-181a* targets, we treated KG1a and MV4-11 cells with Tf-NP loaded with siRNAs for *KRAS*, *NRAS* and *MAPK1* (Supplementary Figure S4A). Following this treatment, we observed a similar anti-leukemic effect. The combined siRNA treatment reduced proliferation of KG1a cells by 32% and MV4-11 cells by 30% compared with scramble siRNA treatment (Supplementary Figure S4B). The reduced proliferation induced by Tf-NP-*miR-181a* treatment was reversed by lentiviral expression of *KRAS*, *NRAS* and *MAPK1* in OCI-AML3 cells (Supplementary Figure S5A–S5C; Supplementary Table S3) attenuating the anti-leukemic activity of Tf-NP-*miR-181a* and thereby supporting the relevance of these targets to leukemogenesis. We also observed a more than 50% reduction of colony formation following Tf-NP-*miR-181a* treatment after 2 weeks (Figure 4B). The average number of colonies formed with mock treatment (buffer only), Tf-NP-sc control and Tf-NP-*miR-181a* treatment were, respectively, 145 ± 7, 145 ± 11 and 44 ± 3 ($P = 0.0002$ compared to Tf-NP-sc) for KG1a, 176 ± 11, 172 ± 8 and 80 ± 6 ($P < 0.0001$ compared to Tf-NP-sc) for OCI-AML3 and 217 ± 42, 180 ± 17 and 82 ± 15 ($P = 0.0001$ compared to Tf-NP-sc) for MV4-11.

Treatment with Tf-NP-*miR-181a* induced apoptosis in both MV4-11 (28.69 ± 5.88% vs. 15.92 ± 0.7% annexinV+, $P = 0.02$) and OCI-AML3 cells (20.15 ± 2.58% vs. 8.54 ± 1.42% annexinV+, $P < 0.0001$) compared to Tf-NP-sc treatment at 96 hours (Figure 4C). Following a combined siRNA treatment with Tf-NP loaded with siRNAs for *KRAS*, *NRAS* and *MAPK1*, we observed similar effects in MV4-11 and OCI-AML3 cells (Supplementary Figure S4C). In addition, after 24 hours of priming cells with *miR-181a*, daunorubicin (DNR) was added to treat the cells for another 72 hours. We observed that *miR-181a* treatment enhanced the apoptotic effect of DNR in MV4-11 (*miR-181a* - > 0.01 μM DNR: 45.27 ± 5.99% vs. scramble - > 0.01 μM DNR: 22.88 ± 4.61% annexinV+, $P = 0.001$) and OCI-AML3 (*miR-181a* - > 0.04 μM DNR: 70.92 ± 5.01% vs. scramble - > 0.04 μM DNR: 53.25 ± 7.06% annexinV+, $P = 0.02$; Figure 4C). We also observed similar effects priming MV4-11 and OCI-AML3 cells with siRNAs for *KRAS*, *NRAS* and *MAPK1* (Supplementary Figure S4C). We then validated our observation in primary patient blasts. Tf-NP-*miR-181a* induced apoptosis in all four patient blast samples compared to Tf-NP-sc controls (patient 1: 17.04 ± 4.22% vs. 6.66 ± 1.73% annexinV+, $P = 0.03$; patient 2: 58.53 ± 0.81% vs. 35.73 ± 2.41% annexinV+, $P = 0.01$; patient 3: 20.86 ± 1.55% vs. 10.32 ± 1.1% annexinV+, $P = 0.025$; patient 4: 39.28 ± 4.19% vs. 26.70 ± 2.95% annexinV+, $P = 0.006$; Figure 4D). When exposed to DNR for 72 hours, the Tf-NP-*miR-181a* treated cells exhibited increased apoptosis compared with control cells (patient 1 exposed to 0.04 μM DNR: 27.28 ± 0.87% vs. 14.75 ± 1.36% annexinV+, $P = 0.01$; patient 2 exposed to 0.01 μM DNR: 75.16 ± 0.71 vs. 55.91 ± 2.42% annexinV+, $P = 0.006$; patient 3 exposed to 0.04 μM DNR: 57.61 ± 3.77% vs. 43.99 ± 4.7% annexinV+, $P = 0.03$; patient 4 exposed to 0.01 μM DNR: 51.61 ± 0.68% vs. 28.06 ± 3.42% annexinV+, $P = 0.005$; Figure 4D).

Systemic delivery of Tf-NP-miR-181a in an AML mouse model

Next, we examined the anti-leukemic activity of Tf-NP-*miR-181a in vivo*. Saline (control), Tf-NP-sc or Tf-NP-*miR-181a* were administrated (1.5 mg/kg/d miR three times/week) through a tail vein 10 days after the engraftment of MV4-11 cells in NSG mice (each group $n = 11$). Randomly, three mice from each group (i.e. saline, Tf-NP-sc or Tf-NP-*miR-181a* treated group) were sacrificed after eight treatment doses. The spleen weights were measured and resulted in 187.3 ± 25.93 mg, 174.3 ± 13.65 mg and 77 ± 50 mg (vs. Tf-NP-sc; $P = 0.03$) in the saline, Tf-NP-sc and Tf-NP-*miR-181a* groups, respectively (Figure 5A). The spleen weight was 58.3 ± 10.5 mg for age-matched blank control mice (Supplementary Figure S6). Cytospins of bone marrow cells and histopathology of sternum, spleen and liver sections from MV4-11 cell engrafted mice treated with either saline or Tf-NP-sc showed infiltration of blast cells. In contrast, cytospins of bone marrow cells and histopathology of sternum, spleen and liver from Tf-NP-*miR-181a* treated leukemic mice were similar to that of the age-matched blank control groups (Figure 5B). Furthermore, the population of leukemic cells in spleen samples, measured by flow cytometry, was significantly reduced in mice treated with Tf-NP-*miR-181a* compared to mice treated with Tf-NP-sc or saline (Figure 5C). We observed a 2.6-fold and a 35-fold increase of *miR-181a* levels in MV4-11 cells harvested and sorted from bone marrow and spleens, respectively, in the Tf-NP-*miR-181a* treated mice compared to Tf-NP-sc (Figure 5D and 5E). In these cells, RAS and MAPK1 proteins were downregulated in the Tf-NP-*miR-181a* treated mice (Figure 5D and 5F).

The median survival time of the remaining mice was 26, 28.5 and 35 days for the animal groups treated with saline, Tf-NP-sc and Tf-NP-*miR-181a*, respectively. Tf-NP-*miR-181a* treatment significantly reduced the disease burden and prolonged survival compared to Tf-NP-sc ($P = 0.0002$) or saline ($P = 0.0001$) treatment (Figure 5G). Interestingly, Tf-NP-sc treatment also had some minor anti-leukemic effects compared to the saline treated control group ($P = 0.04$).

DISCUSSION

MiRs have been implicated in leukemogenesis, and the expression levels of several miRs have been shown to impact the prognosis of AML patients [1–9, 12–14]. Relatively low expression of *miR-181a* is associated with worse outcomes in AML patients [2, 9, 28]. Here, we provided evidence that AML cells with reduced levels of *miR-181a* had a more aggressive AML phenotype, and we validated this clinical observation functionally.

In other types of cancers *miR-181a* has been associated with both tumor suppressor and oncogene functions [20–28], implying context-specific effects. Whereas in colorectal cancer [20] and lymph node

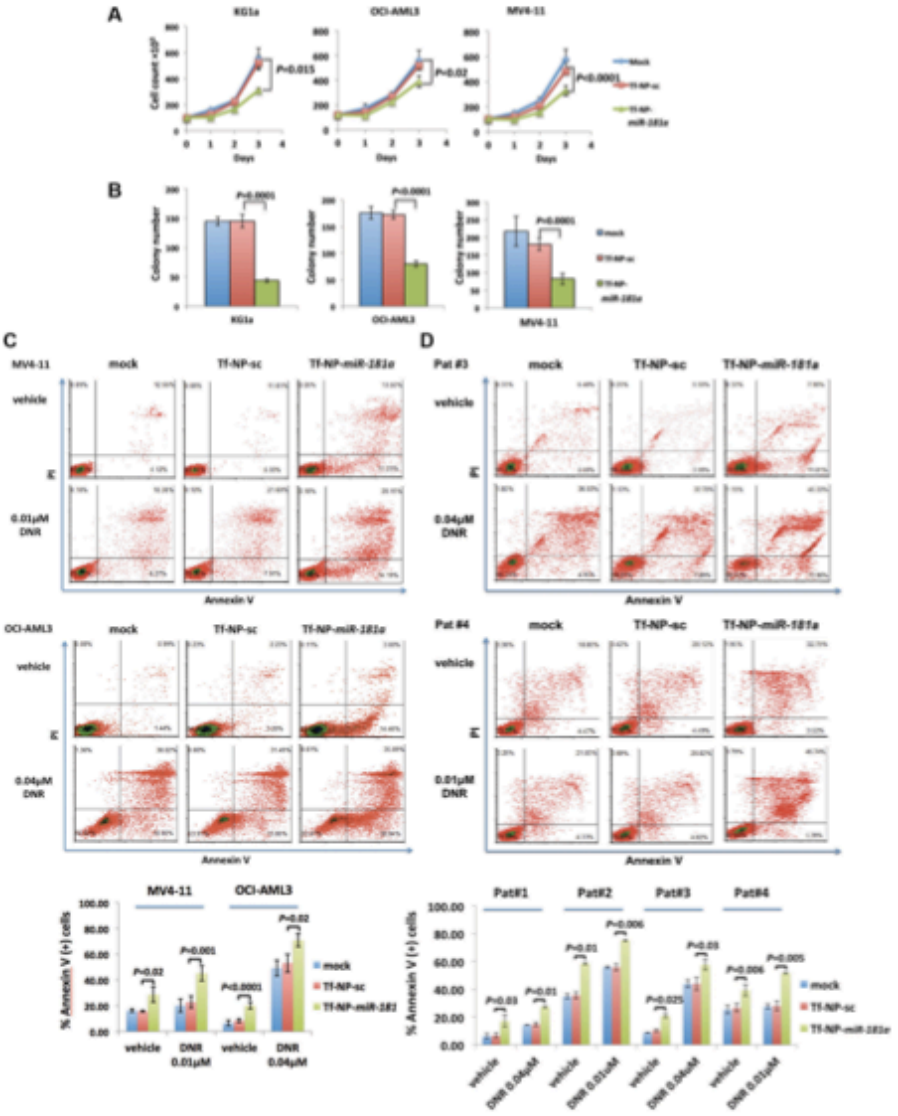

Figure 4: Treatment with Tf-NP-*miR-181a* had anti-leukemic activity in AML cells. Cell growth curve (**A**) and colony numbers (**B**) of KG1a, OCI-AML3 and MV4-11 cells treated with Tf-NP-*miR-181a*, Tf-NP-sc or mock. Error bars represent SD. Annexin V assays in MV4-11 and OCI-AML3 cells (**C**) as well as patient blast cells (**D**) treated with Tf-NP-*miR-181a*, Tf-NP-sc or mock in the presence or absence of daunorubicin (DNR, 0.01 μM for MV4-11, 0.04 μM for OCI-AML3, 0.01 μM for patient 1 [Pat #1] and patient 3 [Pat #3], 0.04 μM for patient 2 [Pat #2] and patient 4 [Pat #4] blasts). DNR was added 24 hours after priming cells with nanoparticle-miR treatment for another 72 hours.

metastasis in oral squamous cell carcinoma [21] a high *miR-181* level seems to be associated with worse clinical outcomes, in glioma this miR has tumor suppressor function [22]. In these brain tumors *miR-181a* was shown to target the anti-apoptotic genes *BCL2* and *MCL1*, and downregulated *miR-181a* reduced glucose deprivation-induced apoptosis and caused mitochondrial dysfunction in astrocytes [22, 49, 50]. The *miR-181*-family has been reported to be an effector in inflammatory response by TNF-α, IL-6, IL-1β, IL-8 and IL-10 [17, 18, 51–53].

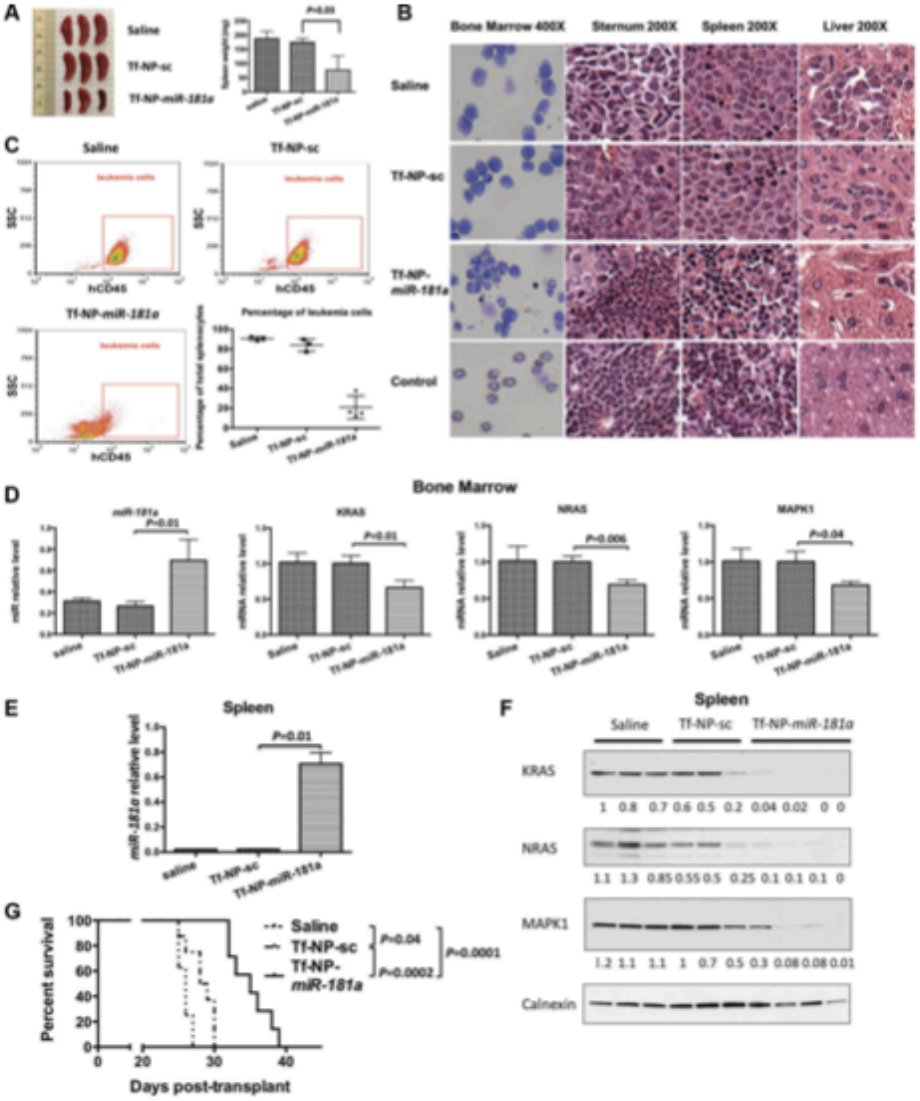

Figure 5: *In vivo* evaluation of Tf-NP-*miR-181a* treatment. (**A**) Spleens and spleen weights from mice sacrificed after 8 doses of treatment from each group: saline, Tf-NP-sc and Tf-NP-*miR-181a* ($n = 3$). (**B**) May-Grünwald/Giemsa staining of bone marrow cells and H&E staining of sections from sternum, spleen and liver of MV4-11 engrafted mice treated with saline, Tf-NP-sc and Tf-NP-*miR-181a*. NSG mice without MV4-11 engraftment were also used as controls. (**C**) Leukemic cell population from the spleens harvested from differently treated mice and assessed by flow cytometry. (**D**) Mature *miR-181a* levels and *KRAS*, *NRAS* and *MAPK1* RNA levels in sorted MV4-11 cells from bone marrow samples harvested from differently treated mice. Error bars represent SD. (**E**) Mature *miR-181a* levels in sorted MV4-11 cells from spleens harvested from differently treated mice. Error bars represent SD. (**F**) KRAS, NRAS and MAPK1 protein expression in sorted MV4-11 cells from spleens harvested from differently treated mice. (**G**) Survival curves of the mice according to the indicated treatment.

With regard to AML, we previously provided preliminary evidence that *miR-181* may target elements of the "inflammasome" that ultimately lead to NF-κB activation and leukemia growth, while Li *et al.* showed that *miR-181* promoted apoptosis, reduced viability and delayed leukemogenesis in MLL-rearranged AML by downregulating the homeobox gene *PBX3* [28]. Bai *et al.* also demonstrated that *miR-181a* may reduce BCL2 and thus enhance chemosensitivity of AML cells [46]. However, the mechanisms through which *miR-181a* attenuates disease aggressiveness and the full spectrum of its targets still remain to be fully understood in AML.

Here, we first demonstrated that *miR-181a* targets the RAS-MAPK1 and RAS-AKT pathways, which have been found to be activated and support AML leukemogenesis [54–58]. Despite extensive efforts, the direct therapeutic targeting of these pathways with small molecule inhibitors remains challenging [59]. Our results show that KRAS, NRAS and MAPK1 proteins may be effectively reduced by utilizing RNA compounds mimicking *miR-181a*. The efficient delivery of *miR-181a* mimics by Tf-NPs decreased the targets and their downstream effectors (AKT, MEK, MYC). Altogether, our results support *miR-181a* replacement as a potential anti-leukemic, RAS targeting strategy in AML. The therapeutic advantage of using miR mimics is in the simultaneous targeting of cross-talking signal transduction pathways (STPs) [38]. Although a use of synthetic mimics may be of relatively difficult in therapeutic application especially compared to the use of anti-miR oligonucleotide, it has been postulated for several types of cancers and is currently being tested in clinical trials (e.g. for *miR-34* in NCT01829971). One of the limitations of miR-based therapies is in the optimal delivery of these oligonucleotides as they are subject to rapid hepatic uptake and metabolism and are easily degraded by endonucleases in biological matrices. Nevertheless, we recently reported a novel anionic lipopolyplex nanocarrier system that was designed for the purpose of allowing for efficient miR delivery to AML cells [47]. Here we show that this system could be adapted to the delivery of *miR-181a* mimics and exert an efficient inhibitory effect on the RAS-MAPK1 and RAS-AKT kinase pathways, thereby resulting in a significant anti-leukemic activity. Interestingly, a very mild anti-leukemic effect and a slight downregulating effect of Tf-NP-scramble treatment on NRAS, KRAS, and MAPK1, as well as on MEK phosphorylation and MYC expression in OCI-AML3 cells was observed. This effect was likely mediated by one of the components of our nanoparticle system, for example, linoleic acid. It has been reported that some fatty acids have anti-tumor activity [60–62].

Other strategies to increase *miR-181a* have also been tested by our group with significant results. In a previous study, we demonstrated that lenalidomide increases endogenous *miR-181a* [45], by enhancing the expression of C/EBPα isoforms, which bind to the *miR-181a* promoter and induce the transcription of *miR-181a*. However, lenalidomide has several unwanted side-effects at the doses necessary to achieve plasma concentrations at which *miR-181a* was increased. Thus, the targeting NPs that we reported here may present the advantage to be more specifically directed to AML blasts, thereby sparing normal tissues and perhaps reducing unwanted toxicity. Our preclinical studies showed encouraging results with no toxicity in NP-treated mice at doses inducing anti-leukemic effects [47]. It should also be underscored that we and others have reported that increased levels of miR-181 lead to enhancement of sensitivity to chemotherapy in AML models [45, 46, 63]. Furthermore, patients with higher levels of miR-181a have a better complete remission rate and longer survival compared with those with lower levels, further supporting a role of this miR as a modifier of the response to chemotherapy [9]. Thus, we envision that potential clinical benefit of *miR-181a* replacement will be more likely if applied in combination with chemotherapy.

In summary, we unveil here a previously unreported activity of *miR-181a* that directly downregulates NRAS, KRAS and MAPK1 and RAS-dependent downstream signals supporting leukemogenesis. We showed that a nanoparticle-based delivery system could be used to efficiently increase otherwise low levels of *miR-181a* and achieve anti-leukemic activity in AML models with no evident toxicity. On the basis of our results, *miR-181a*-NP may warrant further evaluation for potential clinical applications in AML and other RAS-dependent malignancies.

MATERIALS AND METHODS

Cell lines and patient samples

KG1a, MV4-11, HL60, HEK 293T and HEK 293TN cells were obtained from ATCC (Manassas, VA); OCI-AML3 cells were obtained from DSMZ (Braunschweig, Germany). Primary, unselected AML blasts from apheresis samples collected from nine patients were obtained from The Ohio State University (OSU) Leukemia Tissue Bank. Patients signed an informed consent to store and use their tissue for discovery studies according to OSU institutional guidelines.

Lentiviral infections

The lentiviral infections were performed as previously described [7]. The stemloop of *miR-181a* with 200 bp flanking sequence was cloned into the HIV based lentiviral dual promoter vector (pCDH-CMV-MCS-EF1-copGFP+Puro cDNA; System Biosciences, Mountain View, CA). The miRZip anti-*miR-181a* (lenti-anti-181a) and scramble vectors were purchased from System Biosciences.

Luciferase assays

Luciferase assays were carried out as previously described [7]. 293T cells were co-transfected with luciferase vector (pGL4.24), Renilla control vector and *miR-181a* mimic or scramble control. Luciferase activity was normalized to Renilla activity. See supplementary material for more detailed information.

Nanoparticle preparation and treatment

The synthetic double-stranded *miR-181a*, miR-scramble (sc), and *KRAS*, *NRAS* and *MAPK1* siRNAs were purchased from Ambion. Nanoparticle preparation was performed as previously described [47, 64, 65]. Briefly, polyethylenimine was used to capture miRs/siRNAs, and the complex was loaded to pre-made anionic liposomal nanoparticles which consists of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dimyristoyl-sn-glycerol, methoxypolyethylene glycol (DMG-PEG) and linoleic acid. Transferrin was first conjugated with 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (DSPE-PEG2000 maleimide) and then post-inserted to the miR loaded nanoparticle to form the final product. The final concentration of the miRs/siRNAs was 10 nM and was used for all *in vitro* studies. Protein was collected at 24 and 48 hours for western blot analysis.

Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted with TRIzol reagent (Invitrogen). cDNA was synthesized using Superscript III (Invitrogen) or the Taqman miR Reverse Transcription kit (Applied Biosystems, Foster City, CA) for *miR-181a*, *miR-181b*, *miR-140* and *U44*. qRT-PCR was performed with Taqman gene expression assays (Applied Biosystems) following the manufacturer's protocols. *miR-181a*, *miR-181b* and *miR-140* expression were normalized to *U44*. *KRAS*, *NRAS* and *MAPK1* expression were normalized to *GAPDH*. The comparative cycle threshold (C_T) method as previously described was used for relative quantification of gene expression [47].

Western blot analysis

Anti-KRAS (ab55391) antibodies were purchased from Abcam (Cambridge, MA). Anti-NRAS (C-20, sc-519) and Anti-MYC (N-262, sc-764) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-MAPK1, –MEK1/2 (L38C12), –p-MEK1/2 (S217/221,41G9), AKT and p-AKT (S473, D9E) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Equivalent gel loading was confirmed by probing with antibodies against actin (sc-1616; Santa Cruz) or calnexin (C5C9; Cell Signaling). The intensity of the resulting bands was measured by ImageJ 1.48 s (http://imagej.nih.gov/ij). The intensity ratio of each band respective to the corresponding actin intensity was used for relative quantification.

Growth curves

Lentivirally transduced MV4-11 cells (1×10^5/mL) were plated in 12-well plates. KG1a, OCI-AML3 and MV4-11 cells (1×10^5/mL) were plated in 12-well plates and treated with nanoparticles (Tf-NP-sc or Tf-NP-*miR-181a* at a final concentration of 10 nM) or were mock treated (buffer only). Cells were harvested and counted at 24-hour intervals using a Bio-Rad TC20 Automated Cell Counter (Bio-Rad, Berkeley, CA). Each sample was run in triplicate.

Colony assays

Methylcellulose colony formation assays were carried out as previously described [66] and counted after 15 days.

Apoptosis assays

MV4-11 and OCI-AML3 cells and four AML patient blast samples cells were treated with Tf-NP-*miR-181a*, siRNAs, Tf-NP-sc and mock for 24 hours. The cells were then subsequently treated with daunorubicin (DNR; 0.01 μM for MV4-11, 0.04 μM for OCI-AML3, 0.04 μM for patient #1 and #3, 0.01 μM for patient #2 and #4 blasts; Sigma-Aldrich, St Louis, MO) or vehicle control (phosphate-buffered saline; Sigma-Aldrich) for another 72 hours. Annexin V/propidium iodide (PI) stain (BD Biosciences, San Jose, CA) was performed.

In vivo studies

Animal studies were performed according to the Ohio State University institutional guidelines. A total of 5 million lentiviral transduced MV4-11 cells were injected subcutaneously into eight-week female NOD/SCID gamma mice (NSG; The Jackson Laboratory, Bar Harbor, ME). At day 11, 3 mice from each lenti-*anti-181a* and lenti-sc group were sacrificed, and tumors were weighed. At day 23, 3 mice from each lenti-sc and lenti-*181a* group were sacrificed, and tumors were weighed.

For the functional study, six-week-old NSG mice were injected with 0.15 million lentivirally transduced MV4-11 cells intravenously through a tail vein ($n = 6$ in each group: lenti-*anti-181a*, lenti-sc and lenti-*181a*).

For the therapeutic study, six-week-old NSG mice were injected with 0.3 million MV4-11 cells intravenously through a tail vein. The treatment started 10 days after the engraftment. Mice were treated with saline, Tf-NP-sc or Tf-NP-*miR-181a* (1.5 mg/kg/d three times/week).

Randomly, 3 mice of each group were sacrificed after 8 doses of treatment for pathology analysis. Age-matched NGS mice without MV4-11 cell engraftment were used as blank control. The treatment was continued for the remaining mice. Eight mice from each group were monitored for survival. The experiment was repeated for biomarker analysis. Bone marrow and spleen cells were isolated from scarified mice and sorted for human CD45-positive cells for further analysis.

Statistical analysis

Data are presented as mean ± SD of at least 3 independent experiments and analyzed by the two-tailed Student's *t*-test. The mean and SD were calculated and displayed in bar graphs as the height and the corresponding error bar, respectively. Mouse survival was calculated using the Kaplan–Meier method, and survival curves were compared by the log-rank test.

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CONFLICTS OF INTEREST

The authors declare no competing financial interests.

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Clinical and pharmacodynamic activity of bortezomib and decitabine in acute myeloid leukemia

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We recently reported promising clinical activity for a 10-day regimen of decitabine in older AML patients; high *miR-29b* expression associated with clinical response. Subsequent preclinical studies with bortezomib in AML cells have shown drug-induced *miR-29b* up-regulation, resulting in loss of transcriptional activation for several genes relevant to myeloid leukemogenesis, including DNA methyltransferases and receptor tyrosine kinases. Thus, a phase 1 trial of bortezomib and decitabine was developed. Nineteen poor-risk AML patients (median age 70 years; range, 32-84 years) enrolled. Induction with decitabine (20 mg/m² intravenously on days 1-10) plus bortezomib (escalated up to the target 1.3 mg/m² on days 5, 8, 12, and 15) was tolerable, but bortezomib-related neuropathy developed after repetitive cycles. Of previously untreated patients (age ≥ 65 years), 5 of 10 had CR (complete remission, n = 4) or incomplete CR (CRi, n = 1); 7 of 19 overall had CR/CRi. Pharmacodynamic analysis showed *FLT3* down-regulation on day 26 of cycle 1 (*P* = .02). Additional mechanistic studies showed that *FLT3* down-regulation was due to bortezomib-induced *miR-29b* up-regulation; this led to *SP1* down-regulation and destruction of the SP1/NF-κB complex that transactivated *FLT3*. This study demonstrates the feasibility and preliminary clinical activity of decitabine plus bortezomib in AML and identifies *FLT3* as a novel pharmacodynamic end point for future trials. This study is registered at http://www.clinicaltrials.gov as NCT00703300. (*Blood*. 2012;119(25):6025-6031)

Introduction

Despite progress made in understanding the mechanisms of leukemogenesis and the identification of cytogenetic and molecular markers for risk stratification, most adult patients with acute myeloid leukemia (AML) are not cured when treated with conventional chemotherapy, especially elderly patients.[1,2] Thus, novel approaches to improve outcomes for patients with AML are needed.

Bortezomib is a proteasome inhibitor approved for the treatment of multiple myeloma and mantle cell lymphoma, but only transient hematologic improvements were noted in a single-agent phase 1 study of bortezomib in AML.[3] Despite lack of single-agent activity, bortezomib has shown promise when used in combination regimens for AML.[4] We recently demonstrated a unique mechanism of activity of bortezomib: the drug is an indirect transcriptional inhibitor for several target genes that are relevant to AML.[5,6] We showed an important role for bortezomib in disrupting a network that operates on the basis of interactions of *miR-29b*, the transcription factor SP1, and NF-κB(p65). This network affects the expression of several genes in myeloid leukemia cells, including DNA methyltransferase enzymes (DNMT) and the receptor tyrosine kinase (RTK) *KIT*.[5,6] We showed that activating *KIT* mutations, frequently found in core binding factor AML, led to MYC-dependent *miR-29b* repression, resulting in increased levels of SP1 (a *miR-29b* target).[5] Up-regulated SP1 bound NF-κB(p65) and transactivated *KIT*. Therefore, activated KIT ultimately induced its own transcription via *miR-29b*.[5] We demonstrated that bortezomib-induced disruption of the SP1/NF-κB(p65) complex inhibited the growth of leukemic cells via up-regulation of *miR-29b*.[5] The results supported the notion that *miR-29b*/SP1/NF-κB(p65) complex–dependent *KIT* overexpression contributed to the growth of leukemia and could be targeted by bortezomib.[5] Because most AML cells express FLT3 (another member of the RTK family) and because of the relevance of both wild-type and mutated *FLT3* expression for AML cell growth and survival, FLT3 is an important target in AML[7-11]; we hypothesized that the aforementioned mechanisms also extend to *FLT3* expression.

In the current study, we sought to deepen our understanding of the transcriptional inhibitory activity bortezomib and its potential use in patients with AML by combining clinical, pharmacodynamic, and additional in vitro mechanistic experiments. Recently, we reported that the DNA hypomethylating agent decitabine is active in AML. In a phase 2 study conducted at our institution, the complete remission (CR) rate was 47%, the overall response

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rate 64%, and the median overall survival duration approximately 1 year with a 10-day induction regimen of low-dose decitabine in untreated older patients with AML (≥ 60 years, not candidates/refused intensive therapy).[12] Although these results were promising, we viewed the regimen as a framework on which future investigations might build and improve. Given that (1) greater *miR-29b* levels were associated with response to decitabine in that trial[12] and (2) preclinical work showed bortezomib to be an inducer of *miR-29b* expression, bortezomib was a very appealing agent for combination studies with decitabine. Therefore, we performed a phase 1 clinical trial of bortezomib with decitabine in poor-risk AML patients to test feasibility and provide preliminary clinical response data for this combination; we further developed our understanding of the role bortezomib in AML via pharmacodynamic and additional in vitro studies.

Methods

Eligibility criteria and study design

Eligible patients were adults with either (1) relapsed or refractory AML or (2) previously untreated AML who were ≥ 65 years of age. Patients were required to have total bilirubin ≤ 2× the upper limit normal, creatinine ≤ 2.0 mg/dL, alanine aminotransferase/aspartate aminotransferase ≤ 5× upper limit normal, left ventricular ejection fraction at least 40%, and Eastern Cooperative Oncology Group performance status ≤ 2. Exclusion criteria included chemotherapy or radiotherapy within 2 weeks, active other malignancies (within 3 years), active CNS disease or granulocytic sarcoma as sole site of disease, uncontrolled intercurrent illness, and pre-existing neuropathy grade 2 or greater. Informed written consent approved by The Ohio State University (OSU) Human Studies Committee was obtained on all patients before they were entered into the study, in accordance with the Declaration of Helsinki.

Patients were given induction cycles of decitabine 20 mg/m^2 intravenously over 1 hour on days 1-10 with cycles repeated every 28 days until bone marrow (BM) blasts were < 5%, at which time decitabine dosing was cut to 3-5 days/cycle as previously described.[12] Bortezomib was administered immediately after the decitabine dose by intravenous push (IVP). Bortezomib was dose escalated according to the following dose-escalation plan: dose level 1, 0.7 mg/m^2 IVP on days 5 and 8; dose level 2, 0.7 mg/m^2 IVP on days 5, 8, 12, and 15; dose level 3, 1.0 mg/m^2 IVP on days 5, 8, 12, and 15; and dose level 4, 1.3 mg/m^2 IVP on days 5, 8, 12, and 15. Treatment delays of ≥ 10 days were permitted for patients with a BM cellularity of ≤ 10% and no evidence of disease in the marrow, until at least partial restoration of hematopoiesis occurred (defined as BM cellularity > 10% or absolute neutrophil count [ANC] > 1000/μL). Hydroxyurea was permitted to control white blood count to < 40 000/μL, if necessary, before and during cycle 1, but no other antileukemic therapies were permitted. In the absence of a hypoplastic marrow (≤ 10% cellularity), clearly progressive increase in BM blasts (after at least 2 cycles of administration, if possible), ongoing/uncontrolled infection, or serious hemorrhagic complications, dosing was to be continued every 4 weeks without delay. Treatment continued indefinitely until disease progression or unacceptable toxicity occurred, except that bortezomib was discontinued in patients who did not have an objective response after 3 cycles of treatment. Reponses were defined according to the International Working Group criteria for AML, including CR and CR with incomplete count recovery (CRi).[13]

Definition of dosing-limiting toxicity

Adverse events were graded according to the National Cancer Institute (NCI) Common Toxicity Criteria for Adverse Events Version 3.0. Dose-limiting toxicity (DLT) was defined with cycle 1 of therapy. Drug-related nonhematologic toxicity of grade 4 was considered DLT with the exception of alopecia, nausea and vomiting controllable with antiemetic therapy, infection, and fatigue. Given the frequency of infectious complications with conventional chemotherapy in this population and prevalence of disease-related cytopenias, infectious complications were not mandated as DLT unless the severity or duration was longer than that expected with conventional treatment. For DLT, if the toxicity occurred in 2 or more patients at a single-dose level, that dose was deemed intolerable and the next lower dose level was expanded to increase confidence in toxicity assessment at the maximum tolerable dose. Hematologic DLT was defined as follows: failure to recover neutrophil and/or platelet counts by day 42 in patients with < 5% blasts in the BM, absence of myelodysplastic changes, and/or absence of evidence of disease by flow cytometry in the BM. Six additional patients were treated at the maximum tolerable dose.

Cytogenetics, molecular markers, and correlative studies

Standard cytogenetic analyses were performed on BM samples. For patients who consented to and had additional material available for molecular studies, the presence or absence of *FLT3*-ITD and *FLT3*-TKD was determined as previously described.[7,11] Correlative studies included the measurement of *miR-29b*, *FLT3*, *DNMT1*, *DNMT3A*, *DNMT3B*, and estrogen receptor (*ESR*) mRNA expression in BM at pretreatment and at approximately day 26 of cycle 1 (± 2 days) with the use of RT-PCR as previously described.[6,12] In brief, total RNA was extracted with Trizol (Invitrogen) reagent, and cDNA was synthesized from total RNA. Gene expression of *FLT3*, *DNMT1*, *DNMT3A*, *DNMT3B*, and *ESR* were normalized to *ABL1*. For *miR-29b* expression, quantitative RT-PCR was performed by TaqMan MicroRNA Assays (Applied Biosystems) according to the manufacturer's protocol and normalized by *U44* as previously described.[12] Expression of the target genes were measured by use of the ΔCT approach. All Taqman Assays for gene and microRNA expression were purchased from Applied Biosystems

Cell culture and treatment

MV4-11, KG1, and HEK293T cell cultures were in standard fashion. Cells were treated with bortezomib (Millennium Pharmaceuticals) used at concentrations, times, and schedules indicated in the Results section. For additional in vitro mechanistic studies, mononuclear cells from BM samples with > 70% blasts from an AML patient were obtained from OSU Leukemia Tissue Bank. Primary patient blasts were cultured in StemSpan SFEM media (StemCell Technologies), supplemented with StemSpan CC100 (StemCell Technologies) containing FLT3-ligand, stem cell factor, IL-1, and IL-6. Patients signed an informed consent to store and use their tissue for discovery studies according to OSU institutional guidelines.

Transient transfections

Construction of the human SP1 and NF-κB(p65) expression vectors were performed as previously described.[5] On-target plus Smart pool siRNA for *SP1*, *NFKBP65*, and controls were purchased from Thermo Fisher Scientific. Precursor *miR-29b* was obtained from Applied Biosystems. siRNA, miRNA, or plasmid constructs were introduced into the leukemia cell lines MV4-11 and KG1 by Nucleofector Kit (Lonza Walkersville) according to the manufacturer's instructions and as previously reported.[5] Transient transfections of HEK293T cells for the luciferase experiments were performed with the use of Lipofectamine reagent (Invitrogen) according to the manufacturer's description.

ChIP assays

ChIP assays were performed with the EZ ChIP Assay Kit (Millipore) per manufacturer recommendations and as previously described.[5] DNA was quantified by the use of quantitative RT-PCR with SYBR green incorporation (Applied Biosystems). The antibodies used were as follows: SP1 (Cell Signaling) and NFkB p65 (Millipore). Quantitative PCR was used to measure the fraction of *FLT3* promoter DNA enrichment in the immunoprecipitated with SP1 and NFkB p65 antibody.

Table 1. Patient characteristics

Previously untreated patients, age, y/sex	Secondary or de novo	Diagnostic karyotype	Presenting WBC × $10^3/\mu L$	% BM blasts	Response
61/F	De novo	46,XX,del(5)(q13q33)(2)/46,sl,del(11)(p12)(cp2)/46,sl,t(X;6)(p22.1;q13),del(12)(p11.2), del(16)(q12.1)(15)/ nonclonal with clonal abnormalities(1)	4.8	71	CRi
66/F	Secondary	Insufficient Metaphase (46,XX(6)/nonclonal(2))	1.5	48	CR
84/M	De novo	85-88 < 4n > ,XXYY,i(1)(q10),-2,-3,-7,-9,-17,i(17)(q10),+18(cp15)/46,XY(4)/8n(1)	1	76	
65/F	De novo	Normal	5.2	20	CR
68/M	De novo	44,X-Y,-3,del(5)(q22q35),del(6)(q13), del(17)(p11.2)(5)/43,sl,-11,der(19)t(1 1;19) (q12;p13.3)(3)/44,sl,+mar1(cp3)/ 44,sl,add(19)(p13.3)(cp2)/44,sl,+mar2(2)/46,XY(5)	0.5	14	
70/M	De novo	Normal	11.3	65	
73/M	Secondary	46,XY,del(7)(q21)(13)/46,XY(6)/nonclonal(1)	39.8	39	
67/M	De novo	43,XY,-3,del(5)(q13),-7,del(8)(p12), dic(11;12)(p11.2;p11.2), add(15)(p11.1), 16,add(17)(p11.2),+mar(18)/46,XY(1)/nonclonal(1)	0.2	15	
70/M	De novo	46,XY,inv(3)(q21q26)(10)/46,XY(10)	0.5	23	CR
83/M	Secondary	46,XY,del(12)(p11.2p13)(3)/45,idem,-7(22).ish del(12)(ETV6-)	0.8	34	CRu

Cru indicates unconfirmed complete remission for the elderly patient who had peripheral blood count recovery but who refused BM evaluation after treatment, as noted in the text; and WBC, white blood cell.

Luciferase assays

Luciferase assays were conducted with the use of HEK293T cells. A 700-bp upstream region of *FLT3* promoter spanning SP1 and NFkB p65 binding sites was cloned into a pGL2-luciferase-reporter (Promega) vector using the *Hin*dIII restriction enzyme site with primers as indicated in supplemental Methods (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Firefly luciferase and *Renilla* luciferase activities were assessed according to the recommendations detailed in the Dual-Luciferase Reporter Assay System (Promega).

Electromobility shift assay

Nuclear proteins were extracted from MV4-11 cells using the Nuclear Extract Kit (Active Motif) according to the manufacturer's instructions. 5′-biotinylated DNA containing the predicted binding sites for SP1 (SP1-site1 and SP1-site2) and NF-κB (NF-κB-site 1 and NF-κB-site 2) were obtained from Integrated DNA Technologies. For annealing, concentrated complementary oligonucleotides were mixed at a 1:1 molar ratio and incubated at 95°C for 5 minutes. The heat was then gradually reduced over hours until the oligonucleotides reached room temperature 24 hours before start of the experiment. Annealed oligos were diluted to a final concentration of 10 fmol. The Thermo Scientific LightShift Chemiluminescent EMSA Kit (Pierce/Thermo Fisher Scientific) was used according to the manufacturer's instructions. For supershift experiments, SP1 antibody (Santa Cruz Biotechnology) or NFkB p65 antibody (Cell Signaling Technology) were added.

Western blotting

The western blots were preformed as previously described.[5] The antibodies used were as follows: SP1 and actin (Santa Cruz Biotechnology), NFkB p65 (Millipore), and FLT3 (Cell Signaling Technology).

Statistical analysis

Data were compared using the Student 2-tailed t test. $P \leq .05$ was considered statistically significant. All analyses were performed using the R 2.14.1 software package (available at http://www.r-project.org).

Results

Patient characteristics

The enrollment of 19 poor-risk AML patients occurred during the course of 11 months. Clinical and the cytogenetic characteristics of the patients enrolled on the clinical trial are summarized in Tables 1 and 2. The median age was 70 years (range, 32-84 years). Median white blood cell count was 3.9 × $10^3/\mu L$ (range, 1.3-69.9). Previously untreated patients (n = 10) were of median age 70 years (range, 65-84 years) and presented with intermediate or adverse cytogenetic risk according to Cancer and Leukemia Group B criteria[2]; 7 had de novo AML, and 3 had secondary/therapy-related

Table 2. Patient response

Relapsed/ refractory patients, age, y/sex	No. previous inductions	Pretreatment karyotype	Presenting WBC × $10^3/\mu L$	% BM blasts	Response
75/M	3	46,XY,dup(1)(q21q41)(2)/46,XY,del(3)(p21.1p21.3)(2)/46,XY(16)	1.8	4	
66//F	2	46,XX,del(5)(q22q33)(1)/55–56,sl,+1,+2,+8, del(8)(p11.2p23)x2,+9,+10,+11, del(12)(q13q15),+13,+14,+14,add(14)(q32),-17,add(18)(p11.2), add(19)(p13.2), +21,+22,del(22)(q13), +mar1, +mar2,+mar3,+mar4(cp8)/55–56,sdl1,add(4)(q32), -add(14)(q32)(cp5)/46,XX(5)/nonclonal(1)	0.9	42	
67/M	1	Normal	0.6	42	CRi
50/F	2	46X,t(x;10;11),del(12p)	22	86	
77/F	2	Unobtainable (dry tap)	2.1	63	
32/M	3	45,X,-Y,t(8;21)(q22;q22)cp7)/46,XY(13)	1.6	18	
70/M	3	94 < 4n > ,XXYY, +13,+13(9)/46,XY(11)	1.2	31	
73/M	1	Normal	0.4	19	CRi
57/M	4	47,XY,+8[16]/47,idem,t(2;12)(p16;q21)[1]/non-clonal abnormalities[3]	6.8	81	

WBC indicates white blood cell.

AML. Relapsed/refractory patients (n = 9) had a median age of 67 years (range, 32-75 years). Eight had did not respond to previous anthracycline/high-dose cytarabine therapy. All 4 patients who enrolled with AML in untreated first relapse had CR1 duration of < 1 year. Overall, 8 patients consented to a diagnostic assessment of *FLT3* mutational status; none harbored a *FLT3*-TKD, and 1 patient had a *FLT3*-ITD. All 8 patients expressed robust *FLT3* levels as measured by real-time RT-PCR (not shown). Five patients had serial material available for pharmacodynamic studies.

Dose escalation and treatment

Three patients were treated at dose level 1. Four patients were treated at dose level 2 because 1 patient was removed from study as the result of disease-related thrombosis. This event was not judged to be related to the drug because the patient had presented with AML originally with extensive deep-venous thrombosis thought to be related to malignancy and had an inferior vena cava filter placed 11 months earlier. Dose level 3 was expanded to 6 patients because of safety concerns after an infection-related death during induction in 1 patient. Six additional patients were treated at the highest planned dose of bortezomib (1.3 mg/m^2 on days 5, 8, 12, and 15); 1 respiratory death before cycle 2 occurred at this dose. Patients received a median of 2 cycles of treatment (range, 1-14). Only 2 patients received treatment with both drugs beyond 3 cycles. By design, bortezomib treatment was discontinued after 3 cycles in patients without response; neuropathy occurring after cycle 2 required the discontinuation of bortezomib in 3 patients. One patient received the combination of both drugs for 8 cycles, then decitabine alone for 6 additional cycles; the other received the combination for 4 cycles, then decitabine alone for 6 additional cycles.

Toxicities

Typically, induction death in AML is described at 30 days, but given the less-intensive nature of decitabine-based treatments with delayed response and the need for repetitive cycles of administration, 8-week mortality may be a better measure with this agent. Four patients (2 with refractory/relapsed disease, 2 with previously untreated disease) died within 8 weeks of treatment because of infection (n = 2), disease progression (n = 1), and respiratory failure from pulmonary fibrosis (n = 1). Only 1 of these deaths (from disease progression) occurred within 30 days of study entry. Infections or febrile neutropenia were commonly encountered, occurring in 11 patients during cycles 1 and 2 of treatment. Grade 3 or greater toxicities regardless of attribution during the first 2 cycles are listed in Table 3. Through the entire duration of the study, grade 3 or greater neuropathy occurred in 3 patients (none during cycle 1). For 1 of these patients, the neuropathy was autonomic and disabling. No hematologic DLT was observed.

Clinical responses

In previously untreated patients (all ages 65 years and older), CR/CRi occurred in 4 patients; another CR not confirmed by marrow evaluation occurred in a patient who refused BM evaluation after treatment (described in this paragraph), for an overall remission rate of 50% (5/10) in this subset. For the 4 previously untreated patients with documented CR/CRi, the best response and duration of response were as follows: CR, 12 months; CR, 9 months; CR, 10 months (died of myocardial infarction in remission); and CRi, 3 months (incomplete response was ANC < 1000/μL). The fifth responder was an 84-year-old patient who refused BM reevaluation after 2 cycles of treatment, but the patient had complete count recovery with no blood blasts and lived for 17 months before dying of unknown cause (the patient refused further follow-up after discontinuing trial participation). Although we could not classify this patient as a CR by International Working Group criteria because of lack of morphologic documentation by BM aspirate and biopsy, it is likely that this patient achieved CR. Among patients with relapsed or refractory AML, 2 of 9 achieved CRi (CR was incomplete in both cases because of ANC < 1000/μL). Response duration in one was only 2 months, but the other proceeded to allogeneic transplant quickly with no relapse more than 18 months after transplantation. On the basis of the pattern of the response, the patient likely would have met ANC recovery for CR had transplant not immediately occurred. Including the 1 patient with unconfirmed BM CR, the remission rate was 50% (5/10) for those with previously untreated disease and 37% (7/19) for the whole cohort. The median number of cycles to best response was 2 (range, 2-4).

Table 3. Toxicities: grade 3 or greater nonhematologic toxicities regardless of attribution during cycles 1-2

Infections/febrile neutropenia	11 patients
Pneumonia	4
Cellulitis	1
Bloodstream	2
Neutropenic fever	4
Organ toxicities*	23 events
Neurotoxicity	3
Gastrointestinal	3
Pulmonary	3
DVT/PE	2
Hyperglycemia	6
Confusion	1
Rash	1
Acute renal failure	1
Atrial fibrillation	1
Syncope	2
Death	4 patients
Infection	2
Pulmonary fibrosis	1
Disease	1

DVT indicates deep-vein thrombosis; and PE, pulmonary embolism.
*Toxicities that recurred in individual patients in both cycles 1 and 2 are listed as 2 events.

Pharmacodynamic validation studies

The expression levels of *miR-29b* and *FLT3* mRNA were measured in pretreatment and on day 26 of the first cycle in patients who consented to correlative studies and had suitable serial BM samples available (n = 5). At the day 26 posttreatment time point, none of these patients had achieved CR. We observed a trend toward a greater expression of *miR-29b* ($P = .19$) and noted statistically significant lower expression of *FLT3* mRNA ($P = .02$) on day 26 with respect to pretreatment expression levels (Figure 1). The patient with *FLT3*-ITD had 50% lower expression of *FLT3* after 1 cycle (BM blasts % was the same as pretreatment). The median fold-change increase for *miR-29b* expression at day 26 with respect to baseline levels was 2.9 ($P = .2$), whereas the median fold-change decrease for *FLT3* mRNA at day 26 with respect to baseline levels was 0.4 ($P < .01$).

Because we previously showed that *miR-29b* directly or indirectly targets *DNMT1*, *DNMT3A*, and *DNMT3B*,[14] we also assessed the mRNA expression of these genes (supplemental Figure 1A). Posttreatment day 26 down-regulation of all 3 *DNMT* isoforms

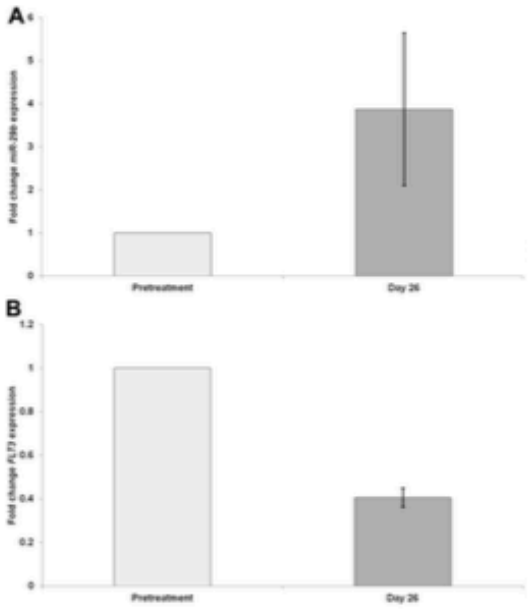

Figure 1. Posttreatment down-regulation of *FLT3* in AML patients. Pretreatment and day 26 expression levels of *miR-29b* (A) and *FLT3* mRNA (B) in patients with serial bone marrow from the decitabine-bortezomib clinical trial (n = 5).

compared with baseline was observed, ie, *DNMT1* (median fold-change: 0.4), *DNMT3A* (median fold-change: 0.2), and *DNMT3B* (median fold-change: 0.4). Furthermore, posttreatment day 26 expression of the *ESR* gene, often hypermethylated and silenced in AML, was increased (supplemental Figure 1B; median fold-change: 2.5). These changes did not achieve statistical significance.

Bortezomib-dependent mechanisms of FLT3 down-regulation

Because down-regulation of *FLT3* expression was observed in all patients at day 26 after treatment, we further investigated in vitro the mechanisms of this pharmacologic effect. We recently showed that a NF-κB(p65)/SP1 complex drives the expression of *KIT*, a member of the RTK family. Thus, we postulated that the SP1/NF-κB(p65) complex could also transactivate the *FLT3* gene. First, we identified 2 putative SP1 and NF-κB(p65) binding sites (sites 1 and 2) within the promoter region of *FLT3* promoter in MV4-11 cells that harbor a *FLT3*-ITD and express *FLT3* at high levels (Figure 2A). Then, using electromobility shift assays, we validated the binding of the SP1/NF-κB(p65) complex to the promoter binding sites for SP1 (only Site 1; Figure 2B) and NF-κB(p65) (both Sites 1 and 2; Figure 2B). The binding affinity of SP1 and NF-κB(p65) on these putative binding sites was confirmed by ChIP assays. Using primers spanning the region of the first SP1 and NF-κB(p65) binding sites, we showed enrichment of both SP1 and NF-κB(p65) on the *FLT3* promoter in MV4-11 cells (Figure 2C).

To assess transactivating activity of SP1/NFκB(p65) on *FLT3* promoter regulatory sequences, we cloned a 700-bp spanning region of the *FLT3* promoter containing the SP1/NF-κB(p65) binding sites into a luciferase-reporter vector. When the *FLT3* luciferase-reporter vector was cotransfected with SP1 or NF-κB(p65) overexpression vector in HEK293T cells, promoter activity was enhanced by SP1 or NF-κB(p65) overexpression compared with negative controls (Figure 2D). Conversely, SP1 or NF-κB(p65) knockdown using siRNAs resulted in the down-regulation

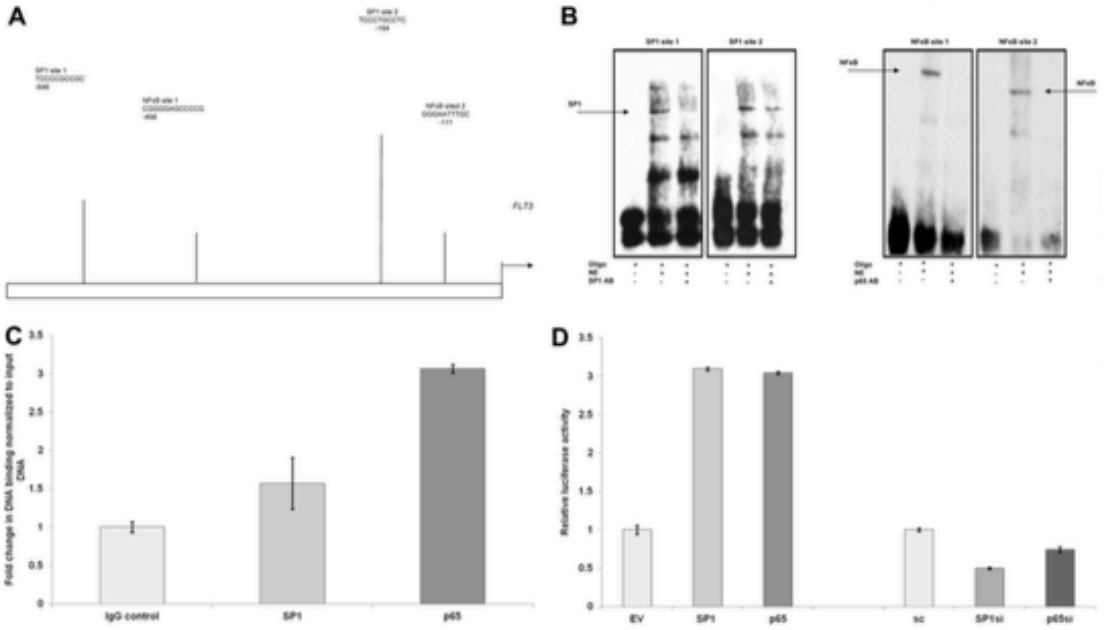

Figure 2. Regulation of *FLT3* expression via the SP1/NF-κB(p65) complex. (A) SP1 and NF-κB(p65) binding sites in the promoter region of the *FLT3* gene. (B) EMSA assays of the 2 identified binding sites for SP1 and NF-κB(p65), demonstrating specific binding of SP1 to the first site and binding of NF-κB(p65) to both sites using specific antibody to supershift the DNA-protein complexes. (C) Chromatin (ChIP) for the region containing binding sites of SP1 and of NF-κB(p65). (D) Luciferase promoter activity reporter assay including 700 bp of the promoter region of the *FLT3* gene containing all identified SP1 and NF-κB(p65) binding sites, demonstrating increased activity after cotransfection with constructs to overexpress SP1 or NF-κB(p65) and decreased activity after siRNA mediated knockdown of SP1 and NF-κB(p65). EV indicates empty vector; and sc, scramble oligo.

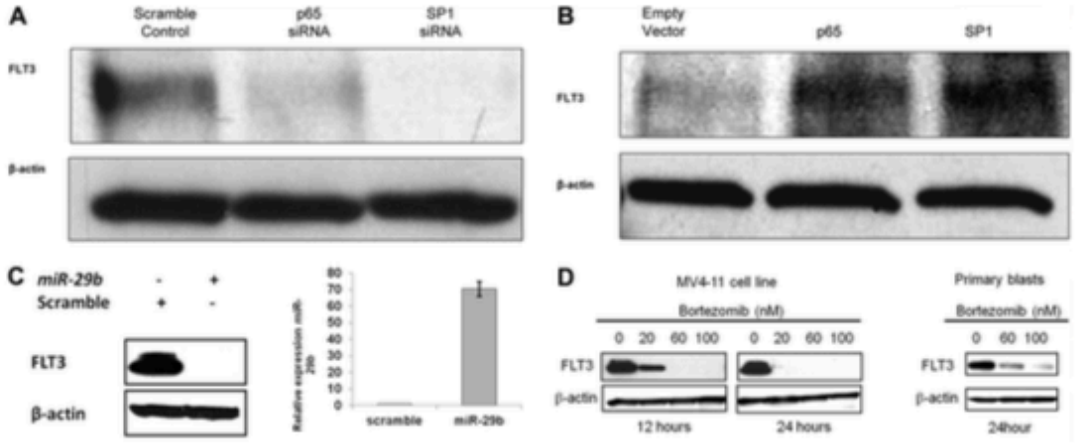

Figure 3. Bortezomib-induced FLT3 down-regulation via interaction with the SP1/NF-κB(p65) complex. (A) SiRNA mediated knock-down of SP1 or NF-κ-B(p65) down-regulates FLT3 expression in MV4-11 cells that harbor a *FLT3*-ITD and express *FLT3* at high levels. (B) Overexpression of SP1 or NF-κB(p65) increases the expression of FLT3 in the KG1 cell line that usually has low expression of FLT3. (C) Increasing *miR-29b* in the MV4-11 cell line decreases FLT3 expression in MV4-11 cells. (D) Bortezomib treatment decreases FLT3 expression in a time- and dose-dependent manner in the MV4-11 cell line and in primary patient blasts (obtained from patients not enrolled on the current clinical trial; samples were procured in the OSU Leukemia Tissue Bank).

of the luciferase activity (Figure 2D). Gain and loss of function experiments further supported the regulatory role of SP1 and NF-κB(p65) on *FLT3* expression. SiRNA-mediated knock-down of SP1 or NF-κB(p65) led to decreased *FLT3* expression in *FLT3*-ITD–positive and *FLT3*-high expressing MV4-11 cells (Figure 3A). In contrast, overexpression of SP1 or NF-κB(p65) led to increased *FLT3* expression in *FLT3* wild-type and *FLT3*-low expressing wild-type KG1 cells (Figure 3B). BecauseSP1 is a bona fide target of *miR-29b*, we also reasoned that *miR-29b* is likely to participate in *FLT3* transcriptional regulation through modulating SP1 expression. Forced *miR-29b* expression indeed resulted in *FLT3* down-regulation in MV4-11 (Figure 3C).

Having validated our hypothesis that the NFκB/SP1 complex up-regulates *FLT3* expression and that *miR-29b* causes down-regulation of this RTK, we reasoned that a pharmacologic intervention that would increase *miR-29b* expression would also down-regulate *FLT3* by interfering with the SP1/NF-κB(p65) complex. We have already reported that bortezomib induced miR-29b and disrupts the SP1/NF-κB(p65) complex.[6] Consistent with these observations, we showed dose- and time-dependent *FLT3* down-regulation in bortezomib treated *FLT3*-ITD–positive MV4-11 cell lines and primary AML blasts (Figure 3D), confirming the observation from patients treated on the clinical trial.

Discussion

We report here the results of a phase 1 trial of bortezomib and decitabine in patients with poor-risk AML. In addition to the clinical results, we provide pharmacodynamic evidence that *FLT3* expression is a novel target for this combination and describe the mechanisms through which bortezomib contributes to *FLT3* down-regulation.

With regard to the clinical trial, the combination of bortezomib and decitabine was tolerable and active in this cohort of AML patients. We observed a 50% CR/CRi rate in previously untreated older AML patients, whereas for patients with refractory or relapsed disease, the CR/CRi rate was 22%. The maximal planned dose of bortezomib in combination with decitabine was reached, but 3 patients experienced serious neuropathy after multiple cycles of therapy. Although the incidence of neuropathy in this trial was similar to that observed with bortezomib in other malignancies, strategies to prolong the duration of exposure to bortezomib and decitabine without increasing the frequency of neurotoxicity must be considered with further development of this regimen. Emerging data suggest that, at least in multiagent regimens, modification of the traditional bortezomib schedule of administration on days 1, 4, 8, and 11 to a once-weekly approach substantially reduces neurotoxicity without a detrimental effect on clinical response end points or survival.

In several studies, authors have noted reduced toxicity with preserved efficacy for once-weekly bortezomib compared with the traditional schedule.[15,16] Most notably, in a phase 3 study in myeloma, clinical outcomes including 3-year progression-free and overall survival were similar between different bortezomib dosing groups (nonrandomized) but with a markedly lower incidence of neuropathy with for weekly dosing versus the traditional schedule.[17,18] Subcutaneous administration of bortezomib appears to be another alternative with reduced neurotoxicity.[18] This consideration is an important one for future phase 2/3 studies of bortezomib and decitabine in AML because hypomethylating agent therapy requires prolonged and repetitive exposure to maximize benefit.

The results of the pharmacodynamic analyses showing a trend for *miR-29b* up-regulation and significant *FLT3* down-regulation, albeit limited by small sample size, led us to further dissect the mechanisms through which bortezomib could target the activity of the *FLT3* gene. We have previously shown that bortezomib interferes with the transcription complex SP1/NF-κB(p65) by increasing *miR-29b* that targets *SP1*.[6] Here, we showed that the SP1/NF-κB(p65) complex transactivated *FLT3* and that the activity of the complex was coregulated by *miR-29b*. Down-regulation of *miR-29b* in AML resulted in greater activity of the SP1/NF-κB(p65) complex (because of elevated levels of SP1),[6] and this in turn caused *FLT3* up-regulation. Reversing this constituted the basis for bortezomib-induced *FLT3* down-regulation in vitro and in vivo. Because overexpression of wild-type or mutated *FLT3* is frequent in AML blasts and because FLT3 activation promotes leukemia cell growth and survival, the finding of pharmacologic

FLT3 transcriptional inhibition in AML patients may represent a novel therapeutic strategy.

Our understanding of the role for bortezomib as a modulator of the SP1/NF-κB(p65) complex[5] has developed over time. Given current understanding of bortezomib's ability to up-regulate *miR-29b*, in turn disrupting expression of genes dependent on this complex and potentially sensitizing patients to decitabine, future clinical studies of decitabine and bortezomib in AML should alter the sequence of administration and test bortezomib given before decitabine, rather than after. We have previously reported that greater expression of *miR-29b* associated with response to decitabine.[12] It is tempting to hypothesize that this occurs because of correspondingly low expression of *miR-29b* targets, including FLT3, that play a relevant role in supporting myeloid leukemia growth and treatment resistance. With the heterogeneity of patients and the small sample size, it is difficult to compare remission rates in untreated older AML patients from this trial to our previously reported study with decitabine alone.[12] However, given that the remission rates appear similar, to determine whether bortezomib increases the clinical efficacy of decitabine by increasing *miR-29b* and down-regulating *miR-29b* targets (ie, FLT3) will require randomization and a larger trial, likely via the use of an alternative route (subcutaneous) or schedule (weekly) of bortezomib administration to ameliorate neurotoxicity concerns. A recently activated Alliance phase 2 trial in previously untreated older AML patients randomized to decitabine versus decitabine plus bortezomib (subcutaneous) will investigate this question (Alliance 11002).

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Authorship

Contribution: W.B. was the principal investigator of the clinical study and takes primary responsibility for the paper; S.S. and G.M. designed the experimental studies; G.M. served as the mentor on this project and provided input into the initial study design, implementation, manuscript preparation, and editing; W.B., A.W., R.K., J.C.B., S.M.D., and G.M. recruited and/or treated the patients; S.S. and A.-K.E. performed the laboratory experiments; S.G. performed statistical analyses; C.G. and C.K. coordinated the research; W.B., S.S., R.G., and G.M. wrote the manuscript; the remaining authors provided materials and/or scientific support for the experiments performed; and all authors agreed on the final version.

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ORIGINAL ARTICLE

Increased anti-leukemic activity of decitabine via AR-42-induced upregulation of *miR-29b*: a novel epigenetic-targeting approach in acute myeloid leukemia

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Histone deacetylase (HDAC) inhibitors either alone or in combination with hypomethylating agents have limited clinical effect in acute myeloid leukemia (AML). Previously, we demonstrated that AML patients with higher *miR* (microRNA)-*29b* expression had better response to the hypomethylating agent decitabine. Therefore, an increase in *miR-29b* expression preceding decitabine treatment may provide a therapeutic advantage. We previously showed that *miR-29b* expression is suppressed by a repressor complex that includes HDACs. Thus, HDAC inhibition may increase *miR-29b* expression. We hypothesized that priming AML cells with the novel HDAC inhibitor (HDACI) AR-42 would result in increased response to decitabine treatment via upregulation of *miR-29b*. Here, we show that AR-42 is a potent HDACI in AML, increasing *miR-29b* levels and leading to downregulation of known *miR-29b* targets (that is, SP1, DNMT1, DNMT3A and DNMT3B). We then demonstrated that the sequential administration of AR-42 followed by decitabine resulted in a stronger anti-leukemic activity *in vitro* and *in vivo* than decitabine followed by AR-42 or either drug alone. These preclinical results with AR-42 priming before decitabine administration represent a promising, novel treatment approach and a paradigm shift with regard to the combination of epigenetic-targeting compounds in AML, where decitabine has been traditionally given before HDACIs.

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Keywords: acute myeloid leukemia; HDACI; AR-42; decitabine; *miR-29b*

INTRODUCTION

The prognosis for the majority of patients with acute myeloid leukemia (AML) receiving standard chemotherapy is poor, novel treatment strategies are needed.[1–3] Aberrant promoter DNA hypermethylation and histone deacetylation are reversible processes implicated in myeloid leukemogenesis, and each are targetable by hypomethylating agents (for example, decitabine) and histone deacetylase (HDAC) inhibitors (HDACIs), respectively.[4–7] Different from other hematologic malignancies such as cutaneous T-cell lymphoma, HDACIs as single agents have resulted in limited clinical activity in AML and combination therapy with hypomethylating agents has not consistently led to a significantly improved response.[4,8–11] Possible explanations for this may be related to differences in pharmacologic potency, rapid metabolism and/or off-target activity (for example, acetylation of non-histone substrates) of available HDACIs.[12–14]

Structural aspects of HDACIs that allow for access to the Zn^{2+} cation in the catalytic pocket of the HDAC enzyme are determinants of inhibitor activity. Researchers at The Ohio State University (OSU) synthesized a new class of HDACIs that are structurally optimized to improve access to the catalytic pocket. These new compounds have been shown to inhibit enzyme activity and cancer cell proliferation, even at nanomolar concentrations.[14] Among these new compounds, AR-42 has been proven to be active in non-Hodgkin lymphoma and multiple myeloma and is in Phase I clinical trials.[15–17] However, AR-42 activity in AML has not yet been investigated.

Altered expression of microRNAs (miRs), small non-coding RNA molecules, has been shown to contribute to the pathogenesis of various human cancers, including AML.[18,19] *miR-29b*, that negatively modulates the expression of genes encoding the transcriptional activator SP1 and DNA methyltransferases (DNMT1, DNMT3A and DNMT3B), is downregulated in AML.[20–22] We and others showed that lower pretreatment levels of *miR-29b* may be associated with worse prognosis[18] and inferior response to the hypomethylating agent decitabine in older (age ⩾60 years) AML patients.[7]

Expression of *miR-29b* is partly regulated by an SP1/NFκB transcriptional complex, which binds to a *miR-29b* enhancer

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region, recruits HDACs and decreases *miR-29b* expression.[22] Thus, here we hypothesized that the inhibition of HDAC activity could disrupt the binding of this complex, increase *miR-29b* expression and in turn induce an improved response to decitabine.

MATERIALS AND METHODS

Cell lines, AML patient samples and cell culture

Kasumi-1 (CRL-2724) and NB4 (ACC 207) cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and German Collection of Microorganisms and Cell Culture (DSMZ, Braunschweig, Germany), respectively. Murine FDC-P1 cells were purchased from ATCC and retrovirally transduced to express the oncogenic D816V *KIT* mutation (FDC-P1-*KIT*^{mut}) as previously described.[22] Kasumi-1, NB4 and FDC-P1-*KIT*^{mut} cells were cultured as previously described.[20–22] These cell lines were chosen because of a low baseline expression of *miR-29b*.[20,22] Primary AML blasts from apheresis samples collected from 10 patients with *de novo* disease were obtained from the OSU Leukemia Tissue Bank. Cytogenetic analysis was available for 9 of the 10 patients (Supplementary Table 1). Samples from the patients 1–2 and 6–10 were used to conduct HDAC inhibition studies, while the patient samples 3–10 were used for miR expression and patients 3–5 were used for gene and protein expression studies. All patients provided written informed consent according to the Declaration of Helsinki to store and use of their tissue for discovery studies according to the OSU institutional guidelines under protocols approved by the OSU Institutional Review Board. The patient samples were cultured as previously described.[22]

HDAC activity inhibition assay

Nuclear extracts of Kasumi-1 and NB4 cell lines and patient AML blasts were prepared using the Nuclear Extract Kit following the manufacturer's protocol (Active Motif, Carlsbad, CA, USA). The effects of 100 nM and 1 μM of AR-42 on HDAC activity were investigated using the HDAC Assay Kit according to the manufacturer's protocol (Upstate, Lake Placid, NY, USA). Doses of AR-42 were chosen based on previous studies in hematologic malignancies.[15,17]

Gene and miR expression

RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA) and quantified using the NanoDrop 2000 Spectrophotometer (Thermo Scientific, Walthan, MA, USA). For both primary miR and messenger RNA (mRNA) gene expression, RNA was reverse transcribed into complementary-DNA using SuperScript III First Strand Synthesis (Invitrogen) according to the manufacturer's recommendations. Real-Time PCR was performed using a 7900HT Fast Real-Time System (Applied Biosystems, Carlsbad, CA, USA). Primer pairs and probes used were human *DNMT1*, *DNMT3A*, *DNMT3B*, *SP1*, *18S* and *primary-miR-29b-1*, as well as mouse *Dnmt1*, *Dnmt3a*, *Dnmt3b*, *Sp1* and *18S* purchased from Applied Biosystems. Taqman Universal PCR master Mix was purchased from Applied Biosystems. The expression of human and murine *18S* were used as internal controls for both mRNA and miR expression.

Western blotting

Whole-cell lysates were run on SDS–PAGE Ready Gel Precast Gels (Bio-rad, Hercules, CA, USA) and transferred to nitrocellulose membrane as previously described.[22] Immunoblotting was performed with rabbit anti-acetylated Histone H3 (06-599, Upstate), rabbit anti-acetylated Histone H4 (06-866, Upstate), rabbit anti-DNMT3A (sc-20703, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-SP1 (sc-59, Santa Cruz), goat anti-β-actin (sc-1616, Santa Cruz), rabbit anti-DNMT1 (ab87656, Abcam, Cambridge, MA, USA), and mouse anti-DNMT3B (ab16304, Abcam).

Cell proliferation assay

Kasumi-1, NB4 and FDC-P1-*KIT*^{mut} cells were seeded in 96-well plates and were treated for 72 h with vehicle, AR-42 (0.3 μM) alone, AR-42 (0.3 μM) followed by decitabine (0.5 μM) after 24 h (AR-42 → decitabine), decitabine (0.5 μM) followed by AR-42 (0.3 μM) after 24 h (decitabine → AR-42) or decitabine (0.5 μM) alone. After 72 h, MTS reagent (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2H-tetrazolium, inner salt; Promega, Madison, WI, USA) was added to each well.

Patient primary blasts were seeded in 96-well plates and were treated for 48 h with vehicle, AR-42 (3, 10, 30, 100, 300 nM or 1 μM) or valproic acid ((VPA); 600 or 2400 μM). After 48 h, MTS reagent was added to each well. Plates were incubated according to the manufacturer's protocol. The absorbance at 495 nM was measured in a Multiskan Spectrum plate reader (Thermo Electron Corporation, Vantaa, Finland). After adjustment for background interference by accounting for wavelength variation secondary to media, data in triplicate from three independent experiments were normalized to the readings from untreated cells.

Leukemogenesis in NOD/SCID mice

Four to 6-week-old NOD/SCID mice (The Jackson Laboratory, Bar Harbor, ME, USA) were i.v. injected through a tail vein with 5×10^6 cells of FDC-P1 cells harboring D816V *KIT*^{mut}. After engraftment, cell-injected mice were treated with either vehicle alone, decitabine intraperitoneally (0.4 mg/kg/day for 4 days in weeks 1 and 3), AR-42 by oral gavage (75 mg/kg/day for 2 days in weeks 1 and 3), decitabine for 4 days followed by AR-42 for 2 days on weeks 1 and 3 (doses for both drugs the same as above) or AR-42 for 2 days followed by decitabine for 4 days for weeks 1 and 3 (doses for both drugs the same as above). Spleen samples were harvested from the treated mice and processed for RNA and cDNA. Real-Time PCR was performed on all samples with murine primer/probes used for all genes and *primary-miR-29b-1* as described above. These studies were performed in accordance with OSU institutional guidelines for animal care and under protocols approved by the OSU Institutional Animal Care and Use Committee.

Statistical methods

Data were represented as mean ± s.d. of at least three independent experiments and analyzed by the two-tailed Student's *t*-test. The means and s.d. were calculated and displayed in bar graphs as the height and the corresponding error bar, respectively. Mouse survival was calculated using the Kaplan–Meier method, and survival curves were compared by log-rank test. A $P<0.05$ was considered statistically significant.

RESULTS

AR-42 inhibits HDAC activity in AML

We first assessed the HDAC inhibitory activity of AR-42 on AML cells. Kasumi-1 and NB4 cells were treated with 100 nM and 1 μM AR-42, and HDAC enzymatic activity was measured after 24 h. HDAC activity was reduced 82% (± 1.8% s.d.; $P<0.01$) and 90% (± 0.4%; $P<0.01$) in Kasumi-1 cells and 85% (± 6.9%; $P<0.01$) and 90% (± 3.2%; $P<0.01$) in NB4 cells following exposure to 100 nM and 1 μM AR-42, respectively (Figure 1a). The doses of AR-42 were chosen based on previous studies in hematologic malignancies.[15,17] The degree of inhibition of HDAC activity caused by AR-42 treatment was comparable to the degree of inhibition achieved by treatment with the hydroxamate analog of AR-42, Trichostatin A (TSA), a known potent HDACI often used as a control for HDACI inhibition assays.[23] In Kasumi-1, concentrations of 100 nM of TSA reduced the HDAC activity by 83%, and 1 μM of TSA reduced the HDAC activity by 88%. In NB4, similar results were demonstrated (Figure 1a). The decrease in HDAC enzymatic activity in cells treated with AR-42 also led to an increase in histone acetylation. Concentrations of AR-42 as low as 30 nM induced histone H3 and H4 acetylation in both the Kasumi-1 and NB4 cell lines at 48 h (Figure 1b).

The HDAC inhibition activity of AR-42 was also demonstrated in AML patient blasts. In these primary cells, we observed a dose-dependent effect of HDAC activity inhibition following AR-42 treatment for 24 h (Figure 1c). Treatment with 1 μM AR-42 reduced the total HDAC activity by 78% (± 11%; $P<0.01$) compared with vehicle, similar to the degree of inhibition (76 ± 5.6%) observed with 1 μM TSA. H3 and H4 histone acetylation was also observed in primary blasts following AR-42 treatment at 300 nM and 1 μM concentrations (Figure 1d).

We also tested 600 and 2400 μM VPA,[24] a known HDACI,[24] as a control in both AML cell lines and additional primary patient samples ($n=5$) and showed that acetylation of H3 and H4 histone

increased in a similar dose-dependent fashion with both compounds (Supplementary Figure 1). Interestingly, similar to Stapnes *et al.*[24] we observed an anti-proliferative effect of the HDACIs, at higher concentrations (that is, >100 nM AR-42 and 2400 μM VPA), and a more heterogenous response at lower concentrations (that is, <100 nM AR-42 and 600 μM VPA; Supplementary Figure 2).

AR-42 upregulates miR-29b expression

We next treated Kasumi-1, NB4 and the murine FDC-P1-*Kit*^{mut} cell lines with AR-42 and determined the effect on *miR-29b* expression. These cell lines were chosen because of their low levels of endogenous *miR-29b*.[20,22] Compared with vehicle-treated control cells, *miR-29b* expression was found to be upregulated 4-fold (±0.93; $P<0.01$) in Kasumi-1 cells, 5-fold (±1.18; $P<0.05$) in NB4 cells and 14-fold (±1.63; $P<0.01$) in FDC-P1-*Kit*^{mut} cells (Figure 2a) after 24 h treatment with 1 μM of AR-42. These results were confirmed in leukemic blasts from eight primary AML patients. Treatment with 1 μM AR-42 increased *miR-29b* expression 6.5-fold (±2.4; $P<0.05$) at 24 h compared with vehicle-treated control blasts (Figure 2b). *miR-29b* upregulation was also observed with 2400 μM VPA both in Kasumi-1 and NB4 cell lines as well as in primary patient blasts. Although in the cell lines VPA-induced increases in *miR-29b* similar to that observed with AR-42 (Supplementary Figure 3), in primary blasts the VPA-induced

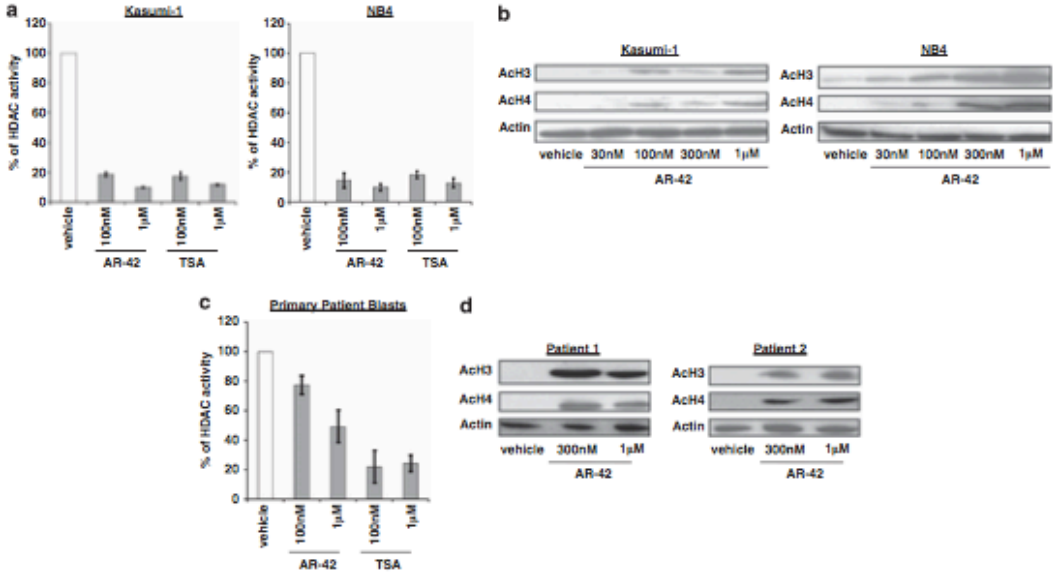

Figure 1. AR-42 treatment inhibits HDAC activity in AML. (**a**) HDAC activity in Kasumi-1 and NB4 cells at 24 h after treatment with vehicle, AR-42 or TSA. (**b**) Increased histone acetylation in Kasumi-1 and NB4 and cells 48 h after AR-42 treatment. (**c**) HDAC activity in primary AML patient blasts 24 h after treatment with AR-42 ($n=2$; patients no. 1 and no. 2). (**d**) Increased histone acetylation in primary patient blasts 48 h after AR-42 treatment (patients no. 1 and no. 2 as indicated).

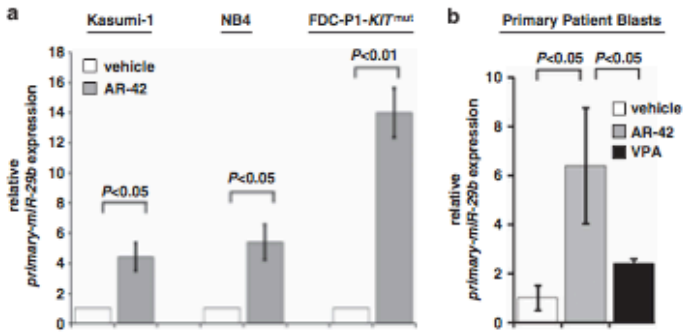

Figure 2. *miR-29b* expression increases following AR-42 treatment. (**a**) In Kasumi-1, NB4 and FDC-P1-*Kit*^{mut} cells, 24 h after treatment with 1 μM AR-42. (**b**) In eight primary AML patient samples (patients nos. 3–10) at 24 h after treatment with 1 μM AR-42 and 2400 μM VPA.

increase in *miR-29b* was 2.5-fold (±0.17) seemingly lower than that induced by AR-42 (6.5-fold (±2.4); Figure 2b).

AR-42 downregulates the *miR-29b* targets DNMT1, DNMT3A, DNMT3B and SP1

It is known that *miR-29b* downregulates *DNMT3A*, *DNMT3B* and *SP1* directly, and reduces expression of *DNMT1* indirectly by targeting its transcription factor SP1.[21] Thus, we assessed the RNA expression levels of *DNMT1*, *DNMT3A*, *DNMT3B* and *SP1* following AR-42 treatment. We compared the Kasumi-1 and NB4 cells treated with 1 μM AR-42 with vehicle-treated controls and observed, respectively, a reduction of *DNMT1* by 80% (±7.2%; $P<0.01$) and 96% (±1.1%; $P<0.01$), *DNMT3A* by 95% (±0.6%; $P<0.01$) and 94% (±0.5%; $P<0.01$), *DNMT3B* by 78% (±10.7%; $P<0.05$) and 90% (±3%; $P<0.05$) and *SP1* by 53% (±0.3%; $P<0.05$) and 82% (±2%; $P<0.05$) after 24 h (Figures 3a and b). Similar results were also obtained using the murine FDC-P1-*KIT*^{mut} cell line treated with 1 μM AR-42, which resulted in a reduction of *Dnmt1* by 63% (±0.5%; $P<0.05$), *Dnmt3a* by 40% (±2% $P<0.2$), *Dnmt3b* by 61% (±1.9%; $P<0.05$) and *Sp1* by 73% (±0.2%; $P<0.05$; Figure 3b). These results were also confirmed at the protein level and although *Dnmt3a* by RNA was not statistically significant, western blotting confirmed the downregulation. DNMT1, DNMT3A, DNMT3B and SP1 proteins were downregulated at 24 h following 1 μM AR-42 treatment in Kasumi-1, NB4 and FDC-P1-*KIT*^{mut} cell lines compared with vehicle-treated controls (Figure 3b). These findings were then validated in primary patient blasts ($n=3$). Twenty-four hours after treatment with 1 μM AR-42, mRNA levels were found to have a reduction of 81% (±5.8%; $P<0.01$) in *DNMT1*, 80% (±13%; $P<0.01$) in *DNMT3A*, 75% (±7.6%; $P<0.01$) in *DNMT3B* and 50% (±13%; $P<0.05$) in *SP1* when compared with vehicle-treated controls (Figure 3c). Likewise, the DNMT1, DNMT3A, DNMT3B and SP1 proteins were downregulated in all three patient samples following 1 μM AR-42 treatment compared with vehicle-treated controls (Figure 3d).

Increased anti-leukemic activity: AR-42 followed by decitabine

We have previously shown that AML patients with higher expression of *miR-29b* had better clinical response to decitabine.[7] Therefore, we hypothesized that an AR-42-induced increase in *miR-29b* expression might result in increased anti-leukemic activity of decitabine. We compared the anti-leukemic activity of AR-42 followed by decitabine with that of both AR-42 and decitabine as single agents, and decitabine followed by AR-42 in Kasumi-1, NB4 and FDC-P1-*KIT*^{mut} cells. The cells were treated for 72 h with vehicle, AR-42 (0.3 μM) alone, AR-42 (0.3 μM) followed by decitabine (0.5 μM) after 24 h (AR-42→decitabine), decitabine (0.5 μM) followed by AR-42 (0.3 μM) after 24 h (decitabine→AR-42) or decitabine (0.5 μM) alone. The lowest cell viability was observed in AR-42→decitabine group in all three cell lines (Figure 4a). Kasumi-1 cells treated with AR-42→decitabine were significantly less viable than those treated with decitabine alone (17% vs 91%; $P<0.01$), AR-42 alone (17% vs 40%; $P<0.01$), and decitabine→AR-42 (17% vs 34%; $P<0.01$; Figure 4a). Similar observations were made for NB4 cells (AR-42→decitabine vs decitabine alone: 59%

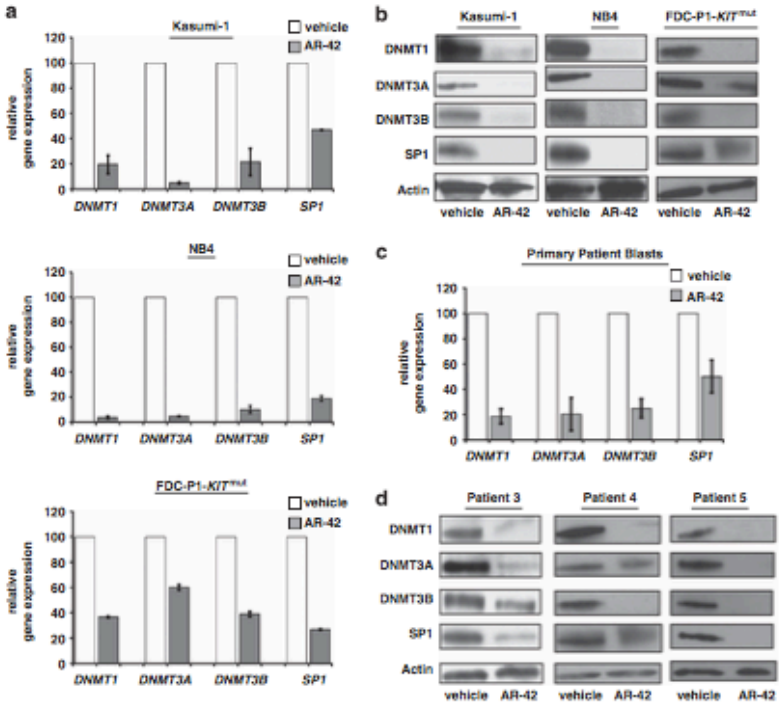

Figure 3. Expression of the *miR-29b* targets DNMT1, DNMT3A, DNMT3B and SP1 decreases following AR-42 treatment. (**a**, **b**) Kasumi-1, NB4 and FDC-P1-*KIT*^{mut} cells treated with 1 μM AR-42 for 24 h: DNMT1, DNMT3A, DNMT3B and SP1, decrease on both mRNA and protein level. (**c**, **d**) Primary patient blasts were treated with 1 μM AR-42 for 24 h with decrease of DNMT1, DNMT3A, DNMT3B and SP1 on both mRNA ($n=3$) and protein level (patients as indicated).

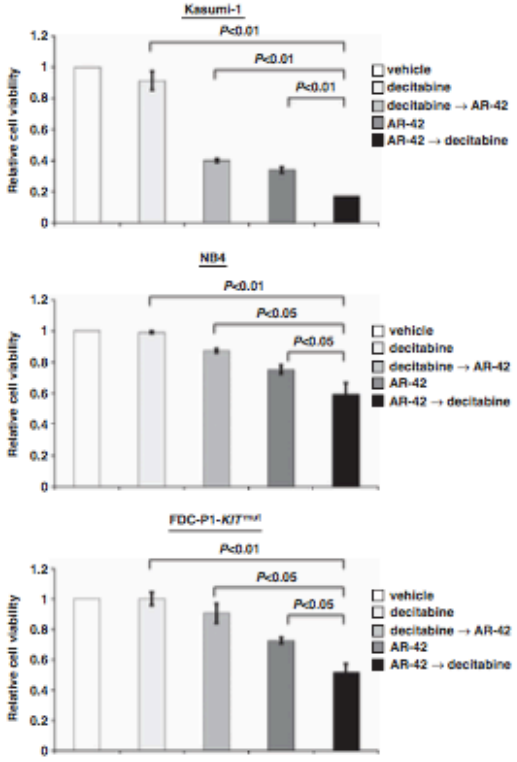

Figure 4. AR-42 followed by decitabine has the strongest activity on cell viability. Kasumi-1, NB4 and FDC-P1-*KIT*^{mut} cells treated with vehicle, decitabine 0.5 μM for 72 h, decitabine 0.5 μM for 72 h with AR-42 0.3 μM added at 24 h, AR-42 0.3 μM for 72 h, or AR-42 0.3 μM for 72 h with decitabine 0.5 μM added at 24 h. Cells treated with AR-42 followed by decitabine showed lowest cell viability.

vs 99% ($P<0.01$); AR-42→decitabine vs AR-42 alone: 59% vs 87% ($P<0.05$); and AR-42→decitabine vs decitabine→AR-42: 59% vs 75% ($P<0.05$)). We also found similar changes in the FDC-P1-*KIT*^{mut} cells (AR-42→decitabine vs decitabine alone: 52% vs 100% ($P<0.01$); AR-42→decitabine vs AR-42 alone: 52% vs 90% ($P<0.05$); and AR-42→decitabine vs decitabine→AR-42: 51% vs 90% ($P<0.05$)).

Next we validated our *in vitro* findings in an *in vivo* AML mouse model. NOD/SCID mice engrafted with FDC-P1-*KIT*^{mut} cells developed AML-like disease[22] and then were treated with either vehicle ($n=7$), decitabine alone at 0.4 mg/kg/day intraperitoneally for 4 days in weeks 1 and 3 ($n=7$), AR-42 alone at 75 mg/kg/day by oral gavage for 2 days in weeks 1 and 3 ($n=10$),[15] decitabine for 4 days followed by AR-42 for 2 days at aforementioned doses ($n=10$), or AR-42 for 2 days followed by decitabine for 4 days at aforementioned doses for both drugs ($n=17$). To evaluate whether AR-42 increased *miR-29b* expression and downregulated *miR-29b* targets *in vivo*, five mice from each group were euthanized 12 h after two doses of AR-42 and compared with vehicle-treated control. RNA was extracted from spleen cells and *miR-29b* as well as *Dnmt1*, *Dnmt3a*, *Dnmt3b* and *Sp1* expression was analyzed. We found that *miR-29b* expression was upregulated 20-fold (±5.4) in the mice treated with AR-42 as compared with those treated with vehicle ($P<0.01$; Figure 5a). *Dnmt1* (72.7%, ±7.4%; $P<0.01$), *Dnmt3a* (65.6%, ±8.8%; $P<0.05$), *Dnmt3b* (93.5, ±0.8%; $P<0.01$) and *Sp1* (74.6%, ±6%; $P<0.05$) were found to be reduced in the AR-42-treated mice compared with vehicle-treated controls (Figure 5b). We also observed a significantly longer survival for mice treated with AR-42→decitabine, compared with vehicle treatment ($P<0.001$), decitabine alone ($P<0.001$), AR-42 alone ($P<0.01$) or decitabine→AR-42 ($P<0.001$; Figure 5a). Indeed by day 60, all of the mice died of disease with the exception of those treated with AR-42→decitabine, among which, 59% (10 of 17) were still alive at this time point (Figure 5c).

DISCUSSION

We previously showed that *miR-29b* has tumor suppressor activity in AML by targeting a variety of genes, including regulators of DNA methylation.[20–22] Furthermore, we reported that AML patients with higher pretreatment levels of *miR-29b* had a better response to decitabine therapy.[7] Thus, here we sought to demonstrate an increase in anti-leukemic activity of decitabine by first increasing *miR-29b* expression. As *miR-29b* is repressed by an SP1/NFκB/HDAC silencing complex in AML,[22] we hypothesized that treatment with a HDACI would increase the expression of this miR in AML cells. Indeed, Sampath *et al.*[25] recently found an upregulation of *miR-29b* following HDAC inhibition in chronic lymphocytic leukemia, suggesting that the expression of this miR is targetable pharmacologically and that epigenetic deregulation of *miR-29b* may also occur in malignancies other than AML.

To prove that HDAC inhibition resulted in *miR-29b* upregulation in AML, we elected to test AR-42, a novel HDACI developed at our institution. The advantage of using AR-42 was its significantly higher HDAC inhibitory potency relative to other HDACI's *in vitro* and *in vivo* cancer models.[26] Furthermore, AR-42 has also been well-tolerated in phase I clinical trials in patients with multiple myeloma and non-Hodgkin lymphoma, with thrombocytopenia and fatigue as the most common adverse events. These side effects are similar to those observed with other HDACIs, which have been also associated with neurological, gastrointestinal and cardiac adverse effects.[27,28] We first demonstrated that AR-42 inhibits HDAC enzyme activity and induced histone acetylation in AML cells at concentrations in the nanomolar to micromolar ranges. We also tested the anti-leukemia activity of the compound. Patient samples seemingly exhibited different susceptibility to AR-42, similar to results previously reported by Stapnes *et al.*[24] for VPA despite the different assays and the time point of analysis utilized in the two studies. AML patients represent a very heterogeneous population with different susceptibilities to HDACIs. Studies of cytogenetic, molecular and *in vitro* growth characteristics of primary blasts associated with HDACI response may provide insight into how to best select AML patients that are more likely to be responsive to this class of compounds.[24,29]

Next we showed that *miR-29b* expression increased upon treatment with AR-42 in AML cell lines and AML patient primary blasts. Upregulation of *miR-29b* induced by AR-42 treatment also resulted in a concurrent downregulation of the known *miR-29b* targets DNMT1, DNMT3A and DNMT3B and SP1. Others have also reported on the effect of HDAC inhibition on DNA methyltransferase expression.[30,31] Xiong *et al.*[30] showed that HDAC inhibition decreased *DNMT3B* mRNA stability in human endometrial cells, and Zhou *et al.*[31] showed that HDAC inhibition decreased DNMT1 expression in breast cancer cells, but neither of these reports included a clear proposed mechanism for their results.

Both DNA hypermethylation and histone acetylation have been shown to contribute to tumor suppressor gene silencing in

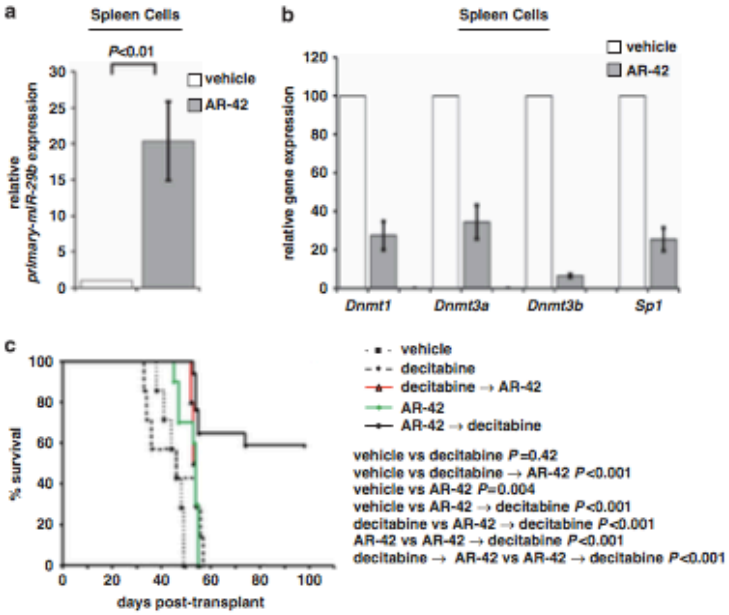

Figure 5. Priming with AR-42 upregulates *miR-29b* and increases survival in murine models. (**a**) *miR-29b* levels were upregulated 20-fold in AR-42 treatment versus vehicle treatment group. (**b**) Dnmt1, Dnmt3a, Dnmt3b and Sp1 were downregulated in AR-42 versus vehicle treatment group. (**c**) Overall survival. FDC-P1-*KIT*^{mut} cells injected into NOD/SCID mice showed 10 of 17 mice with survival at 60 days in AR-42 followed by decitabine group compared with no survival in mouse groups with decitabine treatment alone, decitabine followed by AR-42, or AR-42 treatment alone.

AML. The combination of HDACIs with decitabine has resulted in synergistic effects on apoptosis, DNA hypomethylation and gene re-expression *in vitro*[32] and this combination therapy has been performed to achieve synergism. However, it has been recommended that HDACIs be administered before or concurrently with decitabine because of their ability to induce expression of *p21* and other inhibitors of the cell cycle.[33] Interfering with the cell cycle may decrease the activity of decitabine, as it is necessary for the active metabolite decitabine-trisphosphate to be incorporated into the nascent DNA in order to inhibit DNMT activity, induce DNA hypomethylation and gene re-expression.[34] To date, treatment with HDACIs given following or concurrently with decitabine has demonstrated anti-leukemic activity in AML, but with a relatively low range of clinical response.[4,8–11] In a phase I clinical study conducted by our group, we also did not observe additional clinical benefit following the addition of VPA to decitabine (concurrent) although dose escalation of VPA was limited because of the development of neurological toxicity.[11] In a phase I clinical trial of the HDACI vorinostat, administered either concomitantly or following decitabine in patients with AML or myelodysplastic syndrome, the overall response rate was 41% with the concomitant schedule and 21% with the sequential schedule.[35] Thus, the synergism of post- or concurrent administration of HDACIs with DNA hypomethylating agents demonstrated in preclinical models, could not be fully recapitulated *in vivo*.

In a phase II clinical trial by our group, we reported the results of low dose (20 mg/m^2/day × 10 days) decitabine as a single agent in untreated elderly AML.[7] We showed relatively low toxicity, a complete remission rate of 47%, an overall response rate of 64%, and a median overall survival duration of approximately 1 year. The median pretreatment *miR-29b* expression level in responders was 2.3 times higher (that is, more than double) than the median baseline *miR-29b* levels in non-responders, suggesting relevance for this miR as a predictive marker for response to decitabine treatment. In contrast to our data, Yang *et al.*[36] reported a lack of an association of *miR-29b* levels with clinical response in patients treated with the azanucleoside 5-azacitidine. However, only 10% of 5-azacitidine is reduced to decitabine and incorporated into DNA for hypomethylating activity, while the remaining 90% is incorporated into the RNA. Furthermore, we recently showed that 5-azacitidine limits its own conversion to decitabine by downregulating ribonucleotide reductase.[37] Therefore, these two compounds, although both members of the same class of drugs (azanucleosides), may impact leukemia through different mechanisms, and high *miR-29b* levels may improve response to decitabine but not to 5-azacitidine in AML.

We demonstrated here that an increase of *miR-29b* expression by AR-42 improved the anti-leukemia activity of decitabine. We showed that sequential treatment of AR-42 followed by decitabine decreased cell viability significantly more than each agent alone or the previously recommended sequence of decitabine followed by AR-42. This was validated in a murine AML model where mice treated with AR-42 followed by decitabine survived significantly longer than those treated with single agent therapy or decitabine followed by AR-42. One possible explanation for the better activity of AR-42 followed by decitabine may relate to *miR-29b* targeting DNMT expression. With decreased amounts of DNMT enzyme present, decitabine may more effectively inhibit the activity of the remaining DNMTs resulting in improved treatment response.

Indeed, we previously reported that patients with *DNMT3A* mutations had improved response to decitabine.[38] Thus, it is possible that a clinical benefit from treatment with decitabine may be derived for AML patients from low DNMT3A activity, either because of loss-of-function mutations or because of low gene expression. Another possibility may be that the lower levels of SP1 induced by increased *miR-29b* expression result in decreased transcription of genes known to contribute to AML leukemogenesis such as mutated and/or upregulated receptor tyrosine kinases (that is, FLT3 and KIT) as we have previously demonstrated.[22,39]

Collectively our data support the notion that AR-42 is a potent HDACI that is able to increase *miR-29b* expression and improve clinical response to decitabine in *in vivo* preclinical models. Based on these preclinical findings, clinical trials utilizing AR-42 as a priming agent for decitabine treatment in patients with AML are under development.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

AM, JS, RS, AD, CW, PH, XH, ST and SS performed experiments. AM, HW, XH, KKC, DP, CSC, RG, SS and GM designed experiments and analyzed data. AM, AW, LJL, SJ, KM, CDB, SS and GM wrote the manuscript. All the authors approved the manuscript. AW, RBK, RG, WB, MC, JCB and GM were involved directly or indirectly in care of patients or sample procurement.

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Cancer Therapy: Preclinical

Clinical Cancer Research

Targeted Delivery of *microRNA-29b* by Transferrin-Conjugated Anionic Lipopolyplex Nanoparticles: A Novel Therapeutic Strategy in Acute Myeloid Leukemia

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Abstract

Purpose: *miR-29b* directly or indirectly targets genes involved in acute myeloid leukemia (AML), namely, *DNMTs, CDK6, SP1, KIT,* and *FLT3*. Higher *miR-29b* pretreatment expression is associated with improved response to decitabine and better outcome in AML. Thus, designing a strategy to increase *miR-29b* levels in AML blasts may be of therapeutic value. However, free synthetic miRs are easily degraded in bio-fluids and have limited cellular uptake. To overcome these limitations, we developed a novel transferrin-conjugated nanoparticle delivery system for synthetic *miR-29b* (Tf-NP-*miR-29b*).

Experimental Design: Delivery efficiency was investigated by flow cytometry, confocal microscopy, and quantitative PCR. The expression of *miR-29b* targets was measured by immunoblotting. The antileukemic activity of Tf-NP-*miR-29b* was evaluated by measuring cell proliferation and colony formation ability and in a leukemia mouse model.

Results: Tf-NP-*miR-29b* treatment resulted in more than 200-fold increase of mature *miR-29b* compared with free *miR-29b* and was approximately twice as efficient as treatment with non-transferrin–conjugated NP-*miR-29b*. Tf-NP-*miR-29b* treatment significantly downregulated *DNMTs, CDK6, SP1, KIT,* and *FLT3* and decreased AML cell growth by 30% to 50% and impaired colony formation by approximately 50%. Mice engrafted with AML cells and then treated with Tf-NP-*miR-29b* had significantly longer survival compared with Tf-NP-scramble ($P = 0.015$) or free *miR-29b* ($P = 0.003$). Furthermore, priming AML cell with Tf-NP-*miR-29b* before treatment with decitabine resulted in marked decrease in cell viability *in vitro* and showed improved antileukemic activity compared with decitabine alone ($P = 0.001$) *in vivo*.

Conclusions: Tf-NP effectively delivered functional *miR-29b*, resulting in target downregulation and antileukemic activity and warrants further investigation as a novel therapeutic approach in AML. *Clin Cancer Res; 19(9); 2355–67. ©2013 AACR.*

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Introduction

Acute myeloid leukemia (AML) is one of the most common leukemias and is characterized by a differentiation arrest and an uncontrolled proliferation of malignant blasts. Despite advances in our understanding of disease mechanisms, the outcome of most patients with AML remains poor (1–3). Thus, novel therapeutic strategies are needed.

MicroRNAs (miR) are short noncoding RNAs that regulate the expression of their target mRNA-encoded proteins. The aberrant expression of some miRs has been shown to be involved in AML leukemogenesis and to have prognostic significance (4–7). *miR-29b* has been shown to be downregulated in AML (8). This miR directly or indirectly targets a panel of genes that, when deregulated, contribute to myeloid leukemogenesis. These genes are involved in DNA

Translational Relevance

Patients with AML frequently have a poor outcome. The microRNA *miR-29b* has been shown to have tumor suppressor activity by targeting genes involved in myeloid leukemogenesis and to be associated with better outcome in patients with AML. Thus, increasing *miR-29b* in AML blasts may be beneficial. Here, we developed a targeted, nanoparticle-based system to deliver microRNAs to AML blasts. Following *miR-29b*-nanoparticle treatment, we showed an efficient increase of mature and functional intracellular *miR-29b* levels. The treatment downregulated the *miR-29b* targets DNMTs, CDK6, SP1, and the receptor tyrosine kinases FLT3 and KIT that are frequently mutated in AML. This resulted in decreased leukemia growth and improved survival in an AML-mouse model. Furthermore, we showed that pretreatment with *miR-29b* nanoparticles improved the antileukemic activity of decitabine, a hypomethylating agent often administered in elderly patients. These promising results warrant further development of nanoparticle-based *miR-29b* treatment as a novel approach in AML.

methylation (i.e., *DNMT1*, *DNMT3A*, and *DNMT3B*), cell-cycle progression (i.e., *CDK6*), and apoptosis (i.e., *MCL1*; refs. 8, 9). Furthermore, we recently showed that increasing *miR-29b* levels resulted in decreased expression of the receptor tyrosine kinases (RTK) FLT3 and KIT, which are frequently mutated and aberrantly activated in AML, via disruption of a NF-κB/SP1 transactivating complex, by targeting the transcription factor SP1 (7, 10–13). Moreover, low expression levels of *miR-29b* have been associated with worse outcome in patients with AML (7, 14). Thus, increasing *miR-29b* levels in AML blasts may represent a promising novel treatment strategy for patients with AML with otherwise aberrantly low expression of this miR.

However, synthetic miRs are easily degraded in biofluids and have limited cellular uptake, rendering the clinical development of miR-based therapies relatively difficult. To overcome these limitations, we developed a nonviral system for the delivery of synthetic *miR-29b* mimic molecules using transferrin-conjugated, novel, anionic lipopolyplex nanoparticles, and tested it *in vitro* and *in vivo*.

Materials and Methods

Preparation of nanoparticles

The synthetic *miR-29b*, miR-scramble control (scramble miR molecules), and scramble control labeled with the fluorescent dye FAM (FAM-miR) were purchased from Ambion. The lipid components were 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dimyristoyl-sn-glycerol, methoxypolyethylene glycol (MW~2000; DMG-PEG; Avanti Polar Lipids), and linoleic acid (Sigma-Aldrich). The molar ratio of DOPE/linoleic acid/DMG-PEG was 50/48/2.

We prepared the transferrin-conjugated nanoparticle as shown in Fig. 1. Mimic miRs were mixed with polyethylenimine (MW, ~2000; Sigma-Aldrich) at room temperature (Step 1). The N/P ratio (the ratio of moles of the amine group of PEI to those of the phosphate groups of DNA) was 10:1. To form empty nanoparticles, lipid ethanol solvent

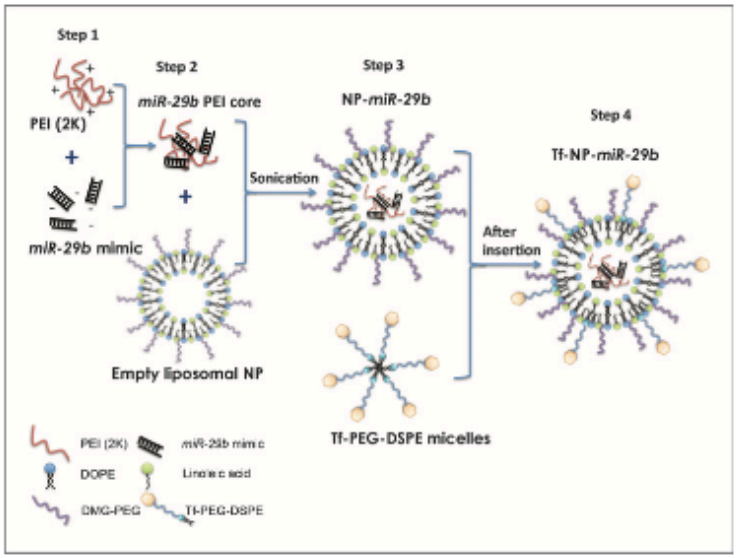

Figure 1. Preparation of miR-loaded transferrin-conjugated nanoparticles (Tf-NP-miR). The preparation of Tf-NP-miR is schematically illustrated. Step 1: negatively charged miR molecules were mixed with positively charged polyethylenimine (PEI) to form a miR-PEI core structure. Step 2: empty nanoparticles were formed by injection of a lipid ethanol solvent into 20 mmol/L HEPES buffer. Step 3: the miR-PEI were mixed with the empty nanoparticles and sonicated to load the miR-PEI core into the nanoparticles. Step 4: NP-miR were modified to incorporate Tf-PEG-DSPE micelles to form the Tf-NP-miRs.

was injected into 20 mmol/L HEPES buffer, pH = 7.4 (Step 2). The percentage of ethanol was less than 5%. The previously prepared empty nanoparticles were then added (Step 3). The mass ratio of lipid to miR was 10:1. Using vortexing and sonication, lipopolyplex nanoparticle-containing the mimic miRs were produced. Finally, a post-insertion method was adopted to incorporate transferrin ligand onto the miR-loaded nanoparticles, as previously described (Step 4; ref. 15).

Characterization of nanoparticles

The size of the nanoparticles was analyzed on a NICOMP Particle Sizer Model 370 (Particle Sizing Systems). The ζ-potential was determined on a ZetaPALS, Zeta Potential Analyzer (Brookhaven Instruments Corp.).

The miR entrapment efficiency was assessed by gel electrophoresis. 0.5% SDS was used to dissolve the nanoparticles. The amount of miR in solution was compared before and after dissolution by SDS by agarose gel electrophoresis of RNA using empty nanoparticles and free miR as controls.

Cell lines, patient samples, and cell culture

Kasumi-1, MV4-11, THP-1, KG1, and KG1a cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA); OCI-AML3 cells were obtained from DSMZ (Braunschweig, Germany). Cell lines were not authenticated by authors after purchase. AML patient blasts were obtained from the Ohio State University (OSU) Leukemia Tissue Bank. All patients provided written informed consent in accordance with the Declaration of Helsinki under an Institutional Review Board-approved protocol for discovery studies according to OSU institutional guidelines for tissue collection and the use of the tissue in research.

Delivery studies

Kasumi-1, OCI-AML3, and MV4-11 cells at a concentration of 3×10^5/mL were treated with *miR-29b*-loaded nanoparticles and controls. For Kasumi-1, OCI-AML3 cells together with the patients' blasts to a final concentration of 100 nmol/L of *miR-29b* mimic molecules were used in all experiments. Because the high treatment sensitivity of MV4-11 cells, a final concentration of 30 nmol/L was used for this cell line in all experiments. After 24 and 48 hours, cells were collected and analyzed by quantitative reverse transcription-PCR (qRT-PCR) and Western blotting as described later.

Laser-scanning confocal microscopy

Cells were incubated with Tf-NP-FAM-miR or NP-FAM-miR at a final concentration of 100 nmol/L for 4 hours at 37°C and washed twice with PBS followed by fixation with 4% paraformaldehyde. Nuclei were stained with 5 μg/μL of Hoechst (Biostatus Limited) for 5 minutes at room temperature. The cells were attached to a poly-D-lysine–coated cover glass slide (Sigma-Aldrich). Green fluorescence of FAM-miR and blue fluorescence of Hoechst were acquired by confocal microscopy (Olympus FV1000).

Flow cytometry

The transferrin receptor cell surface expression was detected using antibodies from BD Bioscience. Flow cytometry was carried out on a FACSCalibur (BD Biosciences). A minimum of 10,000 events were collected and analyzed using Flow Jo software (Tree Star Inc).

RNA extraction and qRT-PCR

Total RNA extraction was carried out as previously described by using Trizol (Invitrogen; ref. 7). Total RNA from leukemic mice was isolated using the MirVana miRNA Isolation kit (Ambion) according to the manufacturer's instructions. Then, cDNA was synthesized using Superscript III (Invitrogen) or the TaqMan miR Reverse Transcription kit (Applied Biosystems) for *miR-29b*, *miR-140*, and *U44*. In addition, qRT-PCR was carried out with TaqMan gene expression assays (Applied Biosystems) following the manufacturer's protocols. Expression of *pri-miR-29b-1*, *pri-miR-29b-2*, *DNMT1*, *DNMT3A*, *DNMT3B*, *SP1*, *CDK6*, *FLT3*, and *KIT* was normalized to *18S*. *miR-29b* and *miR-140* expression was normalized to *U44*. The comparative cycle threshold (C_t) method was used for the relative quantification of gene expression as previously described (8).

Western blot analysis

Western blot analysis was conducted as described previously (7, 16). Anti-DNMT1 (ab87656) and -DNMT3B (52A1018) antibodies were from Abcam. Anti-KIT (SC-17806) and -DNMT3A (SC-20703) antibodies were from Santa Cruz Biotechnology. Anti-SP1 (CS200631) antibodies were from Millipore. Anti-CDK6 (DCS83) and -FLT3 (8F2) antibodies were from Cell Signaling Technology. Equivalent loading was confirmed by Actin (SC-1616; Santa Cruz). The intensity of the resulting bands was measured by ImageJ 1.45s (http://imagej.nih.gov/ij). The intensity ratio of each band respective to the corresponding actin intensity was used for relative quantification and is displayed in the figures.

Growth analysis

OCI-AML3, Kasumi-1 and MV4-11 cells (3×10^5/mL), and AML patient blasts were treated as described earlier. For cell lines, cells were counted at 24-hour intervals using a ViCell counter (Beckman Coulter). Growth curves were generated by MATLAB 7.9.0.529 (R2009b; The Mathworks, Inc.). For patient blasts, after 96-hour incubation, cell viability was measured by MTS assay. CellTiter 96 AQueous One Solution Reagent (Promega) was used according to the manufacturer's instructions. Absorbance was read in a microplate reader Germini XS (Molecular devices). Each sample was run in triplicates.

Decitabine treatment and cytotoxicity studies by MTS assay

Kasumi-1, MV4-11, and OCI-AML3 cells were pretreated with Tf-NP-*miR-29b*, Tf-NP-scramble, or mock (buffer only) 48 hours before decitabine exposure. The

decitabine doses were based on our previous studies (17). After 48 hours incubation, cell viability was measured as described earlier.

Colony formation assays

Methylcellulose colony formation assays were carried out as previously described (18) and counted after 14 days.

Nanoparticle toxicity profiling

Immunocompetent B6.SJL-*Ptprc*^{a}*Pepc*^{b} mice (BoyJ; The Jackson Laboratory) were used for *in vivo* nanoparticles toxicity studies. In a first group, 7-week-old male mice were injected with saline, empty nanoparticles (15 mg/kg/d of lipids), or Tf-NP-*miR-29b* (1.5 mg/kg/d of miR). Blood was collected 24 hours after the injection. The serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), γ-glutamic transferase (GGT), blood urea nitrogen (BUN), and creatinine were assessed by the Clinical Pathology Services at OSU. A second group of mice were treated with either saline or Tf-NP (1.5 mg/kg/d of miR) with 3 doses, every other day. During the treatment, body weight was monitored every other day and for 1 additional week after the treatment. Blood counts were assessed weekly.

In vivo studies

To test the antileukemic activity of Tf-NP-*miR-29b*, we used a leukemic nonobese diabetic/severe combined immunodeficient mice (NOD/SCID-gamma; NSG) mouse model. Six-week-old male NSG mice (The Jackson Laboratory) were intravenously injected through a tail vein with MV4-11 cells (0.3×10^6) as described previously (19). The treatment started 10 days after the engraftment. In the first trial, mice were treated with *miR-29b* mimic ($n = 3$), Tf-NP-scramble ($n = 6$), and Tf-NP-*miR-29b* ($n = 6$; 1 mg/kg/d of miR molecule intravenously on Monday, Wednesday, and Friday for 2 weeks). Mice survival was monitored and recorded. Spleens from the same mice were weighed. In the second trial, mice were treated with saline ($n = 5$), Tf-NP-scramble alone ($n = 7$; 1.5 mg/kg/d miR intravenously), Tf-NP-*miR-29b* alone ($n = 7$), decitabine alone ($n = 7$; 0.4 mg/kg/d, intraperitoneally as previously described; ref. 20), Tf-NP-scramble followed by (→) decitabine, and Tf-NP-*miR-29b*→decitabine as depicted in Fig. 6B. At day 24, blood was collected for gene expression analysis. These studies were carried out in accordance with the OSU institutional guidelines for animal care and under protocols approved by the OSU Institutional Animal Care and Use Committee.

Statistical analysis

Data were represented as mean ± SD of at least 3 independent experiments and analyzed by the 2-tailed Student *t* test. The mean and SD were calculated and displayed in bar graphs as the height and the corresponding error bar, respectively. Mouse survival was calculated using the Kaplan–Meier method, and survival curves were compared by log-rank test. A $P < 0.05$ was considered statistically significant.

Results

Preparation and characterization of the nanoparticle delivery system

To provide a nonviral delivery system for miRs to AML cells, NP-miR (NP-*miR-29b* and NP-scramble) were synthesized. Consistent with a previous report in AML patient blasts (21), a high transferrin receptor surface expression was observed in Kasumi-1, OCI-AML3, and MV4-11 cells (Fig. 2A) and in AML patient blasts (Supplementary Fig. S1). Thus, to facilitate an efficient, targeted delivery, we conjugated the nanoparticles with transferrin (Tf-NP).

Particle size and ζ-potential values are presented in Supplementary Table S1. The average size and ζ-potential of empty nanoparticles were 129.6 ± 1.0 nm (±SD) and −9.8 ± 1.5 mV (±SD) and NP-miR were 137.6 ± 1.0 nm and 22.5 ± 1.4 mV, respectively. After the transferrin conjugation, the size of the miR-loaded Tf-NP was increased to 147.3 ± 4.7 nm and the ζ-potential was 5.8 ± 1.9 mV. The achieved size and charge of the nanoparticles has been previously shown to be optimal for a long-lasting *in vivo* circulation time (22, 23). The mimic *miR-29b* entrapment efficiency of nanoparticles was evaluated by agarose gel electrophoresis. Analysis of the Tf-NP encapsulating miR before the SDS treatment showed no visible band, whereas a clear band comparable with the size and intensity of free miR was observed after dissolving the Tf-NP and releasing the entrapped miR molecules, thereby supporting a high miR entrapment efficiency (Supplementary Fig. S2).

Intracellular uptake of Tf-NP-*miR-29b*

To assess the efficiency of cellular uptake of the miR molecules, we treated 3 AML cell lines with relatively low endogenous *miR-29b* expression (i.e., Kasumi-1, OCI-AML3, and MV4-11; Supplementary Fig. S3) with free FAM-labeled miR (FAM-miR), non–transferrin-conjugated FAM-miR-loaded nanoparticles (NP-FAM-miR), or transferrin-conjugated FAM-miR-loaded nanoparticles (Tf-NP-FAM-miR). Four hours after the treatment, the FAM-label fluorescence was measured by flow cytometry. The mean fluorescence intensity (MFI) levels for Tf-NP–treated Kasumi-1, OCI-AML3, and MV4-11 were, 2.5-, 7.4-, and 4.7-fold higher than the non-transferrin–conjugated nanoparticle-treated cells, whereas free FAM-labeled-miR was barely detectable in the cells (Fig. 2B). This indicated an enhancement of miR uptake using Tf-NP. The qualitative intracellular FAM-miR uptake by AML cells following Tf-NP treatment was confirmed by confocal microscopy that showed an accumulation of FAM-miR mostly in the cytoplasm (Fig. 2C).

Intracellular increase of mature *miR-29b*

The delivery efficiency of the Tf-NP was tested by measuring intracellular levels of mature *miR-29b* (Fig. 3A). Treatment with NP-*miR-29b* and Tf-NP-*miR-29b*, respectively, increased levels of mature *miR-29b* approximately 240- versus 420-fold ($P = 0.009$) in Kasumi-1, 130- versus 240-

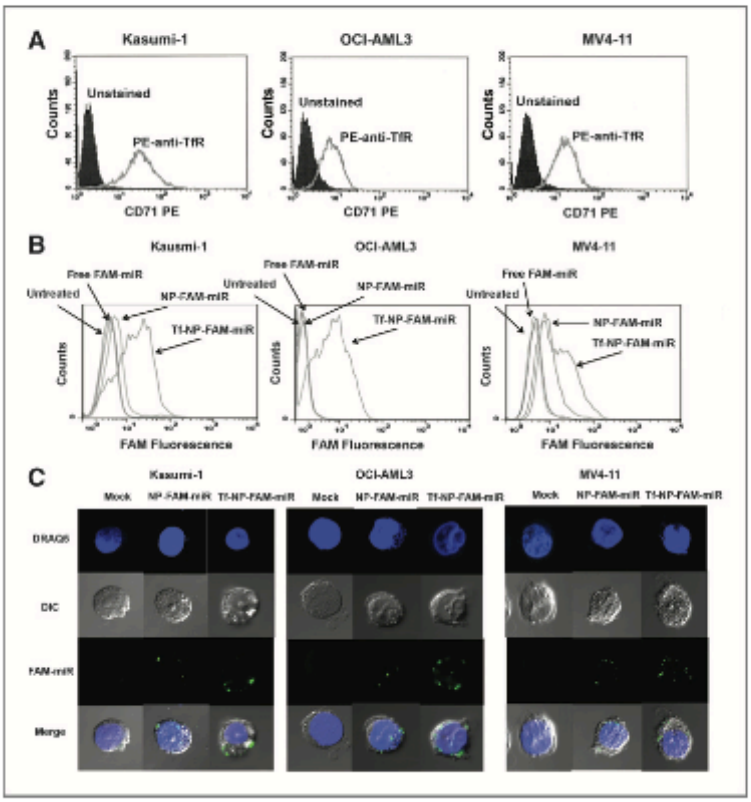

Figure 2. Transferrin targeting enhanced cellular uptake of nanoparticles. A, surface transferrin receptor (CD71) expression on Kasumi-1, OCI-AML3, and MV4-11 cells (unstained cells are shown as control). B, FAM-labeled miR uptake of Kasumi-1, OCI-AML3 and MV4-11 cells. C, confocal microscopy study of Kasumi-1, OCI-AML3, and MV4-11 cells treated with Tf-NP-FAM-miR, NP-FAM-miR, or mock; FAM-labeled miR: green; nucleus: blue.

fold ($P = 0.008$) in OCI-AML3, and 150- versus 220-fold ($P = 0.01$) in MV4-11 compared with mock treatment. Thus, Tf-NP were approximately 2 times more efficient than unconjugated nanoparticles in increasing the *miR-29b* levels. These results also indicated an efficient processing of the delivered *miR-29b* mimic molecules into mature *miR-29b*. No significant change of the expression of an unrelated miR, that is, *miR-140*, was observed (Fig. 3B), thereby supporting the specificity of our delivery system and the lack of interference with the expression of other endogenous miRs in the targeted cells.

Downregulation of *miR-29b* target genes by Tf-NP-*miR-29b* in AML cells

Next, we tested the *miR-29b* targeting activity. We previously reported that *miR-29b* directly downregulates the DNA methyltransferases DNMT3A and DNMT3B and indirectly downregulates DNMT1 by targeting the transcription factor *SP1* that drives *DNMT1* expression (9). Furthermore, *miR-29b* has been shown to target the cell-cycle regulator *CDK6* (8). Indeed, we observed a marked downregulation of DNMT1 by 18.5-, 2.5-, and 5.1-fold, DNMT3A by 4.8-, 15.7-, and 3.4-fold, DNMT3B by 3.6-, 3.5-, and 3.4-fold, SP1 by 4.5-, 3.9-, and 3.3-fold, and CDK6 by 3.9-, 3.5-, and 9.6-fold respectively in Kasumi-1, OCI-AML3, and MV4-11 cells following the treatment with our *miR-29b*-loaded Tf-NP compared with scramble-loaded Tf-NP (Fig. 3C). Thus, the delivered *miR-29b* fulfilled the expected function of the endogenous miR in AML cells.

We recently showed that *miR-29b* also indirectly targets the expression of the RTKs FLT3 and KIT in AML (7, 10). Aberrant activation by activating mutations and/or overexpression of these 2 RTKs is frequently found in AML (11–13). The downregulation of these RTKs following *miR-29b* increase is likely mediated by the disruption of a transactivation complex composed of SP1 and NF-κB, by targeting *SP1* (7, 10). Because we observed a significant downregulation of SP1 on Tf-NP-*miR-29b* treatment (Fig. 3C), we analyzed the FLT3 and KIT expression in Tf-NP-*miR-29b*-treated cells. We observed a downregulation of FLT3 by 3.3-, 2.8-, and 1.9-fold, respectively, as well as a downregulation of KIT by 7.8- 2.5-, and 1.4-fold, respectively, in Kasumi-1, OCI-AML3, and MV4-11 cells (Fig. 3D).

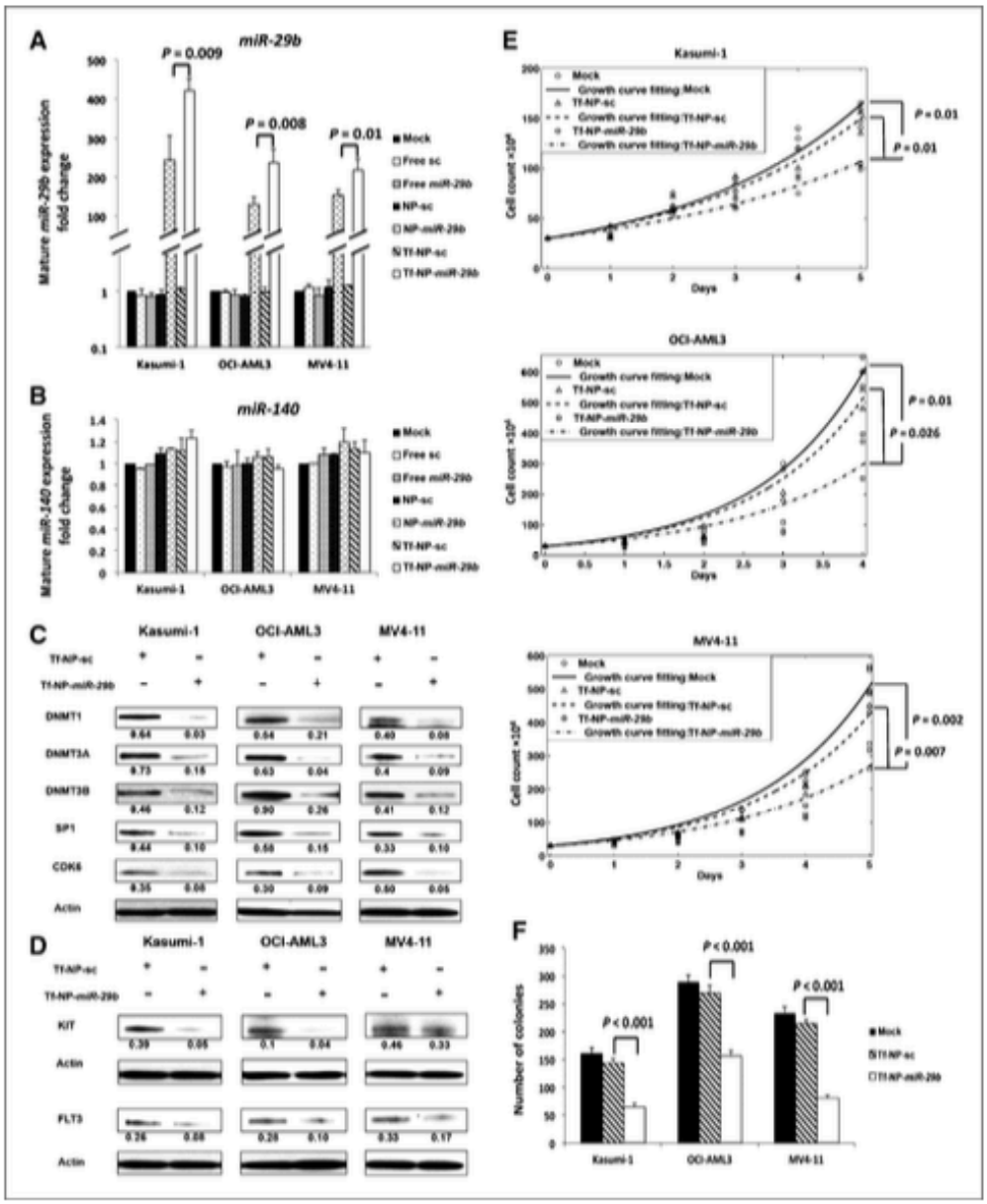

Figure 3. Treatment with Tf-NP-*miR-29b* increased mature *miR-29b* level, downregulated DNMT1, DNMT3A, DNMT3B, SP1, CDK6, FLT3, and KIT, and had antileukemic activity in AML cells. A, mature *miR-29b* expression levels; and B, *miR-140* expression in Kasumi-1, OCI-AML3, and MV4-11 cells. C, DNMT1, DNMT3A, DNMT3B, SP1, and CDK6 expression; and D, FLT3 and KIT expression in Kasumi-1, OCI-AML3, and MV4-11 after treatment with Tf-NP-*miR-29b* compared with Tf-NP-sc treatment. The number below each band represents the ratio of the band's intensity to actin used as a loading control. E, cell growth curve of Kasumi-1, OCI-AML3, and MV4-11 cells treated Tf-NP-*miR-29b*, Tf-NP-sc, or mock. F, colony formation assays in Kasumi-1, OCI-AML3, and MV4-11 cells treated with Tf-NP-*miR-29b*, Tf-NP-sc, or mock.

Intracellular increase of the endogenous *miR-29b* primary transcripts following Tf-NP-*miR-29b* treatment

The endogenous mature *miR-29b* stems from 2 precursors (*pri-miR-29b-1* and *pri-miR-29b-2*) encoded by 2 genes located on human chromosomes 7q32 and 1q23, respectively. We previously reported that an SP1-containing transcription repressor complex downregulated *miR-29b* in AML cells (7). Here, we showed that Tf-NP-*miR-29b* reduced SP1 expression; therefore, we hypothesized that Tf-NP-*miR-29b* may increase the endogenous *miR-29b* expression. We found 1.7-, 2.0-, and 2.3-fold increase in endogenous *pri-miR—29b-1* and 2.1-, 1.8-, and 2.5-fold increase of *pri-miR-29b-2* levels in Kasumi-1, OCI-AML3, and MV4-11 cells, respectively, following treatment with the *miR*–29b-loaded Tf-NP (Supplementary Fig. S4A).

miR-29b inhibits cell proliferation and colony formation in AML cells

We evaluated the antileukemic effects of Tf-NP-*miR-29b* treatment by carrying out growth curves and analyzing the colony forming ability. The Tf-NP-*miR-29b* treatment reduced the growth rate from 32.2% (Tf-NP-scramble) to 25.3% (Tf-NP-*miR-29b*) in Kasumi-1 cells, from 70.9% (Tf-NP-scramble) to 57.3% (Tf-NP-*miR-29b*) in OCI-AML3, and from 53.0% (Tf-NP-scramble) to 43.9% (Tf-NP-*miR-29b*) in MV4-11 cells (Fig. 3E) compared with the Tf-NP scramble treatment. On the last day, the Tf-NP-*miR-29b* treatment was associated with significantly lower cell counts than in the Tf-NP-scramble or mock (buffer only) treatment for Kasumi-1 ($P = 0.01$ for both), OCI-AML3 ($P = 0.026$ and $P = 0.01$, respectively), and MV4-11 cells ($P = 0.007$ and $P = 0.002$, respectively; Fig. 3E). In addition, we observed an approximately 50% reduction in colonies following the Tf-NP-*miR-29b* treatment (Fig. 3F). The average number of colonies (±SD) formed by mock- (buffer only), Tf-NP-scramble-, and Tf-NP-*miR-29b*–treated cells were, respectively, 161 ± 9, 143 ± 9, and 65 ± 6 ($P <$ 0.001 for each comparison) for Kasumi-1 cells, 289 ± 11, 269 ± 13, and 156 ± 10 ($P < 0.001$ for each comparison) for OCI-AML3 cells, and 234 ± 11, 213 ± 7, and 80 ± 5 ($P <$ 0.001 for each comparison) for MV4-11 cells, respectively.

Validation in AML patient blasts

The antileukemic activity of Tf-NP-*miR-29b* was further validated in primary blasts from 3 patients with newly diagnosed AML. Patient 1 had a secondary AML with unknown karyotype (standard cytogenetic analysis failed in this patient). Patients 2 and 3 had a *de novo* cytogenetically normal AML. After Tf-NP-*miR-29b* treatment, we observed an approximate 860-, 400-, and 750-fold increase in *miR-29b* levels, compared with Tf-NP-scramble after 24 hours in blasts sample from all 3 patients (Fig. 4A). No significant change of the expression of an unrelated miR (i.e., *miR-140*) was observed (Fig. 4B). In addition, we observed 2.2-, 2.1-, and 1.9-fold increase in endogenous *pri-miR-29b-1*, and 1.6-, 1.4-, and 2-fold increase in endogenous *pri-miR-29b-2* compared with controls after Tf-NP-*miR-29b* treatment in the blasts from all 3 patients (Supplementary Fig. S4B).

After 48-hour Tf-NP-*miR-29b* treatment, 1.3-, 2.9-, and 6.6-fold DNMT1 downregulation was observed in all 3 patients' blast samples, as well as 3.5-, 4.4-, and 6.4-fold DNMT3A, 6.9-, 9.7-, and 6.7-fold DNMT3B, 7.6-, 5.3-, and 6-fold SP1, and 8.1-, 4.9-, and 2.8-fold CDK6 downregulation compared with Tf-NP-scramble treatment (Fig. 4C). In addition, 1.9- and 2.1-fold FLT3 decrease in patient 1 and patient 2, and 1.9- and 3.3-fold decrease KIT in patient 1 and patient 3 were observed (Fig. 4D). Tf-NP-*miR-29b* decreased cell viability, respectively, by approximately 19% ($P = 0.03$), 15% ($P = 0.017$), and 21% ($P = 0.001$) respectively, compared with Tf-NP-scramble in all three AML patients' blasts (Fig. 4E).

In vivo evaluation of Tf-NP-*miR-29b* in preclinical models

To assess the safety profile of systemic nanoparticle treatment, we evaluated basic hepatic and renal functions in immunocompetent mice after saline, empty nanoparticles, or Tf-NP-*miR-29b* treatment. No significant organ impairment was observed (Supplementary Fig. S5A). Moreover, Tf-NP treatment did not result in body weight changes (Supplementary Fig. S5B) or significant changes in hemoglobin (Hb) level, white blood count (WBC), or platelet (PLT) count (Supplementary Fig. S5C).

Next, we evaluated the *in vivo* therapeutic efficacy of Tf-NP-*miR-29b*. In the first trial, the MV4-11-engrafted mice were treated with free *miR-29b* ($n = 3$; 1 mg/kg/d miR intravenously), Tf-NP-scramble ($n = 6$), or Tf-NP-*miR-29b* ($n = 6$) starting on day 10 after cell injection. The median survival time was 27, 28, and 32.5 days for free *miR-29b*, Tf-NP-scramble, and Tf-NP-*miR-29b*–treated mice, respectively. The survival in the Tf-NP-*miR-29b*-treated group was significantly longer compared with free *miR-29b*-treated group ($P = 0.003$, log-rank test) as well as when compared with the Tf-NP-scramble-treated group ($P = 0.015$, Fig. 5A). Consistent with the longer survival, the spleen sizes in the Tf-NP-*miR-29b*-treated group were significantly smaller than in the free *miR-29b*–treated mice ($P = 0.033$) or in the Tf-NP-scramble–treated group ($P = 0.049$). The mean spleen weight was 29.3 ± 4.1, 26.6 ± 1.6, and 19.3 ± 3.4 mg for the free *miR-29b*, Tf-NP-scramble, and Tf-NP-*miR-29b*-treated mice, respectively (Fig. 5A). To validate these results, we conducted a second independent trial, testing a slightly different schedule and dosing (see Materials and Methods). The engrafted mice were treated with saline ($n = 5$), Tf-NP-scramble ($n = 7$; 1.5 mg miR/kg/d intravenously), or Tf-NP-*miR-29b* ($n = 7$) starting at day 10 after cell injection. The median survival time in this trial was 26, 27, and 34 days for saline, Tf-NP-scramble, and Tf-NP-*miR-29b*-treated mice, respectively. Similar to the first trial, the Tf-NP-*miR-29b* treatment prolonged the survival of the leukemic mice compared with the Tf-NP-scramble–treated group ($P = 0.01$, Fig. 5B).

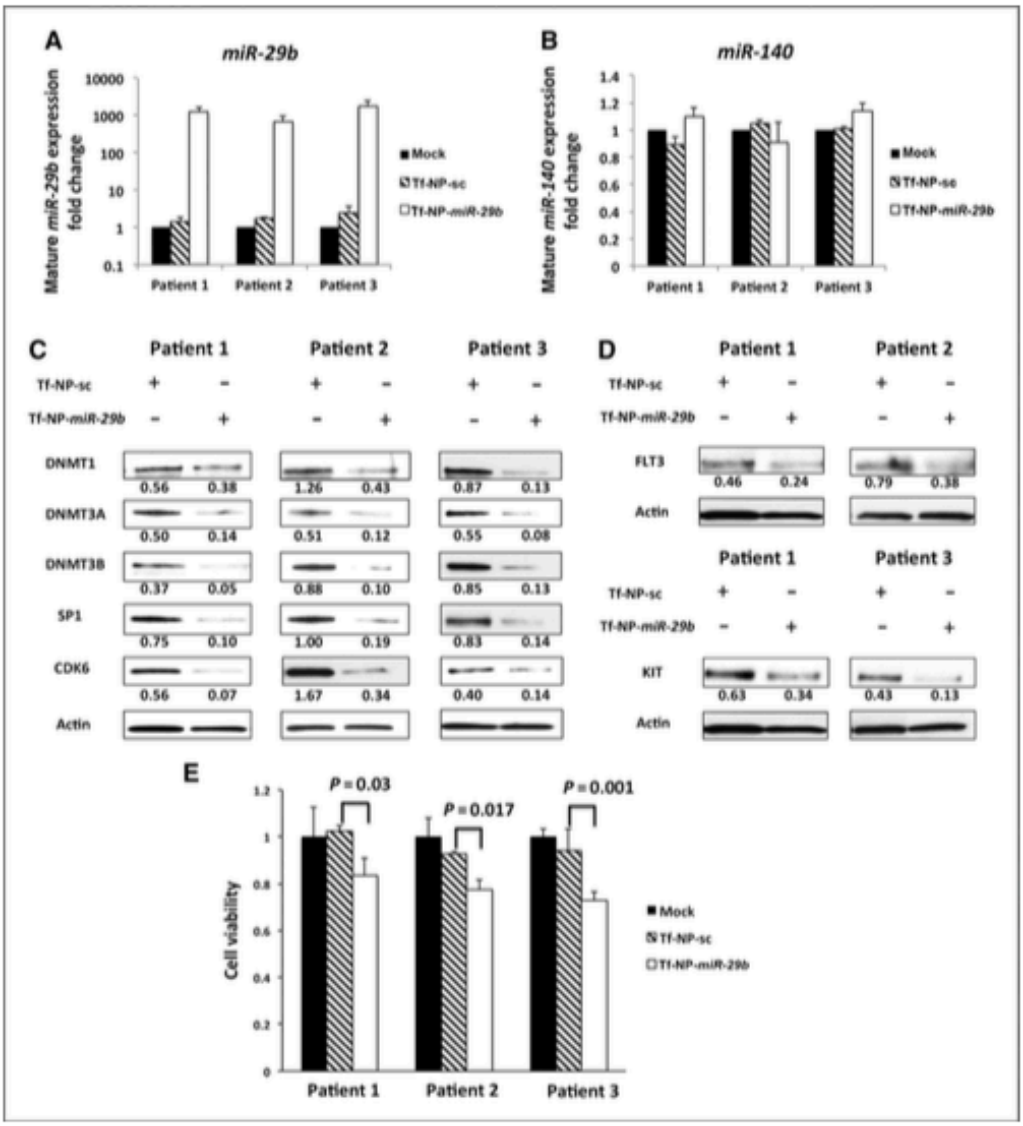

Figure 4. Validation of Tf-conjugated-NP-*miR-29b* treatment effects in AML patient blasts. A, expression levels of mature *miR-29b*. B, *miR-140* expression levels in AML patient blasts from 3 different patients with AML treated with Tf-NP-*miR-29b*, Tf-NP-sc, or mock. C, DNMT1, DNMT3A, DNMT3B, SP1, and CDK6 expression; and D, FLT3 and KIT expression in AML patient blasts. The number below each band represents the ratio of the band's intensity to the respective Actin band intensity. E, cell viability of 3 patient blast samples treated with Tf-NP-*miR-29b*, Tf-NP-sc, or mock.

Blood samples at day 24 (after 6 doses; second trial) showed a 20-fold increase in intracellular *miR-29b* levels in the Tf-NP-*miR-29b*-treated mice compared with the Tf-NP-scramble–treated group ($P = 0.003$, Fig. 5B). Furthermore, we observed a decreased expression of the *miR-29b* targets, *DNMT1* by 1.9-fold ($P = 0.028$), *DNMT3A* by 2.9-fold ($P = 0.02$), *DNMT3B* by 4-fold ($P = 0.002$), *SP1* by 2.9-fold ($P = 0.039$), *CDK6* by 1.6-fold ($P = 0.015$), *KIT* by 3.6-fold ($P = 0.018$), and *FLT3* by 1.5-fold ($P = 0.029$) compared with the Tf-NP-scramble-treated group *in vivo*

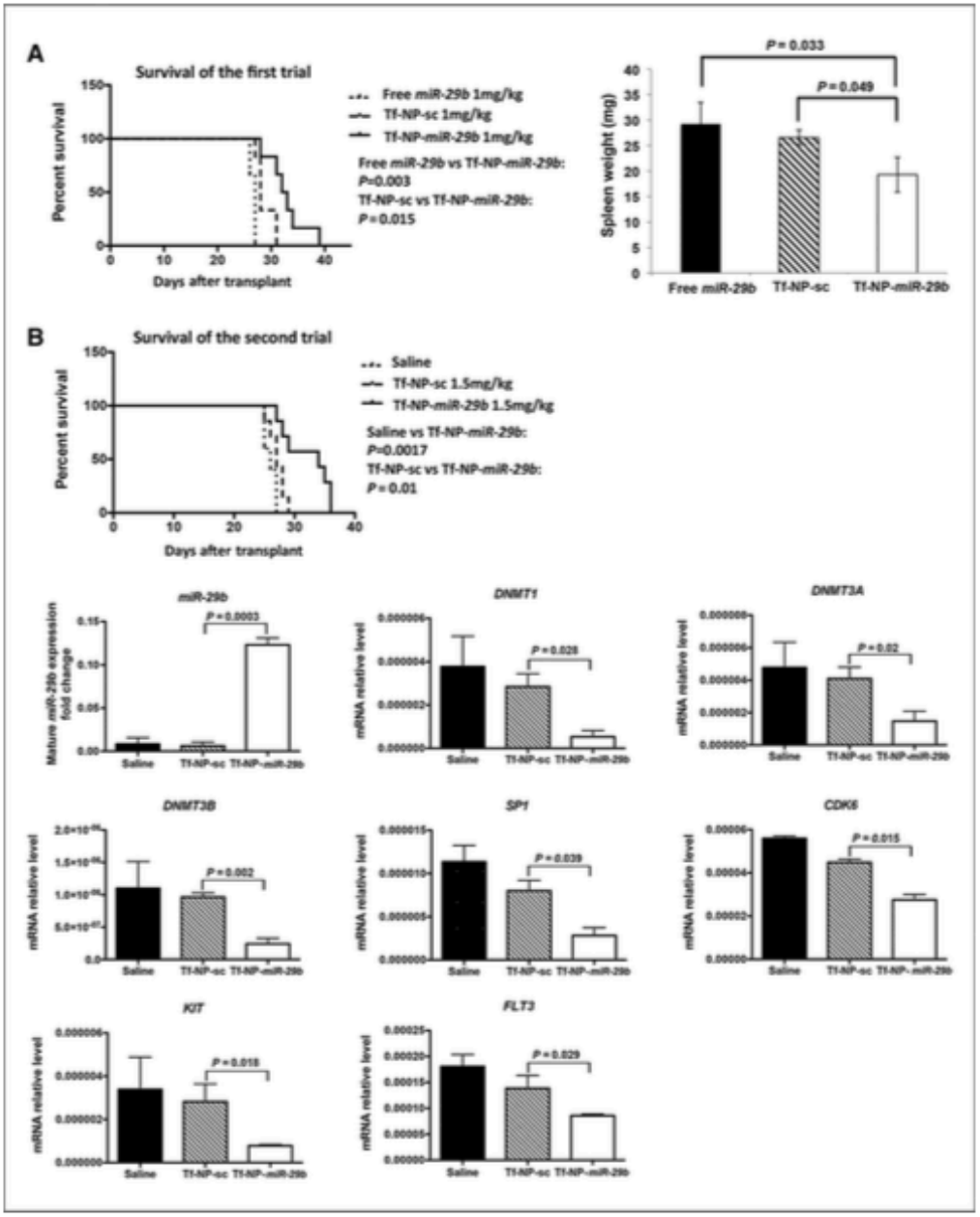

Figure 5. *In vivo* evaluation of Tf-NP-*miR*-29b in preclinical model. A, first trial: leukemic mice were treated with free *miR*-29b mimic, Tf-NP-scramble, or Tf-NP-*miR*-29b. Survival curves of the mice according to distinct treatments are shown (left). Corresponding spleen weights are shown (right). B, second trial: leukemic mice were treated with saline, Tf-NP-sc, or Tf-NP-*miR*-29b. Survival curves of the mice according to distinct treatments are shown (top). Intracellular levels of *miR*-29b, *DNMT1*, *DNMT3A*, *DNMT3B*, *SP1*, *CDK6*, *FLT3*, and *KIT* at day 24.

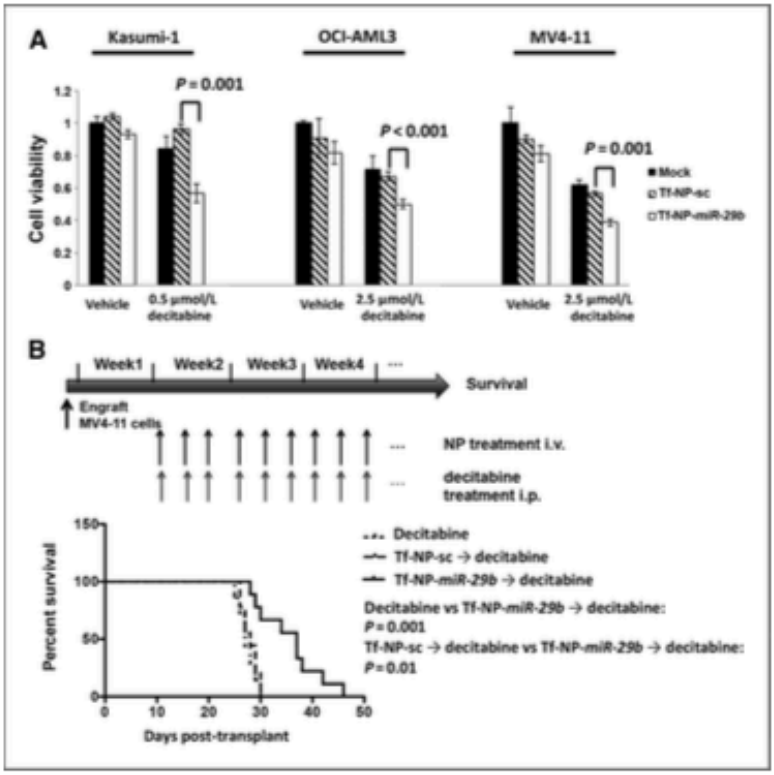

Figure 6. Antileukemic activity of Tf-NP-*miR-29b* followed by decitabine *in vitro* and *in vivo*. A, Kasumi-1, OCI-AML3, and MV4-11 cells were pretreated with Tf-NP-*miR*-29b, Tf-NP-sc, or mock for 48 hours before 48-hour decitabine treatment. B, leukemic mice were treated with decitabine, Tf-NP-sc followed by (→) decitabine, or Tf-NP-*miR-29b*→decitabine 10 days after engraftment. Survival curves of the mice according to distinct treatments are shown.

(Fig. 5B). These findings indicate that the *miR-29b* mimic molecules were successfully delivered to the leukemic cells and decreased *miR-29b* targets *in vivo*.

Antileukemic activity of Tf-NP-*miR-29b* priming followed by decitabine

Because we showed that higher pretreatment *miR-29b* levels were associated with improved clinical response to decitabine (24), we tested here whether Tf-NP-*miR-29b* treatment would improve the antileukemic activity of decitabine in AML cells. As we observed a *miR-29b* target downregulation at 48 hours, we pretreated AML cell lines and primary blasts with Tf-NP-scramble or Tf-NP-*miR-29b* for 48 hours before exposing them to decitabine.

Pretreatment with Tf-NP-*miR-29b* decreased the cell viability by approximately 40% ($P = 0.001$) compared with Tf-NP-scramble pretreatment after treatment with 0.5 μmol/L decitabine in Kasumi-1, approximately 20% ($P<0.001$) after treatment with 2.5 μmol/L decitabine in OCI-AML3 cells, and approximately 18% ($P < 0.001$) after treatment with 2.5 μmol/L decitabine in MV4-11 cells (Fig. 6A).

Next, we evaluated the *in vivo* the Tf-NP-*miR-29b* priming activity. We engrafted NSG mice with MV4-11 cells and treated them with decitabine alone ($n = 7$; 0.4 mg/kg/d intraperitoneally), Tf-NP-scramble ($n = 9$), or Tf-NP-*miR-29b*→decitabine ($n = 9$). The median survival time was 27, 28, and 37 days for the decitabine alone, Tf-NP-scramble → decitabine, and Tf-NP-*miR-29b*→decitabine, respectively. The combination treatment of Tf-NP-*miR-29*→decitabine significantly prolonged the survival of the leukemic mice compared with decitabine alone ($P = 0.001$) and compared with the combination treatment of Tf-NP-scramble-→decitabine ($P = 0.001$) and by trend also when compared with Tf-NP-*miR-29b* alone ($P = 0.06$).

Discussion

The differential expression of some miRs has been associated with myeloid leukemogenesis and/or patient outcome (4–7). The expression level of *miR-29b* has been found to be downregulated in AML blasts compared with normal bone marrow cells (Supplementary Fig. S3; ref. 8). Furthermore, high expression of *miR-29b* has been shown to have antileukemic activity, and to be associated with longer survival in patients treated with conventional chemotherapy and higher odds for achieving a complete remission following decitabine treatment (7, 11, 24). Thus, a therapeutic increase of *miR-29b* in AML blasts could provide substantial clinical benefit. However, the delivery of miRs

remains a challenging goal and, to our knowledge, an efficient miR-delivery system has not been reported for AML blasts.

Currently, the miR-delivery for potential cancer therapy is based on viral (25–28) and nonviral (29–43) systems. Among the reported viral-based systems, the adeno-associated virus (AAV)-based approaches seem promising, as supported by significant therapeutic effects in a murine liver cancer models (26). Nonviral cationic polymer or cationic lipid carrier systems have also been used to deliver miR-expressing plasmids to solid tumors by other researchers (29–41). However, the miR-expressing vectors and the AAV approach share some drawbacks, including limited efficiencies for hematopoietic cells, need for nuclear translocation of large DNA vectors, and limitations in expression of the mature miRs (44). With regard to hematopoietic cells, the shortcomings for both viral and nonviral approaches could be bypassed by engineering a targeting delivery system for mature miRs or miR mimic molecules (45). Most delivery systems for solid tumors use cationic or neutral lipid particles to deliver miR molecules due to their tendency of organ accumulation (34–40, 43). Thus, here we developed a novel anionic lipopolyplex nanocarrier system for miR delivery to AML cells.

The nanoparticles presented here had several remarkable differences from the conventional cationic lipid nanoparticles used in solid tumors that have the tendency to accumulate preferentially in lungs, kidney, and liver due to their charge property (46). The neutral and anionic lipid formulation of our nanoparticles was designed to avoid the nonspecific immune response caused by cationic lipids through activation of TLR4 and NF-κB pathways and, in turn, proinflammatory cytokine production (47, 48). Moreover, the overall neutral surface charge results in reduced plasma protein binding and low rate of nonspecific cellular uptake (23). Low-molecular weight polyethylenimine was selected as a core to condense miR molecules because it is known to be relatively biocompatible and to provide a positive charge, which allows for easily capture of the negatively charged miR molecules, and in turn high entrapment efficiency. The lipid-based carrier was made of DOPE, linoleic acid, and DMG-PEG. The low binding affinity between linoleic acid and small RNA may also enhance the dissociation of miRs from the lipopolyplex after endocytosis to facilitate target gene downregulation (49). Furthermore, the nanoparticles are protected from reticuloendothelial system clearance by 2% (molar ratio) of DMG-PEG to achieve long circulation times (22) and, thus, more efficient delivery in hemtopoietic organs, including bone marrow. To increase the specific effect on tumor cells, nanoparticles may be conjugated with molecules that enhance their targeting specificity (35).

We showed that our nanoparticles were able to efficiently deliver *miR-29b* mimics, increase mature *miR-29b* levels, and effectively target a panel of AML-relevant genes and mechanisms involved in epigenetics, cell-cycle control, and kinase-signaling pathways. Unlike the delivery of siRNA or short hairpin RNAs (shRNA) that are usually designed to target single genes, miRs can concurrently target multiple genes and pathways involved in leukemia that could potentially result in a better antileukemic activity and reduced emergence of resistance mechanisms. Indeed, we showed that Tf-NP-*miR-29b* treatment resulted in an *in vitro* growth inhibition, a reduction of colony formation in AML cells, and in a significant therapeutic activity and prolonged survival in 2, independent AML *in vivo* trials. Interestingly, approximately 80% of the mice treated with Tf-NP-*miR-29b* were still alive at the time when the control-treated mice (i.e., saline, free *miR-29b*, or Tf-NP-scramble) had died. Although, several studies investigating miR-anti-sense/plasmid/mimic delivery-approaches were shown to reduce tumor burden *in vivo* (29–43), only a few of them were able to show that miR-based therapies (i.e., *miR-145*, *miR-34a*, and *miR-107*) prolonged survival in mice with an aggressive cancer (31, 32, 36, 39).

Finally, in this study we also showed that priming AML cells with Tf-NP-*miR-29b* led to an improved antileukemic activity of decitabine *in vitro* and *in vivo*, thereby also supporting our earlier finding that higher endogenous *miR-29b* pretreatment levels associate with improved response to decitabine (20, 24). We now showed that *miR-29b* expression may not only be a predictor of treatment response to decitabine, but *miR-29b* priming may indeed be integral to decitabine-based regimens, especially for those patients with AML with downregulated endogenous *miR-29b*.

In conclusion, we developed a novel transferrin-conjugated nanoparticle system to efficiently deliver synthesized miR mimics to AML blasts. Tf-NP-*miR-29b* treatment increased mature *miR-29b* levels, downregulated known *miR-29b* targets, and showed antileukemic activity by improving survival in *in vivo* AML models. Our nanoparticle delivery approach is a promising new antileukemic strategy that may be rapidly translated into the clinic.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Writing, review, and/or revision of the manuscript: X. Huang, S. Schwind, H. Wang, P. Hoellerbauer, A. Mims, R.B. Klisovic, K.K. Chan, J.C. Byrd, C.D. Bloomfield, M.A. Caligiuri, R.J. Lee, N. Muthusamy, L. James Lee, G. Marcucci
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2.6 Leukemia Initiating Cell Burden as a prognostic marker in AML

In AML, leukemia-initiating cells are assumed to exist within the CD34+/CD38- cell compartment. These cells are more resistant to chemotherapy, enriched in MRD cell populations, and likely responsible for relapse. The here included paper shows that a high CD34+/CD38- cell burden at diagnosis independently associated with worse outcome in AML patients treated with allogeneic HSCT in CR. The inferior outcome was likely mediated by residual leukemia-initiating cells in the bone marrow CD34+/CD38- cell population, escaping the graft versus leukemia effects after allogeneic HSCT. The paper was the first to analyse the prognostic impact of a leukemia-initiating cell population in AML patients consolidated with an allogeneic HSCT and demonstrated the feasibility of the CD34+/CD38- cell burden as a marker for risk stratification and a potential therapeutic target.

Manuscript included in this paragraph:

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RESEARCH ARTICLE

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Prognostic impact of the CD34+/CD38− cell burden in patients with acute myeloid leukemia receiving allogeneic stem cell transplantation

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Abstract

In acute myeloid leukemia (AML), leukemia-initiating cells exist within the CD34+/CD38− cell compartment. They are assumed to be more resistant to chemotherapy, enriched in minimal residual disease cell populations, and responsible for relapse. Here we evaluated clinical and biological associations and the prognostic impact of a high diagnostic CD34+/CD38− cell burden in 169 AML patients receiving an allogeneic stem cell transplantation in complete remission. Here, the therapeutic approach is mainly based on immunological graft-versus-leukemia effects. Percentage of bone marrow CD34+/CD38− cell burden at diagnosis was measured using flow cytometry and was highly variable (median 0.5%, range 0%–89% of all mononuclear cells). A high CD34+/CD38− cell burden at diagnosis associated with worse genetic risk and secondary AML. Patients with a high CD34+/CD38− cell burden had shorter relapse-free and overall survival which may be mediated by residual leukemia-initiating cells in the CD34+/CD38− cell population, escaping the graft-versus-leukemia effect after allogeneic transplantation. Evaluating the CD34+/CD38− cell burden at diagnosis may help to identify patients at high risk of relapse after allogeneic transplantation. Further studies to understand leukemia-initiating cell biology and develop targeting therapies to improve outcomes of AML patients are needed.

1 | INTRODUCTION

Hematopoietic stem cells (HSCs) are found in the bone marrow (BM) and physiologically give rise to all blood cells.[1] The phenotype of HSCs is not well defined, but HSCs have been suggested to be part of the primitive CD34+/CD38− cell population.[2] A high amount of the CD34+/CD38− cells seem to rest in the G0 cell cycle phase,[3] and are able to initiate retransplantable hematopoiesis in animal models.[4]

Acute myeloid leukemia (AML) is a disease originating from the clonal expansion of HSCs or early progenitor cells that have lost the ability to mature.[5] Most researchers agree that similar to the physiological HSCs, AML-initiating cells—often termed leukemia-initiating cells (LICs)—also exist within the CD34+/CD38− stem cell compartment.[6–9] LICs—opposed to the majority of circulating, proliferating leukemic blasts or "bulk cells"—are postulated to survive chemotherapy as minimal residual disease (MRD) and cause AML relapse; their nonproliferative state might be one reason for resistance to chemotherapy.[10–14] When transplanted into NOD/SCID mice, CD34+/CD38− cells of AML patients were able to initiate leukemia and growth of tumors histologically similar to the human donor's neoplasm.[7–15]

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Hematopoietic stem cell transplantation (HSCT) offers a curative treatment option for AML patients if a suitable donor is available. After achievement of complete remission (CR), HSCT is an established form of consolidation therapy for patients at high risk of relapse.[16] The therapeutic effects of HSCT are mainly based on an immunologic graft-versus-leukemia (GvL) reaction.[17]

Previous studies have suggested that a high burden of LICs at diagnosis of AML patients treated with chemotherapy increased their relapse probability and associated with inferior outcomes.[14,18-20] However, it is at present unknown whether a high burden of LICs has a similar prognostic significance in patients undergoing HSCT. As data support the assumption that LICs are also less immunogenic than the leukemic bulk cells,[21] evaluation of the pretreatment LIC or the CD34+/CD38− cell burden in AML patients after allogeneic HSCT might provide important biological and clinical information. The objective of this study was to determine the prognostic significance of the CD34+/CD38− cell burden at diagnosis in patients undergoing HSCT and to analyze associations between the CD34+/CD38− cell burden and clinical, cytogenetic, and molecular characteristics to provide further biological insights.

2 | PATIENTS AND METHODS

2.1 | Patients and treatment

We analyzed 169 adult AML patients who received HSCT in CR at the University of Leipzig between June 2001 and July 2013, and for whom pretreatment BM aspirate material for flow cytometry analysis was available. All patients received standard cytarabine-based protocol chemotherapy and were transplanted in the first or second CR (for details, please see Supporting Information). Written informed consent for participation in these studies was obtained in accordance with the Declaration of Helsinki.

Forty-nine (29%) patients received myeloablative conditioning (MAC), which consisted of cyclophosphamide 60 mg/kg body weight for 2 days and 12 Gy total body irradiation (TBI),[22] whereas 120 (71%) patients received nonmyeloablative conditioning (NMA), which contained fludarabine 30 mg/m^2 for 3 days followed by 2 Gy TBI.[23,24] All patients received granulocyte colony stimulating factor-mobilized peripheral blood stem cells on day 0. Reasons for NMA, as opposed to MAC conditioning, were age (patients over 50 years if receiving unrelated HSCT [$n = 102$] and patients over 55 years if receiving related HSCT [$n = 17$]) and previous autologous HSCT ($n = 1$).

Prior to HSCT, all patients were treated according to age-dependent chemotherapy protocols (under or over 60 years); for details, please see Supporting Information. Patients' characteristics are shown in Table 1 and Supporting Information, Table S1. At HSCT, patients had a median age of 62 years (range 19–75) and according to protocols, patients receiving MAC-HSCT were significantly younger than patients receiving NMA-HSCT (MAC-HSCT, median 40, range 19–55; NMA-HSCT, median 66, range: 47–75 years; $P < .001$). Survival analyses according to conditioning regimes are provided in Supporting Information, Figure S1 and Table S2.

At the time of HSCT, 83% of all patients (84% in MAC-HSCT and 83% in NMA-HSCT) were in their first and 17% (16% in MAC-HSCT and 18% in NMA-HSCT) were in second CR (for CR definition, see Supporting Information). Donors were human leukocyte antigen (HLA)-matched related in 23% of cases (39% in MAC-HSCT and 14% in NMA-HSCT), while 54% (53% in MAC-HSCT and 58% in NMA-HSCT) were HLA-matched unrelated and 23% (8% in MAC-HSCT and 28% in NMA-HSCT) were unrelated with at least one antigen mismatch.

2.2 | Cytogenetics and molecular markers

Pretreatment BM cytogenetic analyses were performed centrally in our institution. The presence of internal tandem duplication in the *FLT3* gene (*FLT3*-ITD), mutations in the *FLT3* tyrosine kinase domain (*FLT3*-TKD), and in the *NPM1* and *CEBPA* genes were determined as described previously.[25-27]

2.3 | Flow cytometry

For all 169 patients, mononuclear BM cells were assessed for surface expression of CD34, CD38, and CD34/CD38 at diagnosis. For details, please see Supporting Information.

2.4 | Definition of clinical end points and statistical analysis

Statistical analyses were performed using the R statistical software platform (version 3.0.2). Utilizing the "OptimalCutpoints" package, an optimal cutoff of 6% was identified that divided the cohort into patients with a high or a low CD34+/CD38− cell burden.

Overall survival (OS) was calculated from HSCT until death from any cause and relapse-free survival (RFS) was calculated from HSCT to relapse or death from any cause.

Associations of CD34+/CD38− burden with baseline clinical, demographic, and molecular features were compared using the Kruskal–Wallis test and Fisher's exact test for continuous and categorical variables, respectively. For time-to-event analyses, survival estimates were calculated using the Kaplan–Meier method. Groups were compared with the log-rank test. Multivariable analysis is described in Supporting Information.

3 | RESULTS

3.1 | Associations of CD34+/CD38− cell burden in BM at diagnosis with genetic and clinical characteristics

The CD34+/CD38− cell burden at diagnosis was highly variable (median 0.5%, range 0%–89% of all mononuclear cells). There was no difference in the CD34+/CD38− cell burden between younger (<60 years) and older (≥60 years) patients at diagnosis ($P = .14$, Figure 1A). However, while there were no differences between age groups in the European LeukemiaNet (ELN),[28] Favorable, Intermediate-I, or Intermediate-II Genetic Groups (Figure 1B–D), older patients in the

TABLE 1 Clinical characteristics of AML patients treated with HSCT according to CD34+/CD38− cell burden at diagnosis (< 6% vs ≥6%)

Characteristics	All patients (n = 169)	Low CD34+/CD38− cell burden (n = 144)	High CD34+CD38− cell burden (n = 25)	P
Age at HSCT, years				.14
Median	62	61	63	
Range	19–75	19–75	30–74	
Sex, n (%)				.67
Male	85 (50)	71 (49)	14 (56)	
Female	84 (50)	73 (51)	11 (44)	
Hemoglobin, g/dL				.45
Median	8.6	8.6	9.4	
Range	4.5–15.7	4.5–15.7	5.3–13.3	
Platelet count, $\times 10^9$/L				.04
Median	63	74	40	
Range	2–327	2–327	13–178	
WBC count, $\times 10^9$/L				.07
Median	7.9	7.5	35.5	
Range	0.7–385	0.7–385	1.1–295	
Percentage of blood blasts, %				.11
Median	26	22	47	
Range	0–98	0–98	2–97	
Percentage of BM blasts, %				.83
Median	56	56	60	
Range	0–95	0–95	0–95	
Karyotype, n (%)				.39
Abnormal	84 (52)	69 (50)	15 (60)	
Normal	79 (49)	69 (50)	10 (40)	
ELN genetic group, n (%)				.001
Favorable	42 (30)	41 (32)	1 (5)	
Intermediate-I	39 (26)	33 (25)	6 (29)	
Intermediate-II	32 (21)	30 (23)	2 (10)	
Adverse	38 (25)	26 (20)	12 (57)	
Disease origin, n (%)				.009
De novo	116 (69)	105 (73)	11 (44)	
Secondary	53 (31)	39 (27)	14 (56)	
MDS	32	25	7	
MPN	10	6	4	
Solid tumor	11	8	3	
NPM1, n (%)				.19
Wild-type	109 (74)	90 (71)	19 (86)	
Mutated	39 (26)	36 (29)	3 (14)	

(continued)

TABLE 1 (continued)

Characteristics	All patients (n = 169)	Low CD34+/CD38− cell burden (n = 144)	High CD34+CD38− cell burden (n = 25)	P
FLT3-ITD, n (%)				.77
Absent	114 (78)	99 (79)	15 (75)	
Present	32 (22)	27 (21)	5 (25)	
CEBPA, n (%)				.13
Wild-type	114 (85)	98 (83)	16 (100)	
Mutated	20 (15)	20 (17)	0 (0)	

Abbreviations: BM, bone marrow; ELN, European LeukemiaNet; FLT3-ITD, internal tandem duplication of the FLT3 gene; HSCT, hematopoietic stem cell transplantation; MAC, myeloablative; MDS, myelodysplastic syndrome; MPN, myeloproliferative neoplasia; NMA, nonmyeloablative; WBC, white blood cell.

ELN Adverse Genetic Group had a trend for a higher CD34+/CD38− cell burden at diagnosis than younger patients (P = .10, median 2.7% vs 1%, Figure 1E).

In further analyses, we used a 6% cutoff to divide the patients according to their CD34+/CD38− cell burden at diagnosis into low [<6%, n = 144 (85%)] and high [≥6%, n = 25 (15%)] burden groups. Subgroup analyses restricted to patients receiving HSCT in the first CR are shown in Supporting Information, Table S3 and Figure S5.

At diagnosis, a high CD34+/CD38− cell burden associated with lower platelet counts (P = .04), and, by trend, higher white blood cell (WBC) counts (P = .07). Patients developing AML as a secondary disease following myelodysplastic syndrome, myeloproliferative neoplasia, or solid tumors were more likely to have a high CD34+/CD38− cell burden at diagnosis than patients with *de novo* AML (P = .009). Patients with a high CD34+/CD38− cell burden were more likely to have a complex karyotype (≥3 cytogenetic abnormalities,[28] P = .02), monosomy 5 or deletion of 5q (P = .004) or to have a monosomal karyotype[29] (P = .004). Among the ELN Genetic Groups, patients with high CD34+/CD38− cell burden at diagnosis were less often classified in the Favorable (5% vs 32%) and more often in the Adverse (57% vs 20%) Genetic Group (P = .001, Table 1). Furthermore, none of the patients with a high CD34+/CD38− cell burden at diagnosis harbored a *CEBPA* mutation (P = .13).

3.2 | Prognostic value of CD34+/CD38− cell burden at diagnosis

A high CD34+/CD38− cell burden at diagnosis associated with shorter RFS (P < .001, Figure 2A) and OS (P = .005, Figure 2B). When the distinct HSCT-conditioning protocols were regarded separately, we

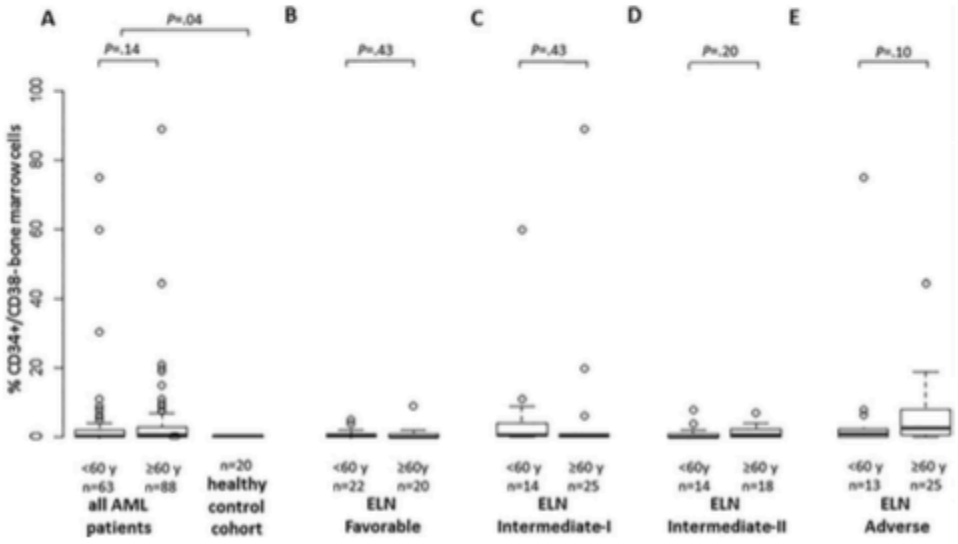

FIGURE 1 CD34+/CD38− cell burden according to age at diagnosis (<60 years vs ≥60 years). (A) All patients (n = 169) versus healthy subjects (n = 20). (B) Favorable ELN genetic risk. (C) Intermediate-I ELN genetic risk. (D) Intermediate-II ELN genetic risk. (E) Adverse ELN genetic risk

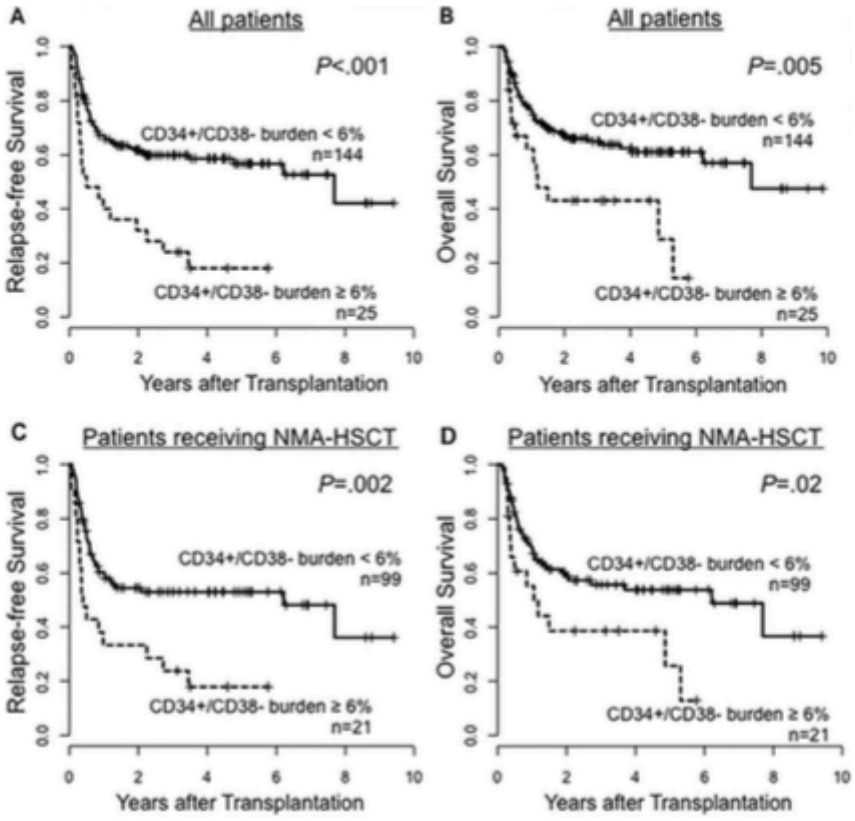

FIGURE 2 Outcome of HSCT-treated AML patients according to the CD34+/CD38− cell burden at diagnosis (<6% vs ≥6%). (A) Relapse-free survival and (B) overall survival for all patients ($n = 169$) and (C) relapse-free survival and (D) overall survival for patients receiving NMA-HSCT ($n = 120$)

observed shorter RFS ($P = .002$, Figure 2C) and OS ($P = .02$, Figure 2D) for patients with a high CD34+/CD38− cell burden treated with NMA-HSCT. In the group of MAC-HSCT-treated patients, only four had a high CD34+/CD38− cell burden at diagnosis preventing further subanalyses of the MAC-HSCT-treated patients. Three years after transplantation, 65% of patients with a low diagnostic CD34+/CD38− cell burden were alive (including 56% of NMA-HSCT-treated patients) and 60% relapse-free (including 53% of NMA-HSCT-treated patients). In contrast, in the group with a high CD34+/CD38− cell burden, only 43% of patients were alive (including 39% of NMA-HSCT-treated patients) and only 24% (including 24% of NMA-HSCT-treated patients) remained relapse-free after 3 years. The CD34+/CD38− cell burden at diagnosis did also impact on RFS and OS when we restricted our analysis to patients with normal karyotype or *de novo* AML (Supporting Information, Figures S2 and S3). Furthermore, in patients with ELN Favorable or Intermediate genetic risk who had a high CD34+/CD38− cell burden at diagnosis, we observed a trend for shorter RFS ($P = .06$) and comparable OS ($P = .11$) to patients within the ELN Adverse Genetic Group (Supporting Information, Figure S8).

In multivariable analysis (Table 2), a high CD34+/CD38− cell burden at diagnosis remained significantly associated with shorter RFS ($P < .001$) and shorter OS ($P = .04$) after adjustment for age at HSCT. The risk of death or an event was about twice as high in patients with a high diagnostic CD34+/CD38− cell burden compared with that of patients with a low CD34+/CD38− cell burden.

4 | DISCUSSION

Similar to normal hematopoiesis, AML cells are thought to emerge from primitive LICs with the ability of unlimited self-renewal.[15,30] The observation that leukemic CD34+/CD38− cells are able to serially transplant AML in NOD/SCID mice[6,14,15] led to the assumption that the CD34+/CD38− BM compartment harbors the LIC population.[30] AML bulk cells are often efficiently eradicated by chemotherapeutic agents. In contrast, LICs show resistance to chemotherapeutic agents.[31,32] LICs exist in a quiescent state within a stem cell niche and have slow dividing properties.[33] Among other biological features, this

TABLE 2 Multivariable outcome analyses of 169 AML patients treated with HSCT according to the CD34+/CD38− burden at diagnosis

	Relapse-free survival			Overall survival		
Variable	HR[a]	95% CI	*P*	HR[a]	95% CI	*P*
Age at the time of HSCT	1.03	1.01–1.05	.007	1.05	1.02–1.08	<.001
CD34+/CD38− cell burden at diagnosis (≥6% vs <6%)	2.44	1.46–4.08	<.001	1.86	1.05–3.37	.04

Abbreviations: CI, confidence interval; HSCT, hematopoietic cell transplantation; HR, hazard ratio; MAC, myeloablative; NMA, nonmyeloablative.
[a]HR <1 (>1) indicate lower (higher) risk for an event for the first category listed for the dichotomous variables and for the higher values of the continuous variables.
Variables considered in the models were those significant at $\alpha = 0.20$ in univariable analyses. Variables considered were sex, disease origin (de novo vs secondary), ELN classification, platelet count at diagnosis, blast count in bone marrow at diagnosis, CD34+/CD38− burden at diagnosis, age at HSCT, disease status at HSCT (first vs second CR), HLA match (antigen match vs mismatch), and HLA donor type (related vs unrelated).

quiescence and the increased expression of multidrug resistance genes may explain the occurrence of relapse after cytotoxic therapy.[21] Furthermore, there is growing evidence that leukemic CD34+/CD38− cells are also less immunogenic than AML bulk cells. Costello et al.[21] showed reduced immunogenicity in vitro with lower lymphocyte proliferation against a CD38− population and decreased IL-2 and IFN-γ secretion. Reasons for this observation might be a lower expression of major immune response molecules in the CD34+/CD38− cell population. These observations led us to explore the outcomes as well as biological and clinical characteristics associated with the CD34+/CD38− cell burden at diagnosis in AML patients undergoing HSCT.

While three previous studies did not find associations of a high CD34+/CD38− cell burden with cytogenetics,[18,19,34] which may be related to the varying numbers and characteristics of patients analyzed, we found that patients with a high CD34+/CD38− cell burden were more likely to have poor-risk cytogenetics. In line with these findings, we also observed an unequal distribution of patients with high and low CD34+/CD38− cell burden in the four ELN Genetic Groups, with patients with a high CD34+/CD38− cell burden being least often classified in the Favorable and most frequently in the Adverse Group (Table 1 and Supporting Information, Table S1). Another study described an association of a CD34+/CD38−/ALDH^{high} LIC phenotype cell burden with poor-risk genetics (ELN adverse genetic risk, monosomal, or complex karyotypes) in a cohort of 98 patients.[35] Patients with a high CD34+/CD38− cell burden were also more likely to have secondary AML, which was also found for the patients harboring a CD34+/CD38−/ALDH^{high} LIC phenotype.[35] Thus, the size of the LIC population might be interconnected and contribute to the known adverse outcome of AML with adverse cytogenetic risk, for example, monosomal or complex karyotypes and secondary AML. Furthermore, none of the patients with a high CD34+/CD38− cell burden at diagnosis was *CEBPA*-mutated compared to 17% of patients with a low CD34+/CD38− cell burden. We did not observe significant associations of a high CD34+/CD38− cell burden with the presence of *FLT3*-ITD or *NPM1* mutations in the entire cohort. However, when we restricted our analysis to patients receiving HSCT in the first CR, none of the patients with a high CD34+/CD38− cell burden at diagnosis were *NPM1* mutated ($P = .01$, Supporting Information, Table S3). A small number of studies analyzed the LIC population burden at diagnosis in the context of molecular markers and, similar to our study, none found an association with the presence of *FLT3*-ITD.[14,18,19,34] Whereas Vergez et al.[19] did not find an association of the presence of *NPM1* mutations with the LIC population burden (defined by CD34+CD38^{low}/−CD123+), Gerber et al.[35] described a decreased frequency of *NPM1* mutations in an LIC phenotype defined by CD34+/CD38−/ALDH^{high}. It is known that *CEBPA* mutations activate self-renewal capacity in committed myeloid progenitor cells,[36] resulting in more mature AML phenotypes, and that *NPM1* mutations occur in more mature CD34− AML cells.[37] As the presence of mutations in both genes impact positively on survival in cytogenetically normal AML,[38] the observed associations may indicate an important biological interaction between *CEBPA* or *NPM1* mutations and the LIC burden at diagnosis in AML patients.

We were able to show that a high CD34+/CD38− cell burden at diagnosis associated with shorter RFS and OS after HSCT in CR. In multivariable analysis, the CD34+/CD38− cell burden retained its prognostic value, independently of other known prognostic factors. Some studies demonstrated that a high burden of LICs at AML diagnosis increased the relapse probability after chemotherapy and associated with worse outcomes in different patient cohorts.[13,18–20,35] In general, these studies described a wide range of diagnostic BM CD34+/CD38− cells, from 0.01% up to 71%, in AML patients, similar to the findings in our study. Van Rhenen et al.[14] found the diagnostic CD34+/CD38− cell burden of AML patients associated with a higher MRD frequency evaluated by flow cytometry after chemotherapy, shorter OS, RFS, and disease-free survival (DFS). Hwang et al.[34] described a higher CD34+/CD38− cell burden at diagnosis in patients who did not achieve a CR after one course of chemotherapy. Khan et al.[20] showed lower CR rates and shorter OS for patients over 60 years of age with a higher diagnostic CD34+/CD38− cell burden in blood, but not in BM. Other studies further characterized the analyzed LIC population. Vergez et al.[19] demonstrated that a high CD34+CD38^{low}/−CD123+ cell burden associated with a lower CR rate, shorter DFS, and shorter OS. Wang et al.[18] performed flow cytometry on FISH-preselected blasts in AML with abnormal karyotype and demonstrated a shorter OS and RFS and higher relapse rates in patients with a high FISH+/CD34+/CD38− cell burden at diagnosis. In these studies, the chosen cutoffs to define a high LIC burden were those with the most significant outcome impact and ranged from 1% to 15%.[14,18–20] This stands in line with our finding with an optimal

cutoff at 6% but also significant outcome differences using a 2%, 7.5%, or 10% cutoff (Supporting Information, Figure S4). Gerber et al.[35] showed lower CR rates and shorter EFS and OS in patients with a more immature LIC phenotype (CD34+/CD38−/ALDH^{high} vs CD34+/CD38−/ALDH^{intermediate} vs CD34−). However, within these studies, only 16%–47% of all patients and only younger individuals underwent HSCT, and none of the studies separately investigated the outcome of a larger cohort of HSCT-treated patients, for whom the GvL effect is thought to provide a continuous impact on residual disease. To our knowledge, this study is the first to show an adverse outcome of patients with a high LIC-containing cell population at diagnosis in a larger cohort treated with HSCT as consolidation therapy. Furthermore, the majority of AML patients we analyzed received an NMA-conditioning protocol,[23,39] for which the therapeutic effect is nearly exclusively based on the GvL effect. The aforementioned reduced immunogenicity of LICs observed by Costello et al.[21] is supported by our finding that HSCT may not be able to fully overcome the described poor prognosis of a high LIC burden at diagnosis. Clinically, not unlike cytotoxic agents, the GvL effect may primarily impact on AML bulk cells, and LICs within their BM niche may at least partly be able to evade the GvL effect. This observation helps to deepen our understanding why after HSCT in CR, some AML patients remain in remission while others do not.

We also compared the CD34+/CD38− cell burden in AML patients at diagnosis to the CD34+/CD38− BM cell counts during disease course (in CR before HSCT, at day 28 after HSCT, at relapse) and to that of 20 healthy individuals (for details, see Supporting Information). The CD34+/CD38− cell counts of AML patients in CR were comparable to or even lower than the CD34+/CD38− cell count in the healthy cohort and no significant difference was observed between patients with a high or a low CD34+/CD38− cell burden at diagnosis. Furthermore, the CD34+/CD38− cell counts were higher during AML relapse and comparable to the CD34+/CD38− cell burden at diagnosis. For details, see Supporting Information, Figure S9. However, as healthy HSCs also show the CD34+/CD38− phenotype, this population alone does not seem to present a suitable marker for risk assessment in CR or MRD detection.

A high CD34+/CD38− cell burden at AML diagnosis independently associated with worse outcomes in patients undergoing HSCT suggesting that determination of the CD34+/CD38− cell burden at diagnosis may provide a simple and widely available method to improve risk stratification in AML patients. This may help to identify patients in need of closer remission monitoring and possibly adjustment of therapeutic approaches, for example, tapering of immunosuppressant agents after HSCT. However, prospective studies to validate our findings are needed.

Given the inferior outcomes of AML patients with a high CD34+/CD38− cell burden at diagnosis that we and others[14,18–20,34,35] observed, regardless of HSCT as a consolidating therapy, new strategies to target LICs may improve survival. For example, the ability to target the CD34+/CD38− cell population was shown for Gemtuzumab ozogamicin,[34] and in vitro combination with tipifarnib suggested synergistic effects, especially on the LIC population.[40] Even though some evidence points to an intraindividual and interindividual heterogenic LIC phenotype,[35] some surface markers such as CD123,[34,41,42] CD96,[43] or CD117[44] may be able to discriminate between healthy HSCs and AML LICs, and ways to therapeutically exploit these phenotype differences are under investigation.[45,46] As CD123 seems to be expressed on LICs rather than HSCs, another promising therapy are T-cells expressing CD123-specific chimeric antigen receptors with high effector activity against AML cell lines and patient samples in vitro without affecting granulocyte or erythroid colonies.[47] Further potential therapeutic targets may be identified from genes and proteins differentially expressed in LICs compared to AML bulk cells or healthy HSCs.[9,48–50] Combining therapeutic approaches derived from these studies with chemotherapy and/or HSCT may help to improve outcomes—especially for those patients who have a high diagnostic CD34+/CD38− cell burden.

In conclusion, our data demonstrate that the negative prognostic impact of a high CD34+/CD38− cell burden at diagnosis seems not to be easily overcome by the GvL effect after HSCT in AML patients. In multivariable analysis, a high CD34+/CD38− cell burden at diagnosis was an independent factor for shorter RFS and OS, likely mediated by LICs within the CD34+/CD38− cell population escaping the GvL effects of HSCT. However, HSCT versus non-HSCT studies will be needed to evaluate whether patients with a high CD34+/CD38− cell burden at diagnosis might benefit from a HSCT as consolidation therapy, despite their worse outcome than patients with a low burden at diagnosis. Determination of the CD34+/CD38− cell burden at AML diagnosis may help to improve risk stratification, adjust disease monitoring and treatment, especially that it is widely available and relatively inexpensive. Finally, novel therapeutic agents targeting AML LICs within the CD34+/CD38− cell population may help to improve outcomes of these patients.

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CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interest with the contents of this article.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

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3. CONCLUSUION and FUTURE PERSPECTIVES / ZUSAMMENFASSUNG und AUSBLICK

3.1 Conclusion: molecular characteristics in AML

The publications discussed in the here presented work on molecular biomarkers have contributed to a better understanding of leukemogenic pathways, let to improved risk assessment and demonstrated possible novel treatment strategies in AML. Besides the importance for translational and functional research in the presense and future improved molecular characterisation of AML patients has the potential to adjust risk assessment at diagnosis or during disease course as demonstrated in multiple examples in this work. Some molecular markers have already been integrated in today's risk stratification which underlines the importance of these findings. Apart from coding genes and proteins, miRs may function as targets for new treatment approaches, e.g. by upregulation/ downregulation following sequenical treatment prior to cytotoxic therapy. Promising data is provided in animal studies and early clinical phase trials in examples in this work. Furthermore, the burden of leukemia initiating cells has been shown to provide high prognostic significance and can also contribute to risk assessment, highlighting athe importance of clinically evaluating therapeutic approaches to tartget this cell population.

3.2 Clonal evolution

Even though substantial advancements have been achieved in understanding AML biology - that also led to the development of novel targeted therapies - and patients' risk stratification has continuously been improved, outcomes for most AML patients' remain unsatisfactory. Novel methodologies, such as next-generation-sequencing to explore moleculargenetic and epigenetic changes that drive AML leukemogenesis are beeing implemented and help to understand this clinically and genetically very heterogeneous disease.

In the last years substantial effort has been put into further understanding pre-AML conditions and the definition of pre-leukemic stages has evolved.[97-99] Patients diagnosed with myelodysplastic syndrome, individuals with germline mutations in certain transcription factors (such as *RUNX1* or *CEBPA*), or with preceding therapies for unrelated neoplasms such as

alkylating chemotherapy or radiation show an elevated risk to develop AML.[97,98,100] Apart form these conditions, recently it was noted that some mutations found in AML patients (*e.g.* in the *DNMT3A*, *TET2*, *IDH1* or *IDH2* gene) can also be found in older 'healthy' individuals who have an increased risk of developing frank AML.[99,101,102] In 'healthy' individuals these mutations have been linked to cloncal hematopoiesis, and the term 'clonal hematopoiesis of indeterminate potential' (CHIP) was coined, indicating a pre-leukemic condition similar to monoclonal gammopathy of undetermined significance (MGUS) or monoclonal B-cell lymphocytosis (MBL). Understanding the connection of these mutations linked to the phenomenon of clonal hematopoiesis, AML and the impact on prognosis and treatment may provide important information to improve patients' outcomes in the future and today represents an active field of research.

3.3 Further defining the leukemia initiating cell population

Since AML is thought to be initiated and maintained by a population of leukemia initiating cells, in the past years growing efforts have been put into identifying the phenotype (*e.g.* the CD34+/CD38- subpopulation[84] or expression of the G protein-coupled receptor 56 [GPR56][103]) of these cells. Additionally, there is increasing evidence that a high expression of stem cell gene signatures is associated with poor clinical outcomes.[104,105] Since also the diagnostic burden of these cells has been associated with outcome it seems crucial to identifiy and target this populations to improve remission rates and long term survival for AML patients. Additionally, better characterisation of the leukemia initiating cell phenotyp may improve methods for MRD assessment, since it may allow for a more sensitive and clinical significant detection of remaining disease burden during or after AML treatment.

These recent developments are examples of current focuses in the field of translational AML research that may in the future be able to further improve AML risk stratifications, treatment, and outcomes.

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5. INDEX OF ABREVIATIONS / ABKÜRZUNGSVERZEICHNIS

A

AML	acute myeloid leukemia
APL	acute promyelocytic leukemia
ASXL1	additional sex combs like 1
ATRA	all-trans retinoid acid

B

BAALC	brain and acute leukemia, cytoplasmic

C

CBF	core-binding factor
CD	cluster of differentiation
CDK6	cyclin dependent kinase 6 gene
CEBPA / C/EBPα	CCAAT/enhancer-binding protein alpha gene / protein
CHIP	clonal hematopoiesis of indeterminate potential
CIR	cumulative incidence of relapse
CN-AML	normal cytogenetics
CR	complete remission

D

DFS	disease-free survival
DNMT	DNA methyltransferase

E

EFS	event-free survival
ELN	European LeukemiaNet
ERG	ETS-related gene
ETV6	translocation-Ets-leukemia virus

F

FAB	French American British
FLT3-ITD	internal tandem duplikation in the Fms related tyrosine kinase 3 gene
FLT3-TKD	tyrosine kinase domain of the Fms related tyrosine kinase 3

G

GvL	graft versus leukemia
GPR56	G protein-coupled receptor 56

H

HSCs	hematopoietic stem cells
HSCT	hematopoietic stem cell transplantation
HOX	homebox

I

IDH	isocitrate dehydrogenase

K

KRAS	kirsten rat sarcoma

M

MAPK	mitogen-activated protein kinase
MPL	monoclonal B-cell lymphocytosis
MGUS	monoclonal gammopa
miR	microRNA
MN1	meningeoma-1
MHY11	myosin heavy chain 11
MRD	measurable residual disease

N

NPM1	nucleophosmin-1
NRAS	neuroblastoma rat sarcoma

O

OS	overall survival

R

RAS	rat sarcoma
RUNX1	Runt-related transcription factor 1

S

SP1	specificity protein 1

T

TP53	tumor protein 53

W

WHO	World Health Organisation

6. Eidesstattliche Erklärung zur vorgelegten Habilitationsschrift

Hiermit erkläre ich an Eides statt,

1. dass die vorliegende Habilitationsordnung der Medizinischen Fakultät der Universität Leipzig anerkannt wird;
2. dass die Habilitationsschrift in dieser oder ähnlicher Form an keiner anderen Stelle zum Zweck eines Graduierungsverfahrens vorgelegt wurde;
3. dass die Habilitationsschrift selbstständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt wurden;
4. dass die Einhaltung der Satzung der Universität Leipzig zur Sicherung guter wissenschaftlicher Praxis“ in der Erstfassung vom 17. April 2015 Grundlage der Forschungstätigkeit war.

Dr. med. Sebastian Schwind

Leipzig, 10.10.2017

8. COMPLETE LIST OF PUBLICATIONS

Peer-Reviewed Publications

1 Garzon R, Liu S, Fabbri M, Liu Z, Heaphy CEA, Callegari E, **Schwind S,** Pang J, Yu J, Natarajan MN, Havelange V, Volinia S, Blum W, Rush LJ, Perrotti D, Andreeff M, Bloomfield C.D, Byrd JC, Chan K, Wu LC, Croce CM, Marcucci G: *MicroRNA-29b* induces global DNA hypomethylation and tumor suppressor gene reexpression in acute myeloid leukemia by targeting directly DNMT3A and 3B and indirectly DNMT1. *Blood.* 2009; 113:6411-8.

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8. CURRICULUM VITAE

PERSÖNLICHE DATEN

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SCHULISCHE AUSBILDUNG

1983-1989	Wetzlargrundschule in Berlin
1989-1996	Leonardo-da-Vinci Gymnasium in Berlin

STUDIUM

1996-2003	Studium der Humanmedizin an der Feien Universität Berlin
März 1999	Famulatur in der Gefäßchirurgie, Reutingen
März 2000	Famulatur in der Traumalogie, Berlin
August 2000	Famulatur in der Endokrinologie, Berlin
März 2001	Famulatur in der plastischen Chirurgie, Kap Stadt, Südafrika
September 2001	Famulatur in der klinischen Pharmakologie, Berlin
Oktober 2002- Februar 2003	Praktisches Jahr (1.Tertial) in der Inneren Medizin, Kantonsspital Aarau, Aarau, Schweiz
Februar 2003- Juni 2003	Praktisches Jahr (2. Tertial) in der Anästhesiologie Klinikum Neuköln, Berlin
Juni 2003- September 2003	Praktisches Jahr (3. Tertial) in der Allgemeinchirurgie, Universitätsklinikum Benjamin Franklin, Berlin

PROMOTION

Titel: „The prognostic Impact of *microRNA-181a* expression levels in patients with cytogenetically normal acute myeloid leukemia";
Note: summa cum laude

BERUFLICHER WERDEGANG

2004- 2005	Medical Advisor/ Product Manager bei Sanofi-Synthelabo, Berlin Internal Medicine und Neurology Drugs
Seit November 2005	Arzt in Weiterbildung Department für Innere Medizin, Neurologie und Dermatologie Selbststäige Abteilung für Hämatologie und Internistische Onkologie, Hämostaseologische Ambulanz
April 2008– August 2012	Research Fellowship an der Ohio State University (OSU), Columbus, OH; USA; Comprehensive Cancer Center

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European Hematology Association (EHA)

GUTACHTERTÄTIGKEIT

Leukemia
Leukemia Research
Onkologie
The International Journal of Biochemistry & Cell Biology

AUSZEICHNUNGEN

2009 ASCO Cancer Foundation Merit Award
2010 AACR-GlaxoSmithKline Outstanding Clinical Scholar Award
2011 EHA-ASH Translational Research Training in Hematology Award
2012 AACR-Aflac, Incorporated Scholar-in-Training Award
2013 EHA Travel Grant
2014 Pomblitz-Award of the University of Leipzig, Germany

DRITTMITTEL

Stiftung Leukämie des Kompetenznetzes „ Akute und chronische Leukämien“
Title: „ Die funktionelle Bedeutung differentieller ERG Expression in der Akuten Myeloischen Leukämie“
15.000,00 EUR,
Duration: 01.05.2013 bis 30.05.2014

Jose Carreras Leukämie Stiftung
Titel: „Die funktionelle Relevanz und therapeutische Implikationen aberranter ERG Expression in der akuten myeloischen Leukämie“
198.040,00 EUR
Duration: 01.09.2014 bis 30.08.2016

Zusammen gegen den Krebs e.V.
Titel: “Die Rolle von long non-coding RNAs (lncRNAs) in Neoplasien mit *IDH* und *DNMT3A*-Mutationen”
166.600,00 EUR
Duration: 01.12.2015 bis 30.04.2018

Zusammen gegen den Krebs e.V.
Titel: “Die Synthese und Testung von Zweit-Generationsinhibitoren des Transkriptionsfaktors ERG”
179.347,20 EUR,
Duration: 01.12.2015 bis 30.04.2018

Jose Carreras Leukämie Stiftung
Titel: „Der Einfluss des molekularen Resterkrankungsnachweises im Krankheitsverlauf auf die Prognose von Patienten mit akuter myeloischer Leukämie“
156.448,00 EUR
Duration: 01.02.2017 bis 30.01.2019

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