Abstract

**Purpose:** In acute myeloid leukemia (AML) without retinoic acid receptor (RAR) rearrangement, the effect of all-trans-retinoic acid (ATRA) is still poorly understood despite an association of NPM1 mutation and ATRA response. Recently, preferentially expressed antigen in melanoma (PRAME) has been shown to be a dominant repressor of RAR signaling.

**Experimental Design:** Thus, we further investigated ATRA response mechanisms, especially the impact of PRAME expression on ATRA responsiveness. We profiled gene expression in diagnostic samples derived from our AML HD98B trial, in which ATRA was administered in addition to intensive chemotherapy.

**Results:** Our data revealed a PRAME expression-associated gene pattern to be significantly enriched for genes involved in the retinoic acid metabolic process. In leukemia cell line models, we could show that retinoic acid-regulated cell proliferation and differentiation are impacted by PRAME expression. In patients with primary AML, repressor activity of high-PRAME levels might be overcome by the addition of ATRA as indicated by better outcome in 2 independent studies (P = 0.029).

**Conclusions:** PRAME seems to impair differentiation and to increase proliferation likely via blocking RAR signaling, which might be reversed by ATRA. PRAME therefore represents a promising target for both ATRA treatment and possibly future immunotherapeutic approaches in AML. *Clin Cancer Res; 19(9); 2562–71. ©2013 AACR.*

Introduction

The translocation t(15;17) in acute promyelocytic leukemia (APL) was among the first recurrent aberrations detected by chromosome banding analysis in acute myeloid leukemia (AML; ref. 1). APL also became the first human leukemia successfully treated by molecularly targeted therapy. Using all-trans-retinoic acid (ATRA), which targets the rearranged retinoic acid receptor-α (RARα) in the fusion gene PML-RARA, the repressive potential of PML-RARA on the transcription of wild-type RARA target genes could be successfully overcome (2).

Today, molecular insights have also grown significantly in non-APL AML based on the advancement of novel technologies allowing comprehensive genomic, transcriptomic, and epigenomic analyses (3–5). This improved understanding of the molecular aberrations underlying the disease started to translate into daily routine and AML guidelines support genotype-specific treatment approaches (6). For example, NPM1 mutations represent the most frequent genetic alteration found in 45% to 64% of cytogenetically normal acute myeloid leukemia (CN-AML). Recently, this entity has been included in the revised World Health Organization classification as novel provisional molecularly defined AML entity. "Targeted" therapies investigated within clinical trials provided evidence that ATRA in combination with chemotherapy might be a powerful therapeutic approach in NPM1-mutated AML (7). However, so far, little is known about the functional role of ATRA in NPM1-mutated AML.

Preferentially expressed antigen in melanoma (PRAME) is a dominant repressor of RAR signaling. It binds to RAR in the presence of retinoic acid, and thus prevents ligand-induced receptor activation and target genes transcription (8). Retinoic acid induces the transcription of a set of target genes by binding of its active receptor RAR. This results in differentiation and cell-cycle arrest of responsive cells, and therefore, loss of retinoic acid responsiveness might be beneficial to cancer cells (9). PRAME is found to be over-expressed in many solid tumors as well as in hematologic malignancies like AML (10). In accordance, downregulating
In this work, we now further highlight an important role of PRAME in the regulation of cell proliferation and differentiation in AML cells. In addition, based on data from 2 independent clinical trials, we provide evidence that PRAME might be a predictive marker for ATRA response in patients with non-APL AML treated with a combination of chemotherapy and ATRA.

Materials and Methods

AML patient samples

Diagnostic peripheral blood and bone marrow samples from adult patients with AML (n = 80) were provided by the German-Austrian AML Study Group (AMLSG) with informed consent obtained from all patients entered within the AML HD98B trial (7). Clinical information and response to ATRA are provided as Supplementary Table S1 (n = 39 patients had been randomized into the ATRA treatment arm). Moreover, we investigated previously published data (17) from an independent cohort of patients (n = 69) treated within a randomized phase II study on ATRA in combination with induction and consolidation therapy (AMLSG 07-04; clinicaltrials.gov identifier NCT00151242). Here, younger patients with AML (≤60 years) were randomized upfront for open-label treatment with ATRA (n = 28 patients were randomized into the ATRA treatment arm and compared with n = 41 patients from the non-ATRA arm).

Leukemia samples were enriched for leukemic cells by Ficoll separation (Biochrom) and stored for RNA preparation at −80°C. For cellular assays, Ficoll-separated peripheral blood mononuclear cells (PBMC) were tested freshly or following cryopreservation in AB serum (IKT) containing 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich).

Gene expression profiling analysis in primary leukemia samples

Gene expression profiling (GEP) was conducted as previously described using cDNA microarrays (18). The complete gene expression microarray dataset is available at Gene Expression Omnibus (GEO; accession number: GSE16432). For supervised analysis, cases were grouped on the basis of PRAME expression and the highest and lowest quartiles compared with ClassComparison using BRB-Array Tools Version 3.6.1 (developed by Dr. R. Simon and A.P. Lam, National Cancer Institute, Bethesda, MD; available at http://linus.nci.nih.gov/BRB-ArrayTools.html) and R Version 2.6.0 (available at www.r-project.org). Gene signatures were further evaluated using the Molecular Signatures Database (MSigDB, http://www.broadinstitute.org/gsea/msigdb; ref. 19), and Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.abcc. nciifcrf.gov/; ref. 20). For the correlation with survival data expression, values have been dichotomized by the median expression of the respective gene across all AML samples, and Kaplan–Meier curves were plotted using GraphPad Prism 4 (GraphPad Software Inc.).

For further evaluation of the impact of PRAME expression on ATRA responsiveness, we evaluated a previously published independent data set profiled on Affymetrix
HG-U133 plus 2.0 microarrays (GEO accession number: GSE15434; ref. 17).

Western blot analysis and quantitative real-time PCR

Western blot analysis was conducted according to standard protocols as previously described using a polyclonal PRAME antibody (Abcam) and a β-actin antibody as loading control (Sigma-Aldrich; ref. 21).

The mRNA expression of PRAME was measured by quantitative real-time PCR (qRT-PCR) using light cycler SYBR Green I technology and previously published primers according to the manufacturer’s instructions (22). The amount of mRNA of PRAME was normalized to the housekeeping gene TATA-box binding protein.

Culture of cell lines

The human cell lines K562, THP-1, KG-1, and Kasumi-1 were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). Cell lines were cultured under standard conditions in RPMI-1640 medium (Biochrom) containing 10% to 20% fetal calf serum (FCS), 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 U/mL streptomycin.

Cell culturing using ATRA

ATRA was obtained as a powder (Retinoin, Sigma-Aldrich), dissolved in DMSO and stored at −20°C. For cell culture experiments, the ATRA stock was further diluted in RPMI-1640 medium containing 10% FCS, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 U/mL streptomycin.

Conventional RT-PCR

mRNA was isolated from PBMC of healthy controls or tumor samples using the mRNA QuickPrep Micro Purification Kit (Amersham Pharmacia Biotech). Two microgram of each mRNA sample was subjected to CDNA synthesis (Superscript II Gibco BRL). PCR for PRAME was conducted as previously described using the indicated conditions and reagents (22).

siRNA-Mediated PRAME knockdown

Four different siRNAs specific for PRAME as well as a negative scrambled siRNA (scrRNA) control were obtained from Qiagen and tested toward their ability to decrease PRAME expression in the K562 cell line. Briefly, 2 to 3 × 10⁶ cells were pelleted, resuspended in 100 μL Cell Line Nucleofector Solution V (Lonza Cologne), and nucleofected with up to 200 nmol/L siRNA using the program V-001 for KG-1, and 24 hours after nucleofection, different concentrations of ATRA were added and cells kept in culture for up to 6 days (medium was changed every 3 days with or without ATRA).

BrdUrd-proliferation assay

After transfecting the cell lines and adding ATRA at different concentrations, cells were labeled with bromodeoxyuridine (BrdUrd) for 2 or 24 hours according to the manufacturer’s instructions (BD Biosciences). Thereafter the BrdUrd Flow Kit staining protocol was carried out for intracellular staining to determine the proliferation ability of the transfected or nontransfected cells.

Flow cytometry-based measurement of differentiation and apoptosis markers

CD66b is a cell surface antigen expressed in mature myeloid cells and was used as a marker for lineage-specific cell differentiation. Following transfection with either PRAME plasmid or short hairpin RNA (shRNA) directed against PRAME, flow cytometry was conducted to determine the fraction of CD66b-positive cells. Samples were stained with 10 μL of CD66b antibody (BD) without ATRA at 4°C for 20 minutes and analyzed by flow cytometry using a fluorescence-activated cell sorting (FACS) Calibur analyzer. Each sample was run with the corresponding isotype control to define the gate of positive cells.

For the detection of apoptosis, we used Annexin V and propidium iodide (PI) staining. Cells were stained with Annexin V and/or PI according to the manufacturer’s protocol (BD). Data analysis was conducted using the Weasel software.

Gene expression profiling following siRNA-mediated PRAME knockdown and ATRA treatment in the K562 cell line model

For the cell line models, GEP was conducted using Affymetrix microarray technology according to the manufacturer’s recommendations as previously reported (Human Genome U133plus2.0 Array, Affymetrix; ref. 21). SiRNA- and scrRNA-treated as well as ATRA-treated K562 cells were profiled in triplicate stemming from independent experiments. Fluorescence ratios were normalized by applying the Robust Multi-array algorithm using the BRB Array Tools software package (see above). The complete microarray data are available at the GEO (accession number: GSE43258).

Results

PRAME expression-associated GEP in AML

We previously observed that non-APL AML cases, and especially those who are NPM1-mutated, might benefit from a combination of chemotherapy and ATRA (7).
Furthermore, a recent report showed that retinoic acid-associated regulation of cell proliferation and differentiation was impacted by PRAME expression (16). PRAME is a ligand-dependent corepressor of RARα, RARβ, and RARγ signaling (8). On the basis of these observations, we wanted to investigate the role of PRAME on ATRA response in non-APL cases. Thus, we aimed to define a PRAME expression-associated gene pattern in our primary AML cases as such a gene pattern might provide further insights into the association of PRAME expression with deregulated RAR signaling in AML.

In a first step, we verified that PRAME mRNA expression correlated with PRAME protein levels in myeloid leukemia cells by Western blot analysis (Fig. 1A) and that PRAME expression was well measured by our microarray by validating PRAME expression levels by qRT-PCR (Fig. 1B) in a random set of cases (n = 29). qRT-PCR results for PRAME correlated well with our microarray findings (P < 0.001; Fig. 1B), and interestingly PRAME was not correlated with any of the molecular markers NPM1 mutation, CEBPA mutation, or FLT3 internal tandem duplication (ITD; see Supplementary Table S1). We next grouped our primary AML cases based on PRAME expression (n = 80 cases with valid information on PRAME expression) and identified genes and gene sets characterizing the AML cohorts defined by either low or high-PRAME expression. ClassComparison analysis revealed 1,051 clones to be significantly differentially expressed between the PRAME expression subgroups at the nominal P = 0.05 level of significance in univariate tests (760 clones were positively and 291 negatively correlated; Fig. 1C and Supplementary Table S2). Notably, this PRAME-associated signature was found enriched for gene set modules associated with gene expression changes in the leukemia cell line U937 following immune response and myeloid differentiation in K562 (e.g., MSigDB module 52, P < 0.001; Supplementary Table S3), and functional annotation using DAVID revealed an enrichment of annotation terms associated with RAR signaling. For example, there was a correlation with the annotation terms "response to vitamin A," "response to retinoic acid," and "retinoid binding," "retinoid metabolic process," "retinol binding," and "retinoic acid metabolic process" (P < 0.05; Fig. 1D).

**PRAME expression in cell lines**

Next, we studied PRAME expression in leukemia cell lines to identify models for further functional analysis of the role of PRAME in retinoic acid signaling. On the basis of a previous GEP study analyzing different myeloid leukemia-derived cell lines (23), we could identify cell lines with either higher (HL-60, MONO-MAC-1, K562, UT-7, and THP-1) or lower PRAME expression levels (OCI-AML2, KG-1, NB4, ME1, MV4-11, and Kasumi-1; 35-fold mean PRAME expression difference between cell line groups; Fig. 1E and Supplementary Table S4). Following validation of PRAME expression levels by qRT-PCR, we decided to use K562 and THP-1 as "PRAME-high" and KG-1 and Kasumi-1 as respective "PRAME-low" model to study the impact of ATRA.

**Response to ATRA in leukemia cell lines is dependent on PRAME expression level**

Higher doses of ATRA similar to the doses used for the clinical treatment of patients with AML (note: the highest concentration of 10^{-6} mol/L used in the manuscript is still in the range of peak plasma levels seen in patients treated with ATRA; ref. 24) led to reduced cell numbers and cell proliferation of leukemic blasts in vitro. Notably, in PRAME-low AML cells (Kasumi-1), a reduction of the proliferation rate in half as measured by BrdUrd incorporation was observed already at low ATRA concentrations and could not be further decreased by increasing ATRA levels (Fig. 2A). In contrast, in PRAME-high (THP-1) AML cells increasing ATRA concentrations were associated with continuously decreasing proliferation rates (Fig. 2B). Although the proliferation of PRAME-low cells could not be intensively inhibited with higher ATRA concentrations, with regard to the impact on cell numbers, these cells were nevertheless quite sensitive to ATRA (Fig. 2A). In contrast, PRAME-high cells showed a dose-dependent effect for both the reduction of cell numbers and the proliferation rate (Fig. 2B).

ATRA treatment-induced cellular differentiation, reflected by the expression of the myeloid differentiation marker expressed on granulocytes CD66b (25), for which the cell surface expression was measured by FACS analysis, was dependent on the expression of PRAME. The PRAME-low AML cell lines, KG-1 and Kasumi-1, reacted already with a high differentiation rate at a low ATRA concentration, and the expression of CD66b did not further increase using higher ATRA doses (Fig. 2C). Although the percentage of CD66b-expressing cells was very low in the PRAME-high leukemia cells, treatment with higher ATRA concentrations increased the percentage of CD66b-expressing cells (Fig. 2D). These results suggest that PRAME may block differentiation and increase proliferation via PRAME-mediated inhibition of RAR signaling, which might be overcome with increasing doses of ATRA. In contrast, in PRAME-low cases, even low ATRA doses lead to a differentiation initiation, suggesting that the RAR-signaling pathway is responsive and not blocked in these cells.

To further evaluate the ATRA-induced decrease in cell numbers, we also investigated a potential role of apoptosis in the PRAME-high and -low cell lines. Although no relevant changes in the apoptosis rate were observed in PRAME-high cells treated with increasing ATRA concentrations (Supplementary Fig. S1), the operating mode of ATRA in myeloid leukemia cells seems to be primarily the modulation of proliferation and differentiation.

**siRNA-Mediated PRAME knockdown impacts ATRA response**

To further study the role of PRAME expression, we transfected the PRAME-high leukemia cell line K562 with 4 different shRNA molecules (siRNA) targeted against PRAME. Using nucleofection, we could achieve an average transfection rate of 45% to 50% (26) resulting in an approximately 50% reduction of PRAME expression for siRNA#8 following 24 and 72 hours posttransfection (Fig.
3A). Using this siRNA, we then treated siRNA- and scrRNA-
transfected K562 cells with ATRA at a high concentration of
10^{-7} mol/L. Following siRNA#8-mediated PRAME knock-
down proliferation rate and differentiation, potential of the
leukemia cells were again analyzed by measuring cell counts
and CD66b cell surface expression (see above). Cell
demonstrated that PRAME knockdown increased the ATRA-induced differentiation more than 4-fold (Fig. 3B), thereby further suggesting a PRAME-induced block of RAR signaling. In line, cell counts were also more effectively decreased by low-dose ATRA treatment in PRAME-high leukemia cells following siRNA-mediated PRAME knockdown as compared with scrRNA-transfected K562 controls (Fig. 3C).

**PRAME overexpression in PRAME-low cells mitigates ATRA response**

To determine whether ATRA response could be mitigated in PRAME-low cells, we overexpressed PRAME in KG1 cells by transfecting a plasmid coding for PRAME. Despite a respectively low-transfection efficiency (20%-30%, data not shown), PRAME overexpression led to more than 2-fold increase of cells numbers (Supplementary Fig. S2). We again investigated changes following ATRA treatment. Using increasing ATRA concentrations, we observed a larger reduction of cell numbers in PRAME transfected than in mock-treated cells, although due to low transfection rates, results have to be cautiously interpreted. Therefore, these data provide only a hint for a respective PRAME-mitigated ATRA response.

### siRNA-Mediated PRAME knockdown associated gene expression changes

To gain additional insights into the effects following PRAME knockdown in PRAME-high cells, we profiled gene expression in siRNA- and scrRNA-transfected K562 cells and conducted a ClassComparison analysis for paired samples, which revealed 485 genes to be significantly differentially

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**Figure 2.** Proliferation and differentiation of PRAME-high or -low AML cells in response to ATRA treatment. A, PRAME-low AML cell line Kasumi-1 shows a low proliferation rate measured by BrdUrd treated with ATRA at different concentrations in contrast to PRAME-high THP-1 cells (B). The proliferation of PRAME-low cells did not intensively decrease with higher ATRA concentrations in contrast to PRAME-high cells where a markedly reduced proliferation could be detected in these cells treated with higher ATRA concentration. C, CD66b cell surface FACS analysis showed also a relatively dose-independent differentiation of PRAME-low cells as a high differentiation rate was already observed at low ATRA concentration and the differentiation/expression of CD66b did not increase with high ATRA doses. D, in contrast, PRAME-high cells showed a low differentiation rate at low ATRA concentrations, but the CD66b expression could be increased using high ATRA doses.
expressed ($P < 0.05$, univariate $t$ test). As anticipated, the top candidate was PRAME, which in line with the qRT-PCR data was found to be repressed approximately 2-fold by siRNA#8 (Fig. 4A and Supplementary Table S5).

Interestingly, the PRAME knockdown-associated signature was similar to the primary leukemia-derived PRAME–expression-associated gene pattern as it showed an enrichment of gene sets associated with myeloid differentiation and CD34+ stem cells (e.g., module 15; Supplementary Table S6). In accordance, DAVID functional annotation clustering revealed, for example, "stem cell development" and "stem cell differentiation" among the significant gene ontology terms ($P = 0.0096$ and $P = 0.017$, respectively; Enrichment Score: 1.44; data not shown). Functional annotation also revealed, for example, the terms "negative regulation of RAR signaling pathway" and "regulation of RAR signaling pathway" ($P = 0.0058$ and $P = 0.0097$, respectively). Notably, PRAME seems to be also involved in epigenetic mechanisms, as MSigDB analysis showed an overlap with HDAC3 and HDAC1 knockdown-associated gene expression changes (Supplementary Table S6), and in line the DAVID functional annotation showed significant results, for example, for acetylation, chromatin remodeling, and

Figure 3. siRNA-mediated PRAME knockdown in PRAME-high cell line: impact on ATRA-induced differentiation. A, transfection of PRAME-high leukemic cells (K562) with different siRNAs directed against PRAME. The most significant reduction in PRAME expression measured by RT-PCR was obtained with siRNA#8 following 24 and 72 hours after transfection (~50% reduction, scrRNA). B, CD66b cell surface expression of leukemic cells treated with ATRA at a concentration of $10^{-8}$ M/L following PRAME knockdown using siRNA#8. After 72 hours, cell differentiation as indicated by CD66b expression could only be mildly increased in PRAME-high K562 cells treated with scrRNA. However, PRAME knockdown significantly increased the ATRA-induced differentiation with results resembling findings in PRAME-low cells, thereby further suggesting a PRAME-blocked RAR differentiation. C, in accordance, cell growth as measured by cell counts was also more effectively inhibited by low-dose ATRA treatment in PRAME-high leukemia cells following siRNA-mediated PRAME knockdown.

Figure 4. Gene expression changes associated with siRNA-mediated PRAME knockdown and ATRA treatment. A, ClassComparison findings comparing siRNA knockdown and scrRNA K562 samples are depicted as volcano plot that combines the $t$ test $P$ value with the magnitude of the expression change following PRAME knockdown. On the $y$-axis the negative log$_{10}$ of the $P$ value and on the $x$-axis the log of the fold change following siRNA-mediated PRAME knock down (downregulated genes are plotted on the left). B, volcano plot depicting ClassComparison findings for ATRA-treated/untreated K562 samples.

and "stem cell differentiation" among the significant gene ontology terms ($P = 0.0096$ and $P = 0.017$, respectively; Enrichment Score: 1.44; data not shown). Functional annotation also revealed, for example, the terms “negative regulation of RAR signaling pathway” and “regulation of RAR signaling pathway” ($P = 0.0058$ and $P = 0.0097$, respectively). Notably, PRAME seems to be also involved in epigenetic mechanisms, as MSigDB analysis showed an overlap with HDAC3 and HDAC1 knockdown-associated gene expression changes (Supplementary Table S6), and in line the DAVID functional annotation showed significant results, for example, for acetylation, chromatin remodeling, and
bromodomains, although results have to cautiously interpreted \( P < 0.05 \) for all terms, ES > 1.54 in all cases; data not shown).

Next, we also evaluated the ATRA response at the gene expression level in K562 (ATRA response signature). Although we observed significant expression changes for 427 genes, more than 80% of genes were upregulated following ATRA treatment \( (P < 0.05, \text{Fig. 4B and Supplementary Table S7}) \). Notably, we observed a prominent upregulation of a \( \text{HOX} \) gene signature including, for example, \( \text{HOXA1}, \text{HOXA3}, \text{HOXB3}, \text{MEIS1}, \) and \( \text{PBX1} \). Similarly, in line with the location of miR-10a within the \( \text{HOX} \) gene cluster, we observed a significant enrichment of miR-10a/10b target genes \( (P < 0.05, \text{Supplementary Table S8}) \). Thus, in \( \text{PRAME} \)-high K562 cells ATRA-induced upregulation of \( \text{HOX} \) genes, a gene pattern characteristic for \( \text{NPM1} \)-mutated AML (17), might confer a favorable impact on the response to conventional chemotherapy similar to the favorable response to chemotherapy seen in \( \text{NPM1} \)-mutated AML.

Impact of \( \text{PRAME} \) expression on clinical outcome in patients treated with ATRA

Finally, we went back to our primary AML cases treated with ATRA and investigated the impact of \( \text{PRAME} \) expression on the treatment outcome. Although we only had limited samples with information on \( \text{PRAME} \) expression, we nevertheless observed a trend for improved overall survival in patients with ATRA-treated \( \text{PRAME} \)-high AML as compared with all other as well as patients with ATRA-treated \( \text{PRAME} \)-low AML \( (P = 0.11 \) and \( P = 0.16, \) respectively; log-rank test; Fig. 5A and B).

To further evaluate this potential association, we investigated the impact of \( \text{PRAME} \) expression on the clinical outcome of an independent cohort of younger patients with AML treated with ATRA within the randomized phase III study AMLSG 07-04. In this cohort, ATRA treatment in \( \text{PRAME} \)-high patients expressing was significantly associated with improved outcome in comparison with all other cases, and ATRA-treated \( \text{PRAME} \)-high patients tended to have a better outcome than ATRA-treated \( \text{PRAME} \)-low AML \( (P = 0.029 \) and \( P = 0.13, \) respectively; log-rank test; Fig. 5C and D).

Discussion

\( \text{PRAME} \) is an important target structure in tumors as this tumor-associated antigen is frequently expressed in different solid tumors and hematologic malignancies, whereas no expression is seen in most normal tissues or CD34-positive hematopoietic stem cells (10, 22, 27). Although the function and clinical relevance of \( \text{PRAME} \) is still not completely understood, in our current work, we could show that cell proliferation and differentiation was associated with the level of \( \text{PRAME} \) expression and can be influenced...
by ATRA, thereby suggesting that PRAME expression has an impact on the clinical outcome of patients with ATRA-treated AML (7).

PRAME, a dominant repressor of RAR signaling, binds to the RAR in the presence of retinoic acid, thus preventing ligand-induced receptor activation and target gene transcription (8). Retinoic acid induces transcription of a set of target genes through the binding and activation of its receptor, resulting in a differentiation and cell-cycle arrest in responsive cells. Therefore, loss of retinoic acid responsiveness might provide beneficial selection bias for cancer cells (9). In accordance, Oehler and colleagues showed that PRAME overexpression can contribute to leukemogenesis by inhibiting myeloid differentiation through the blockage of the RARα-signaling pathway (16). Thus, the regulation of RAR signaling by PRAME seems to be also very likely in primary AML, but this of course warrants further investigation.

In our study, analysis of different AML cell line models suggests that PRAME expression does not only impact differentiation, but also proliferation of the leukemic cells. Likely playing an important role, in PRAME-low AML cell lines, a high differentiation rate could already be achieved following the treatment with ATRA at low concentrations, thereby suggesting that the RAR-signaling pathway is responsive and not blocked in these cells. Similarly, in PRAME-low AML cases, excess ATRA might have no additional beneficial effect as RAR signaling-induced differentiation is not blocked to begin with and other yet unknown mechanisms account for the differentiation block, which cannot be overcome by ATRA. In accordance, differentiation reflected by the expression of CD66b could not be further increased by using higher ATRA doses. In contrast, AML cells characterized by high-PRAME expression levels showed only a low increase in the differentiation rate when treated with low-dose ATRA concentrations. However, CD66b expression could be notably increased by treating the cells with higher ATRA concentrations. This suggests that in these cells, PRAME inhibits cellular differentiation and by increasing ATRA concentrations the PRAME-induced block might be overcome and RAR signaling restored, thereby again enabling ATRA-dependent cell differentiation induction.

With regard to a potential impact on apoptosis, it recently has been shown that the TRAIL response to retinoic acid can be blocked by PRAME in CML (28). In CML, BCR-ABL-mediated upregulation of PRAME is leading to decreased TRAIL expression, thereby impairing TRAIL-induced apoptosis. However, in our patients with AML, we observed no inverse correlation of PRAME and TNFSF10 (encoding for TRAIL) expression (see Supplementary Table S2), thereby suggesting different mechanisms of apoptosis deregulation in AML. Notably, we also detected no relevant differences for the induction of apoptosis in PRAME-high or PRAME-low cell lines treated with ATRA. Therefore, in AML mechanisms of TRAIL action seem to be mainly influencing PRAME-mediated proliferation and differentiation changes, but not apoptosis.

On the basis of these observations, further studies are warranted to unravel the exact mechanisms of ATRA action in PRAME-high non-APL cases. One might hypothesize that PRAME overexpression phenocopies PML-RAR translocations in that both interfere with RAR signaling under physiologic concentrations of retinoic acid, and like in APL, ATRA might then trigger degradation of the inhibitory RARα–PRAME complex. Although retinoid acid-induced transcriptional activation is directly coupled to proteasome-mediated RARα degradation, this pathway accounts for retinoic acid-induced degradation of both RARα and APL-associated RARα fusions, and such RARα proteolysis is best seen with high doses of retinoic acid (29).

With regard to a potential impact of PRAME on RAR signaling in primary patients with AML (n = 80), our microarray-based observations in an ATRA-treated elderly AML cohort (n = 39) was in line with our in vitro data. These pointed to a PRAME-dependent effect of ATRA in AML, and in accordance, we observed a trend toward improved outcome within a previously published phase III study investigating the effect of ATRA in untreated elderly patients with AML (61 years or older; ref. 7). In the respective HD98B trial, we could show that addition of ATRA to conventional therapy can significantly improve the complete remission rate, event-free survival, and overall survival in patients with AML with the genotype NPM1 without concomitant FLT3-ITD mutation. This genotype has been associated with favorable outcome (6), and also seems to be a predictive marker for a response to ATRA (7). In the current analysis, we did not find a correlation of PRAME expression with neither NPM1 nor FLT3-ITD mutational status (see above). However, the ATRA response signature revealed a prominent induction of a gene expression pattern similar to the one observed in NPM1-mutated AML (17). These gene expression changes might confer a cellular state rendering the leukemia cells again more sensitive to chemotherapy, similar to the good chemotherapy response observed in NPM1-mutated FLT3-ITD-negative AML irrespective of ATRA treatment.

Although the treatment with ATRA in high-PRAME-expressing patients did not reach statistical significance in the elderly AML cases, an independent study of younger patients with AML within the AMLSG 07-04 trial with high-PRAME expression (n = 13) showed a significant clinical benefit when treated with ATRA as compared with all other patients (n = 56, P = 0.029).

In conclusion, PRAME seems to play a critical role in cell proliferation and differentiation and in addition may be of therapeutic use. In AML cases with higher PRAME expression levels, the addition of ATRA to chemotherapy and probably also the use of ATRA as maintenance therapy might further contribute to the elimination minimal residual disease in PRAME-high AML cases. Thus, in future, a combination of immunotherapy and ATRA as targeted therapy against PRAME-mediated blockage of RAR signaling might further improve patient management in PRAME-expressing AML cases.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: I. Bullinger, K. Döhner, M. Schmitz, J. Greiner
Development of methodology: A.C. Russ, V. Schneider, J. Greiner
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Bullinger, R.F. Schlenk, U. Boitzenhardt, S. Hofmann, A. C. Russ, A. Babiak, J. Greiner
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Bullinger, R.F. Schlenk, S. Hofmann, A. C. Russ, J. Greiner
Writing of the manuscript: L. Bullinger, R.F. Schlenk, M. Götz, S. Hofmann, A. Babiak, S. Collard, J. Greiner
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R.F. Schlenk, M. Götz, L. Zhang
Study supervision: L. Bullinger

References

PRAME-Induced Inhibition of Retinoic Acid Receptor Signaling-Mediated Differentiation—A Possible Target for ATRA Response in AML without t(15;17)

Lars Bullinger, Richard F. Schlenk, Marlies Götz, et al.