Frequent loss of RAF kinase inhibitor protein expression in acute myeloid leukemia

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Abstract

RAF kinase inhibitor protein (RKIP) is a negative regulator of the RAS-mitogen activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) signaling cascade. We investigated its role in acute myeloid leukemia (AML), an aggressive malignancy arising from hematopoietic stem and progenitor cells (HSPCs). Western blot analysis revealed loss of RKIP expression in 19/103 (18%) primary AML samples and 4/17 (24%) AML cell lines but not in ten CD34+ HSPC specimens. In *in-vitro* experiments with myeloid cell lines, RKIP overexpression inhibited cellular proliferation and colony formation in soft agar. Analysis of two cohorts with 103 and 285 AML patients, respectively, established a correlation of decreased RKIP expression with monocytic phenotypes. RKIP loss was associated with RAS mutations and in transformation assays, RKIP decreased the oncogenic potential of mutant *RAS*. Loss of RKIP further related to a significantly longer relapse free survival (RFS) and overall survival (OS) in uni- and multivariate analyses. Our data show that RKIP is frequently lost in AML and correlates with monocytic phenotypes and mutations in *RAS*. RKIP inhibits proliferation and transformation of myeloid cells and decreases transformation induced by mutant *RAS*. Finally, loss of RKIP seems to be a favorable prognostic parameter in patients with AML.

Keywords

RAF kinase inhibitor protein; acute myeloid leukemia; *RAS* mutation

Introduction

Acute myeloid leukemia (AML) is an aggressive malignancy caused by transformation of hematopoietic stem and progenitor cells (HSPCs). It is a heterogeneous disorder characterized by differentiation defects and uncontrolled growth of the leukemic clone ultimately leading to bone marrow failure. Classification of AML is based on clinical, morphological, immunological and genetic parameters.[1-4] Despite intensive treatment strategies including stem cell transplantation, the prognosis of patients with AML is still dismal with the majority succumbing to resistant disease.[3]

The RAS-MAPK/ERK pathway is activated by a spectrum of cytokine receptors in response to ligand binding and plays an important role with respect to proliferation, survival and differentiation of hematopoietic progenitors. Transmission of signals from the cell surface to intracellular effectors within this pathway is mediated by RAS-induced activation of a three-tiered kinase cascade comprising RAF, MEK and MAPK/ERK.[5, 6] Oncogenic mutations affecting this pathway are frequently observed in human cancers with *NRAS* or *KRAS* mutations occurring in about 20% of AML patients.[5] Constitutive RAS-MAPK/ERK signaling is also initiated by somatic mutations in genes encoding the upstream *FLT3* and *c-Kit* receptor tyrosine kinases in an additional 25-40% of AML cases.[3, 7, 8]

RKIP - also known as PEBP1, phosphatidylethanolamine binding protein 1 - has been identified as a negative regulator of the RAS-MAPK/ERK signaling cascade. It inhibits the interaction between C-RAF (also termed RAF1) and MEK thereby preventing C-RAF mediated MEK phosphorylation which is necessary for signal propagation.[9] More recently, RAS-MAPK/ERK independent functions have been described. RKIP suppresses

the activity of the NF-κB-Snail circuitry and inhibits the epithelial to mesenchymal transition program, which is a pivotal step in tumor invasion and the formation of metastasis.[10-12] In several solid neoplasms, RKIP expression is indeed frequently reduced or absent, and while this has no effect on the growth of the primary tumor it correlates with an increased risk of metastatic disease and enhanced invasiveness of cancer cells *in vitro*.[13-16]

We previously described loss of RKIP in patients with therapy-related AML and *C-RAF* germline mutations.[17, 18] RKIP silencing was shown to be a somatic, leukemia-specific event and contributed to C-RAF driven malignant transformation. However, it is unknown whether loss of RKIP is restricted to this small subset of cases or whether it is of broader significance for myeloid leukemogenesis.

Patients and methods

Patient samples and cell lines

One hundred and three blood and bone marrow samples from patients with AML (AML cohort 1) were collected at the Division of Hematology, Medical University of Graz, Graz, Austria, and processed as described.[18, 19] Ninety-five samples were obtained at diagnosis and eight at relapse of AML, all had a blast cell count of ≥80%. AML was classified according to French-American-British (FAB) and World Health Organization (WHO) guidelines.[1, 2] Cell lines were obtained from the German National Resource Center for Biological Material (DSMZ, Braunschweig, Germany) and regularly screened by variable number of tandem repeat profiling for authenticity.[20] Normal CD34+ HSPC specimens were isolated from leukapheresis harvests of six patients with non-myeloid

malignancies and four umbilical cord blood samples. The study was approved by the institutional review board of the Medical University of Graz, Graz, Austria, and informed consent was obtained from all individuals. For microarray analysis, a total of 285 AML patients were collected at the Erasmus University of Rotterdam, The Netherlands, as previously reported (AML cohort 2).[21] This cohort is also characterized with respect to mutations in *NRAS*, *KRAS*, *NPM1*, *CEBPA*, the *FLT3* tyrosine kinase domain (*FLT3*-TKD) as well as to *FLT3* internal tandem duplications (*FLT3*-ITD).

Protein and gene analyses

Western blot analyses were performed as described[17, 18] using the following antibodies: anti-RKIP (Upstate, Billerica, MA, USA), anti-Beta-Actin, anti-FLAG M2 (both from Sigma-Aldrich, St Louis, MO, USA), anti-ERK1/2, anti-phospho-ERK1/2 (all from Cell Signaling Technology, Beverly, MA, USA) and anti-GAPDH (Ambion/Applied Biosystems, Foster City, CA, USA). Data generated with Affymetrix U133A GeneChips have been used for analysis of *RKIP* and *Snail* mRNA expression.[21] *RKIP* sequencing, as well as methylation specific PCR and copy number specific quantitative real-time PCR were carried out as reported earlier.[17, 22] Codons 12, 13 and 61 of *NRAS* and *KRAS* were analyzed by PCR and direct sequencing as detailed in Supplementary Table 1. Quantitative real-time PCR expression analysis was performed as described[23] using the TaqMan® Gene Expression Assay (Applied Biosystems) as outlined in Supplementary Table 1.

Cell culture and transfection of hematopoietic cell lines

U937 and THP-1 were maintained at 37°C/5%CO_2 in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS). 32D cells were cultured in the same way, but with additional supplementation of 10% WEHI-conditioned medium as a source of IL-3.[24] Cells were transfected using Amaxa Nucleofection (Lonza, Cologne, Germany) according to the manufacturer's protocol. For generation of cell lines with stable expression of *pMSCV-FLAG-hRKIP* or empty vector, $2x10^6$ THP-1 cells were transfected with 4 µg and $1x10^6$ U937 cells with 2 µg of DNA, respectively. Selection was performed using 250 ng/ml puromycin for THP-1 and 300 ng/ml for U937, respectively. 32D cells expressing the same set of constructs have been generated as described.[24]

Analysis of cell growth, apoptosis and proliferation

For determination of viable cells, proliferation and apoptosis, THP-1 were seeded at a density of $1x10^4$ cells/ml in media supplemented with 5% FCS and at a density of $2x10^5$ cells/ml in media with 0.05% FCS, respectively. 32D were seeded at a density of $5x10^5$ cells/ml in media supplemented with 10% FCS/1% WEHI-conditioned medium and U937 were seeded at a density of $2x10^5$/ml in media supplemented with 0.05% FCS. The amount of viable cells was measured at the indicated time points using a Casy Cell Counter (Roche Innovatis, Bielefeld, Germany) or via a trypan blue exclusion assay, respectively. Propidium iodide (PI)/bromodeoxyuridine (BrdU) assays were performed as described previously.[25] Incubation with 25 µM BrdU was performed for 1h. Apoptotic cells were defined by DNA content (subG1 peak).[26]

Focus formation and soft agar colony assay

Early passage NIH3T3 cells were seeded at low density and transfected the following day with 0.1 µg *HRAS V12* or 0.1 µg *KRAS V12*, respectively, with or without 1.2 µg *RKIP* using Lipofectamine 2000 (Invitrogen, Paisley, UK) according to the manufacturer's protocol. Cells were then cultured in 10% donor bovine serum for 2 weeks, fixed in methanol, stained in Giemsa solution and foci were counted. Soft agar assays were carried out as described.[17, 18]

Statistical analysis

The frequency of patients with RKIP loss in the AML FAB M4/M5 vs. non-M4/M5 subtypes were compared using Fisher's exact test. Cluster analysis was performed on Affymetrix U133A GeneChips expression profiling data as previously described.[21] For comparison of *RKIP* mRNA expression between patients with AML FAB M4/M5 and non-M4/M5, between cluster 5 and non-cluster 5, and between patients with and without mutations in *RAS, FLT3, NPM1* and *CEBPA*, respectively, the Mann-Whitney-Wilcoxon test was applied. *P*-values presented are the mean of three probe sets of *RKIP* expression. For analysis of *in-vitro* experiments, paired and unpaired Student's t-tests, respectively, were calculated from at least 3 independent experiments. Survival rates were obtained from the Kaplan-Meier estimator. The effect of risk factors was assessed by a marginal and a conditional proportional hazards model (Cox model). Marginal effects of dichotomous risk factors were tested by the log-rank test. The effect of natural logarithm of RKIP expression and of RKIP loss conditional on age, cytogenetics, *RAS* mutation

status and the logarithm of white blood cell count (WBC) was tested by the Wald criterion, which was also the basis for the confidence limits of the relative hazard rates. R 2.12.0 (www.r-project.org) was used for analysis. All tests were performed two-sided and a *P*-value of < 0.050 was considered statistically significant.

Results

RKIP is frequently lost in AML

In a first approach, we examined RKIP protein levels by Western blot analysis in 103 AML patient samples (cohort 1), 17 AML cell lines and ten normal CD34+ HSPC specimens. Beta-Actin was chosen as loading control due to its uniform expression among AML samples and CD34+ HSPC specimens. Expression levels were determined densitometrically, the ratio RKIP/Beta-Actin calculated and subsequently normalized to a calibrator sample, which was set to a value of 100%. The four AML cell lines with minimal or non-detectable RKIP staining demonstrated expression levels of below 5%. However, as patient samples contained up to 20% non-leukemic cells, loss of RKIP was arbitrarily defined as an expression of less than 25% as compared to the calibrator. This cut-off was further supported by the distribution of individual RKIP expression levels, as it clearly discriminated the group of samples defined as RKIP loss (Supplementary Figure 1). Whereas normal expression of RKIP could be demonstrated in all CD34+ HSPC specimens, 19/103 (18%) patient samples and four out of 17 (24%) cell lines demonstrated loss of RKIP (Figure 1 and Supplementary Figure 1). Also, analysis of myeloid cell lines showed that RKIP expression was not modulated by growth factors (Supplementary Figure 2). To assess whether RKIP protein loss correlates with decreased

mRNA expression, we performed quantitative real-time PCR of selected AML patient samples and cell lines. As outlined in Supplementary Figure 3, a significant correlation between RKIP expression at the protein and mRNA levels was observed. Searching for possible causes of RKIP downregulation, we screened for mutations in the *RKIP* gene, promoter methylation, *RKIP* gene deletions as well as correlation with the expression of *Snail* - a known transcriptional repressor of *RKIP*[27] - but did not find any aberrations (Supplementary Table 2). Finally, we were unable to correlate RKIP loss with the activation status of the RAS-MAPK/ERK pathway in primary AML cells and cell lines (Supplementary Figure 4c).

RKIP inhibits proliferation and colony formation of myeloid cells

To test the functional consequences of RKIP loss in normal and malignant myeloid cells, we stably expressed a FLAG-tagged *RKIP* transgene in 32D murine hematopoietic cells and in the AML cell lines U937 and THP-1 (Figure 2a and Supplementary Figure 5). THP-1 carries a NRAS G12D mutation (Catalogue of Somatic Mutations in Cancer; www.sanger.ac.uk/genetics/CGP/cosmic) and shows RKIP loss (Figure 1c), whereas U937 harbors wildtype *RAS*[28] and shows normal RKIP expression. Initially, we tested for effects on the RAS-MAPK/ERK activation status and observed decreased levels of phosphorylated ERK following (re-)expression of RKIP (Supplementary Figure 4a and b). Furthermore, RKIP induced a significant decrease in the number of viable cells in all three cell lines tested under conditions of reduced growth factor supply (P=0.0013 for 32D, P=0.0044 for U937 and P=1.46 x 10^{-6} for THP-1, Figure 2b). Noteworthy, this

effect was less pronounced when supplementation of growth factors was increased (Supplementary Figure 6). Next, we performed PI/BrdU cell cycle/proliferation assays in the THP-1 and U937 cell lines (Figure 2c). Enhanced RKIP levels resulted in a significant decrease of proliferating cells in S-phase in both cell lines studied (P=0.00060 for U937 and P=3.156 x 10^{-5} for THP-1, respectively), whereas an increase of apoptotic cells, as measured by the subG1 peak, was observed in U937 only (P=0.013). Importantly, also the oncogenic potential of THP-1 cells, as assayed by colony growth in soft agar, was significantly diminished following RKIP reconstitution (P=0.0019, Supplementary Figure 7). In our experiments, U937 and 32D cells stably overexpressing RKIP as well as their vector transfected controls failed to form colonies. However, despite several attempts, stable cell lines with RKIP knocked down by miRNA could not be obtained with any of the cell lines tested.

RKIP shows decreased expression in patients with mutant RAS and antagonizes RAS driven malignant transformation

In a next step, we sought to identify mutations that co-exist with RKIP loss. Therefore, we analyzed *RKIP* expression in a previously described transcriptomic data set of 285 AML patients (cohort 2).[21] Thirty-four of 283 (12%) evaluable patients had mutations in either *NRAS* or *KRAS*, 95/283 (34%) in *NPM1*, 19/283 (7%) in *CEBPA*, 32/282 (11%) in the *FLT3*-TKD and 77/283 (27%) a *FLT3*-ITD. Importantly, only mutations in *RAS* demonstrated a significant association with decreased expression of *RKIP* (P=0.0020, Figure 3a and Table 1) suggesting cooperation of mutant *RAS* and RKIP loss in AML. To test for a functional synergism, we employed NIH3T3 cells, a well established system to

study RAS transformation.[29, 30] Transfection of either *HRAS V12* or *KRAS V12* induced cellular transformation as assessed by the appearance of transformed cell foci which were scored after 11 days of culture. Co-transfection of RKIP significantly inhibited focus formation (P=0.0010 for *KRAS* and P=0.0034 for *HRAS*, Figures 3b and c).

Loss of RKIP correlates with AML with monocytic differentiation

We then sought to correlate RKIP protein expression in 103 AML samples of cohort 1 with subtypes defined by the FAB classification. Samples with loss of RKIP expression were almost exclusively found in subgroups with a monocytic phenotype (FAB M4/M5; P<0.0010, Figure 4a). To corroborate these results in an independent cohort, we analyzed *RKIP* gene expression in a previously described transcriptomic data set of 285 AML patients (cohort 2).[21] Expression of *RKIP* mRNA was significantly lower in AML FAB M4/M5 as compared to non-M4/M5 groups (P=1.6 x 10^{-8}, Figures 4b and c). Intriguingly, a highly significant decrease of *RKIP* mRNA expression was also found in a specific cluster of AML samples, designated "cluster five" in Valk et al.[21] (P=1.4 x 10^{-15}, Figures 4b and d). Interestingly, this cluster has not yet been characterized by any specific genetic alterations but consists mainly of AMLs with monocytic differentiation.

Loss of RKIP is a favorable prognostic factor in AML

Finally, we correlated loss of RKIP with treatment outcomes in patients with AML. Among those 103 AML patients of cohort 1, who were analyzed for RKIP protein expression, 68 had received at least one cycle of standard AML induction chemotherapy and were therefore evaluated. Achievement of complete remission (CR), relapse free

survival (RFS) and overall survival (OS) were chosen as parameters and defined as described.[3] Sixteen out of 68 (24%) patients demonstrated RKIP loss whereas 52/68 (76%) had normal RKIP expression. Similar to cohort 2, mutations in either *NRAS* or *KRAS* were present in 10/67 (15%) patients. The presence of RKIP loss was not correlated with conventional AML risk factors including patient age, WBC at diagnosis and cytogenetic risk group, respectively (data not shown). Univariate analysis revealed RFS to be significantly longer in patients with RKIP loss as compared to patients with normal RKIP expression (hazard ratio [HR] = 0.3; 95% confidence interval [CI] = 0.11-0.78; *P*=0.0090). This finding could be confirmed in multivariate Cox regression analyses that included the risk factors mentioned above as well as the *RAS* mutation status (HR = 0.116; 95% CI = 0.032-0.429; *P*=0.0012). Furthermore, OS of patients with loss of RKIP protein was significantly longer in univariate (HR = 0.36; 95% CI = 0.15-0.862; *P*=0.017) and multivariate analyses, respectively (HR = 0.171; 95% CI = 0.048-0.604; *P*=0.0061). Noteworthy, the favorable impact of low RKIP expression on OS and RFS could be confirmed when RKIP expression was used as a continuous variable obtained from densitometry data (RFS: HR = 1.547; 95% CI = 1.144-2.092; *P*=0.0046, OS: HR = 1.361; 95% CI = 1.053-1.758; *P*=0.019). Importantly, in agreement with previous studies,[31, 32] monocytic differentiation of AML had no prognostic value in this cohort of AML patients (data not shown). Kaplan-Meier analyses for RFS and OS according to the RKIP protein expression status are shown in Figure 5 and results of Cox regression analysis in Table 2. No difference was observed in the probability to achieve CR between patients showing normal or lost RKIP expression (data not shown).

Discussion

Aberrant RAS-MAPK/ERK signaling is of seminal importance in the pathogenesis of AML[5, 33]. In this study, we have identified loss of the C-RAF-MEK inhibitor RKIP as an additional frequent molecular event affecting this crucial signaling pathway in AML. So far, several lines of evidence have established RKIP as a metastasis suppressor in solid neoplasms. In prostate cancer, RKIP expression was significantly reduced in metastatic as compared to primary tumor cells both at the mRNA and protein level. Overexpression of RKIP in prostate cancer cell lines did not affect cell proliferation and colony formation but was associated with decreased invasiveness *in vitro*.[13, 34] In addition, RKIP sensitized prostate and breast cancer cells to chemotherapy-induced apoptosis while loss of RKIP conferred resistance to anticancer drugs.[12, 35, 36] Treatment of prostate cancer cells with the novel proteasome inhibitor NPI-0052 was reported to induce RKIP expression via inhibition of NF-κB and Snail leading to chemoimmunosensitation to cisplatin and TRAIL.[37] In contrast to the data on solid tumor cells, we observed growth inhibition by (re-)expression of RKIP in 32D murine hematopoietic cells and the AML cell lines U937 and THP-1, the latter characterized by RKIP loss and a mutation in *NRAS*. Interestingly, these effects were most pronounced under conditions of limited growth factor supply. This is in accordance with previous reports of decreased RKIP/CRAF binding following mitogen activation, which in turn dampens the inhibitory effects of RKIP on the MAPK/ERK pathway.[9, 38] Therefore, RKIP seems to come into play in situations where cells are missing the abundance of extracellular stimuli and have to rely on intracellular "driver mutations" as is the case with leukemic blasts. The observation that RKIP inhibits transformation induced by mutant RAS may further strengthens this hypothesis. PI/BrdU

cell cycle/proliferation assays, performed in U937 and THP-1 cells, demonstrated a significant decrease in proliferation in both leukemic cell lines. Noteworthy, increased apoptosis was observed in U937 only, suggesting the existence of anti-apoptotic molecular aberrations in THP-1 cells. The *NRAS* mutation present in this cell line is a likely candidate as suppression of apoptotic cell death has been shown for mutant *RAS* before.[39, 40] Intriguingly, the oncogenic potential of THP-1 cells as assayed by colony growth in soft agar could also be significantly diminished by stable (re-)expression of RKIP. Taken together, these data suggest an important role of RKIP in the regulation of proliferation and malignant transformation of myeloid hematopoietic cells.

In our study, RKIP decreased the phosphorylation of ERK in 32D and THP-1 cells, which further supports its inhibitory role on the RAS-MAPK/ERK pathway.[9] Interestingly, analysis of AML cell lines and patient samples did not reveal any significant correlation between RKIP loss and phosphorylation of ERK, an observation that has been reported in breast cancer and malignant melanoma previously.[14, 41] This finding may suggest the involvement of additional RKIP effector pathways in primary leukemic cells.

In a next step, we sought to determine whether RKIP expression levels correlate to other genetic abnormalities frequently observed in AML. We therefore analyzed gene expression data of a previously published cohort of 285 AML patients[21] and correlated *RKIP* expression to mutations in either *NRAS* or *KRAS*, *NPM1*, *CEBPA*, in the *FLT3*-TKD and to *FLT3*-ITD. Importantly, only patients with mutant *RAS* exhibited a significant decrease in *RKIP* expression suggesting co-existence of mutations in *RAS* and RKIP loss. To further test for a possible involvement of RKIP in RAS driven

transformation, we performed NIH3T3 transformation assays, a model frequently used to study the biology of oncogenic RAS.[29, 30] RKIP significantly decreased focus formation induced by mutated *RAS* suggesting synergistic effects of mutant *RAS* and loss of RKIP in cellular transformation. Importantly, as RKIP has proven unable to interact with RAS itself,[9] the effects on RAS driven transformation are most likely to be mediated via inhibition of the downstream effectors RAF/MEK. This is further supported by the fact that transformation induced by mutant C-RAF is inhibited by RKIP as well.[17] Inhibition of proliferation and colony formation by RKIP in the *RAS* mutated THP-1 cell line further highlights the relevance of this cooperation in an AML specific cellular model. These findings are insofar of interest as *RAS* mutations per se are considered insufficient to induce AML. They have been shown to establish a myeloproliferative disorder in mice with a penetrance of up to 100%, however, these mice never developed frank AML.[42, 43] Additional genetic alterations like inactivation of NF1[44] or expression of the *PML-RARα* fusion gene[45] were necessary to enable transition to AML. Recently, the classical "two-hit model" describing AML pathogenesis as a result of two single alterations in pathways affecting differentiation as well as proliferation/self-renewal has been challenged. Genome wide studies have indicated the presence of multiple driver mutations in AML cells. For instance, a *NRAS* mutation was identified in an AML specimen with a normal karyotype together with eleven other mutations in the coding region of several genes including *IDH1* and *NPM1*.[46] In a study by Haferlach et al., up to three alterations in the RAS-MAPK/ERK pathway were found in AML with a *CBFß-MYH11* rearrangement with 22% showing at least two alterations in this pathway.[47] These data indicate that by far more than two molecular events may be relevant for the pathogenesis of many AML

cases and distinct alterations affecting the same pathway may be a common finding in this disease.

A preferential association of molecular abnormalities with particular phenotypes can be found in several subtypes of AML. The translocation t(8;21)(q22;q22) is associated with AML with minimal maturation (FAB M2) whereas translocations involving the RARA gene define cases of acute promyelocytic leukemia. Such associations may reflect a selective disruption of the developmental process of the affected lineage.[48-50] Alternatively, they are the result of a disturbance in the process of lineage selection as some leukemias arise in cells with multipotent developmental capacity.[51, 52] In the majority of AMLs, however, the morphologic subtype cannot be assigned to a distinct molecular abnormality. Here we demonstrated that loss of RKIP highly correlates with a monocytic phenotype in two independent AML cohorts of 103 and 285 patients, respectively. Our data therefore support and extend previous notions that molecular alterations in the RAS-MAPK/ERK pathway are associated with a monocytic subtype, both in AML[8, 53] and myelodysplastic/myeloproliferative neoplasms such as chronic and juvenile myelomonocytic leukemia.[54, 55] Gene expression data were available in the cohort of 285 AML patients and reduced *RKIP* expression was mainly confined to a specific cluster of samples ("cluster 5" in Valk et al.[21]). This cluster comprises almost exclusively patient samples with a monocytic subtype but has not been characterized by a recurrent molecular alteration so far. Based on our data, loss of *RKIP* may constitute a molecular marker defining this distinct AML subset.

Treatment outcomes of patients with AML are extremely variable and depend on characteristics particular to the AML clone, such as cytogenetics or WBC counts, as well

as on patient related factors like age and co-mobordities.[3] Recent years have seen the discovery and exploration of numerous genetic aberrations important for leukemogenesis with mutations in *NPM1* and *CEBPA*, respectively, now defining provisional entities in the 2008 WHO classification.[2, 3] Advances in the molecular characterization of AML have facilitated the establishment of more detailed risk scores, which proved to be particularly useful in patients without distinct cytogenetic profiles.[56, 57] Further extension of these scores by inclusion of new molecular markers with prognostic significance will be a pivotal step in the development of personalized risk profiles for patients diagnosed with AML. In this study we present loss of the RKIP protein expression as a potential new prognostic marker. In uni- and multivariate analyses including established risk factors as well as the *RAS* mutation status, loss of RKIP proved to be an independent favorable prognostic marker. Although there was no difference between RKIP loss and normal RKIP expression in the probability to achieve a CR, patients with loss of RKIP proved to have a significantly longer RFS cumulating in a significantly better OS. Noteworthy, the reasons for improved therapeutic outcome in AML patients with loss of RKIP remain unclear. Leukemic cells with loss of RKIP might less likely harbor additional mutations recognized as poor prognostic parameters. Unfortunately, the small sample size of this cohort did not allow analysis of the prognostic value of RKIP loss in the context of multiple other genetic aberrations. Hence, analysis of RKIP loss in larger patient cohorts with well documented clinical and molecular data will be of great interest. Alternatively, one might speculate that RKIP influences sensitivity to cytotoxic agents used in the treatment of AML. Whatever reason, the fact that a genetic alteration

contributes to leukemogenesis does not preclude its role as a biomarker predicting a good treatment response as has been shown for *NPM1* mutations recently.[58]

In summary, we report loss of RKIP as a frequent molecular event in AML with monocytic differentiation and describe RKIP as a negative regulator of proliferation and transformation in myeloid cells. Loss of RKIP further correlates with mutant *RAS* and inhibits its oncogenic potential. Finally, loss of RKIP seems to be a favorable prognostic factor in patients with AML.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

AZ designed the research and performed experiments, collected, analyzed and interpreted data and wrote the manuscript; KL, OW, MH, CB, IF, AD, DP, DM, OR and KB performed experiments and collected data; FQ performed statistical analysis; ET and RD

provided key research tools, collected, analyzed and interpreted data; and AW, WK, JT and HS designed the study, collected, analyzed and interpreted data, and wrote the manuscript.

Supplementary Information accompanies the paper on the Leukemia website

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Tables

Table 1 Correlation of RKIP expression with molecular aberrations

Table 2 Multivariate Cox regression analysis for OS and RFS

Figures

Figure 1 Loss of RKIP expression is a frequent event in AML. (**a,b**) Representative Western blots of AML patient and normal CD34+ HSPC samples, respectively, showing loss of RKIP in samples 2976 and 3605. (**c**) Representative Western blot of AML cell lines showing RKIP loss in ML-2, THP-1, TF-1, and HEL. Graphs demonstrate the x-fold change in RKIP expression as compared to a calibrator sample, which was set to a value of 100%.

Figure 2 RKIP decreases cellular proliferation in hematopoietic myeloid cells. (**a**) FLAG-*RKIP* and empty vector were overexpressed in 32D and U937 cells and re-expressed in THP-1 cells, respectively, by stable transfection. (**b**) Cells were seeded and maintained as described in Materials and Methods. Viable cells were counted after two days for 32D, after three days for U937 and after 6 days for THP-1, respectively. For comparison of the three cell lines, the vector transfected controls have been set at a value of 1 and the relative decrease of viable cells in the RKIP transfected cells has been

693 calculated using the ratio viable cells RKIP/viable cells vector. (**c**) PI/BrdU cell
694 cycle/proliferation assays were performed in THP-1 and U937 cell lines to evaluate the
695 percentage of cells in S-phase (1), G0/G1-phase (2), G2/M-phase (3) and apoptotic cells
696 (subG1 [4]), respectively. Black bars, vector transfected controls; white bars, RKIP
697 transfected cells. The graphs summarize the results of at least three independent
698 experiments. Data are expressed as means ± SD and *P*-values have been calculated using
699 Student's t-test. *indicates $P<0.050$ and **indicates $P<0.010$.
700
701
702 **Figure 3** RKIP inhibits transformation induced by mutant *RAS*. (**a**) Box plot
703 displaying *RKIP* mRNA expression levels in AML patients with *RAS* mutations (mt) and
704 without (wt). The *P*-value has been calculated using the Mann-Whitney-Wilcoxon test.
705 (**b**) For focus formation assays, NIH3T3 fibroblasts were transfected with the indicated
706 constructs (0.1 µg *RAS*/ 1.2 µg *RKIP*). Foci were stained and scored after 11 days in
707 culture. (**c**) The average number of foci per µg of DNA ± SD from three independent
708 experiments is given. *P*-values have been calculated using paired Student's t-test.
709
710
711 **Figure 4** Loss of RKIP is associated with a monocytic phenotype. (**a**) Samples with
712 loss of RKIP were correlated to FAB subgroups in the AML cohort of 103 AML patients.
713 Bars show the percentage of cases with and without RKIP loss in either subgroup. The *P*-
714 value was obtained by comparing subgroups with monocytic differentiation (FAB
715 M4/M5) with all other subgroups using Fisher's exact test. (**b**) *RKIP* mRNA expression

using microarray data from 285 AML patients is significantly decreased in a specific subgroup of AML samples with monocytic differentiation (cluster five). Omniviz Correlation View of 16 clusters representing all 285 AML patients.[21] The FAB classification (first column) and the expression levels of the three different *RKIP* mRNA probe sets present on Affymetrix U133A GeneChip arrays are depicted along the diagonal of the Correlation View. FAB subtype M4 is indicated in yellow and subtype M5 in light blue. Height of the black bars denotes probe set expression levels. (**c,d**) Box plots displaying expression levels of the mean of all three *RKIP* mRNA probe sets in these AML samples. Mann-Whitney-Wilcoxon test *P*-values were corrected for the number of clusters by the Bonferroni method. Differences in expression between individual probe sets were found to be significant as well (data not shown).

Figure 5 Loss of RKIP protein expression is a favorable prognostic marker in patients with AML. Kaplan-Meier estimates for OS (**a**) and RFS (**b**), respectively, according to RKIP protein expression status. Statistical difference was evaluated with the log-rank test.