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BRIEF REPORT

Epigenetic silencing of AKAP12 in juvenile myelomonocytic leukemia

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ABSTRACT

A-kinase anchor protein 12 (AKAP12) is a regulator of protein kinase A and protein kinase C signaling, acting downstream of RAS. Epigenetic silencing of *AKAP12* has been demonstrated in different cancer entities and this has been linked to the process of tumorigenesis. Here, we used quantitative high-resolution DNA methylation measurement by MassARRAY to investigate epigenetic regulation of all three *AKAP12* promoters (i.e., α, β, and γ) within a large cohort of juvenile myelomonocytic leukemia (JMML) patient samples. The *AKAP12α* promoter shows DNA hypermethylation in JMML samples, which is associated with decreased *AKAP12α* expression. Promoter methylation of *AKAP12α* correlates with older age at diagnosis, elevated levels of fetal hemoglobin and poor prognosis. *In silico* screening for transcription factor binding motifs around the sites of most pronounced methylation changes in the *AKAP12α* promoter revealed highly significant scores for GATA-2/-1 sequence motifs. Both transcription factors are known to be involved in the haematopoietic differentiation process. Methylation of a reporter construct containing this region resulted in strong suppression of *AKAP12* promoter activity, suggesting that DNA methylation might be involved in the aberrant silencing of the *AKAP12* promoter in JMML. Exposure to DNMT- and HDAC-inhibitors reactivates *AKAP12α* expression *in vitro*, which could potentially be a mechanism underlying clinical treatment responses upon demethylating therapy. Together, these data provide evidence for epigenetic silencing of *AKAP12α* in JMML and further emphasize the importance of dysregulated RAS signaling in JMML pathogenesis.

Introduction

Juvenile myelomonocytic leukemia (JMML) is a rare childhood myelodysplastic/ myeloproliferative disorder with an incidence of 1.2 cases per million children per year. The average age at diagnosis is 1.8 y and, if left untreated, this malignancy is rapidly fatal for most affected children. Until now, the only curative treatment for JMML is allogeneic haematopoietic stem cell transplantation (HSCT).[1] Still, about 35% of patients relapse after HSCT, which demonstrates a strong need for a better understanding of the molecular mechanisms that are involved in the pathogenesis of JMML.[2]

Promoter hypermethylation and subsequent gene inactivation are frequent events in myeloid malignancies, including JMML.[3-6] Two recent studies linked aberrant DNA methylation patterns to an aggressive phenotype of JMML.[7,8] Using a candidate gene approach, a prognostic score based on four frequently hypermethylated genes (*BMP4, CALCA, CDKN2B*, and *RARB*) could be identified, which retained prognostic value independently of other clinical risk factors.[7] Several studies have shown that aberrant methylation patterns may be governed by

deregulated signaling.[9,10] In this way, it becomes increasingly clear that abnormal epigenetic marks are closely linked to aberrant cell signaling, a characteristic of various cancer entities.[11] A hallmark of JMML is its hypersensitivity to growth stimuli, such as the granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-1 (IL-1), and the tumor necrosis factor α (TNF-α).[12] GM-CSF hypersensitivity has been linked to a constitutively active Ras signaling pathway, which is believed to be the major driver of the disease.[13,14] Indeed, in >80% of JMML patients different members of the RAS/MAP kinase (MAPK) pathway are targeted by mutations in a mostly mutually exclusive fashion. Gain of function mutations occur in *KRAS* and *NRAS* (15–20%), and in the non-receptor tyrosine phosphatase *PTPN11* (35%), whereas loss of function mutations occur in *NF1* (15–20%) and *CBL* (15%).[15-18]

The A-kinase anchor protein 12 (AKAP12) is a key regulator of protein kinase A and C signaling and has been described to suppress the PKC-Raf/MEK/ERK pathway, located downstream of RAS.[19] Epigenetic suppression of *AKAP12* has been reported in various childhood myeloid leukemias.[20] None of

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the six JMML patients investigated in that study showed aberrant methylation of the *AKAP12* promoter. Yet, the suppressive role of AKAP12 on RAS downstream signaling and the dependence of JMML on activated RAS signaling suggested a more detailed study on the potential role of *AKAP12* in JMML pathogenesis. In the present study, we investigated the epigenetic regulation of *AKAP12* in a cohort of 81 children diagnosed with JMML.

Results and discussion

Hypermethylation of the AKAP12α promoter in JMML

AKAP12 is a known tumor suppressor gene that can be silenced by deletions or by epigenetic mechanisms. Aberrant DNA methylation in the promoter region has been shown to specifically silence *AKAP12* in Src- and Ras-transformed fibroblasts.[10] Re-expression of *AKAP12 in vitro* and *in vivo* suppresses oncogenic growth,[21] while overexpression of *AKAP12* leads to cell cycle arrest.[22] In line with this, cells react with increased *AKAP12* expression levels upon mitogenic stimuli.[23,24] The *AKAP12* gene locus encodes for three isoforms α, β, and γ.[25] Recent whole-genome bisulfite studies have shown that the vast majority of CpGs in the adult mammalian genome are methylated. The only exceptions are gene regulatory regions like enhancers and promoters that are associated with (potentially) active genes.[26,27] Gene regulatory regions are enriched for CpG islands, which are usually protected from methylation and thus can be found most frequently in the vicinity of constitutively active genes. All three *AKAP12* isoforms are under the control of independent promoter regions, each containing one or more CpG islands. In gastric, colorectal, and hepatic cancer one or both major *AKAP12* isoforms (α, β) are targeted by promoter DNA hypermethylation and are transcriptionally repressed.[23,28-30]

We used quantitative DNA methylation analysis at single CpG unit resolution to test if the *AKAP12* promoter is epigenetically silenced in JMML. All three promoter regions corresponding to known *AKAP12* isoforms were tested using the MassARRAY technology in a cohort of 81 JMML cases and leukocytes from healthy individuals as controls (Fig. 1A). It is well known that methylation changes occurring in gene regulatory regions in a physiological or pathological context usually start from the borders of a given regulatory region and that is why one can generally observe the strongest methylation changes in CpG island shores but also in the distal regions of a given promoter or enhancer.[27,31] This effect is nicely recapitulated in Fig. 1A. The distal region of the *AKAP12α* promoter covered by amplicon $\alpha 1$ was found to be significantly hypermethylated in a subset of JMML samples as compared to the control samples ($P < 0.0001$) while the region covered by amplicon $\alpha 2$ showed only a slight increase in DNA methylation levels. In contrast, the regions covered by amplicons β and γ showed no relevant methylation changes and a slight hypomethylation, respectively (Fig. 1A, B). The average DNA methylation of amplicon $\alpha 1$ showed a large spread in JMML (range: 16–78%), as compared to healthy cells (21–32%). We subsequently defined a genomic region as being hyper- or hypomethylated, respectively, if the average methylation levels exceeded two standard deviations from the mean of normal granulocytes. According to these criteria, 42% of JMML cases showed hypermethylation in the *AKAP12α1* promoter region. Additionally, we analyzed the *AKAP12α1* promoter methylation status of CD34$^+$ cells from umbilical cord blood of healthy neonates, which are thought to represent the most appropriate healthy counterpart of JMML-initiating cells. These cells displayed an even lower promoter methylation profile than the healthy controls with an average CpG methylation of 16% in amplicon $\alpha 1$.
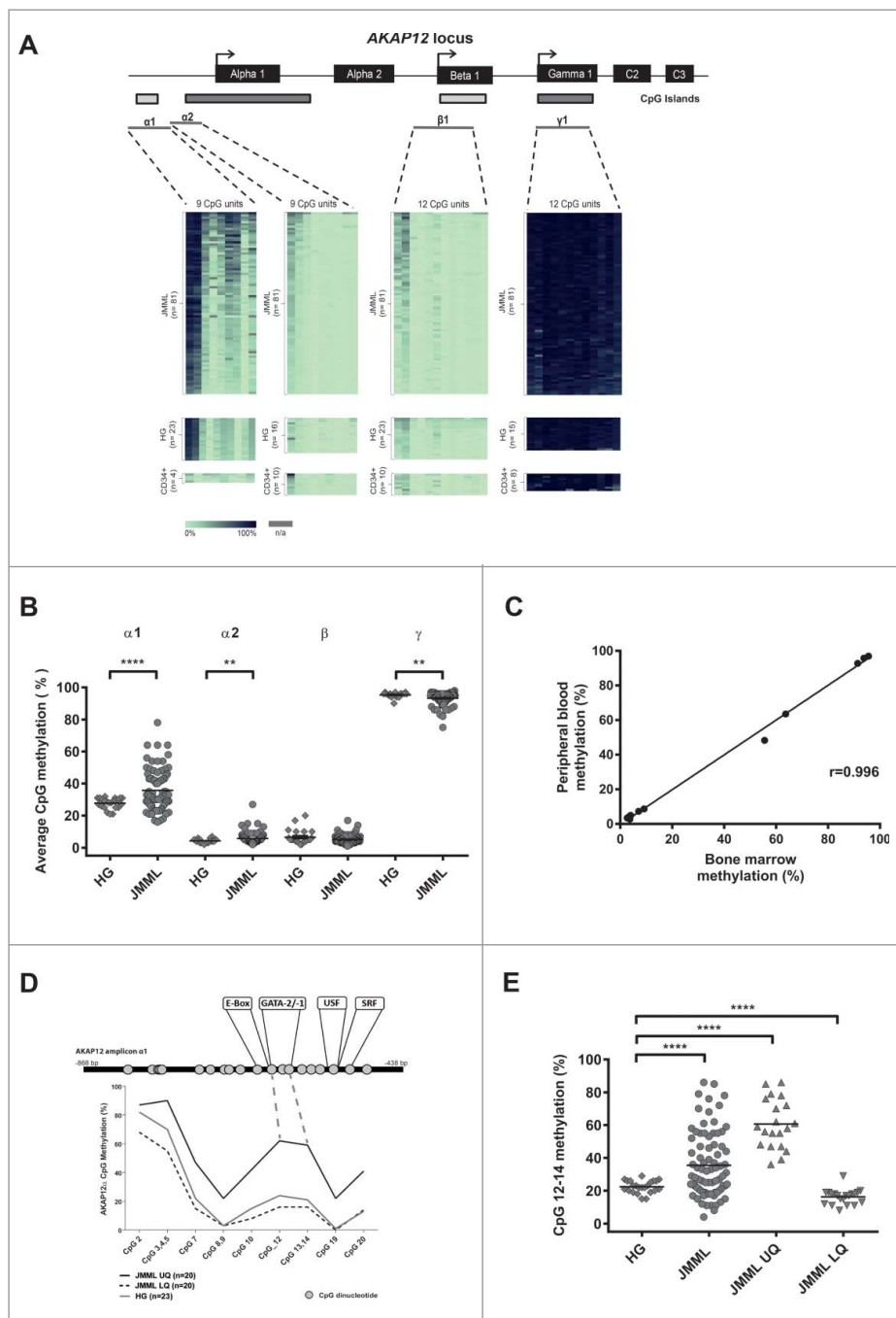
The promoter region of *AKAP12β* was unmethylated in JMML samples as well as in healthy controls. The promoter of *AKAP12γ* was strongly methylated in samples from healthy individuals, which correlates with the fact that its expression is known to be testis-specific.[25,32] In JMML cases, a slight hypomethylation was observed ($P = 0.002$; Fig. 1B). Indeed, compared to healthy controls, 23% of the JMML samples showed reduced promoter CpG island methylation within amplicon $\gamma 1$. We did not observe any correlation between *AKAP12γ* hypomethylation and *AKAP12α* hypermethylation (data not shown).

Aberrant AKAP12α methylation in JMML is independent of the cell source

Since the clinical samples from 81 JMML patients were obtained either from bone marrow (BM) or from peripheral blood (PB), we investigated whether the source of the leukemic material had an influence on the observed methylation pattern. Therefore, matched pair analyses of malignant leukocytes isolated from BM and from PB were carried out. Highly similar DNA methylation values within the *AKAP12α* promoter region (amplicon $\alpha 1$) were observed between cells that originate from different tissue sources of the same patient (r = 0.996; Fig. 1C). Moreover, DNA methylation profiles did not differ between granulocytes and mononuclear cells, derived from BM or PB. Together, these data indicate that the heterogeneity of DNA methylation levels observed in this JMML cohort is most likely explained by differences in clonality levels between samples. Anyhow, we cannot fully rule out the possibility that varying levels of contamination with lymphocytes or inherent epigenetic instability may contribute to the observed heterogeneity of DNA methylation patterns.

Identification of potentially disrupted gene regulatory regions of AKAP12

In order to identify key regulatory regions within the differentially methylated *AKAP12α* promoter region, a search for highly differentially methylated CpG sites was performed. In Fig. 1D the DNA methylation status of *AKAP12α* is given at single CpG unit resolution. In the JMML samples representing the lower quartile of DNA methylation, the methylation levels almost parallel those of healthy controls. The upper quartile shows most strongly elevated methylation levels in amplicon $\alpha 1$ between CpG 12 and CpG 14 located 614–587 bp upstream of the *AKAP12α* transcription start site (TSS). The scatter plot depicted in Fig. 1E compares CpG methylation levels in this specific region between the upper and lower methylation quartile of JMML samples (JMML UQ and JMML LQ, respectively) and also to healthy individuals. A wide range of methylation values can

Figure 1. Quantitative DNA methylation analysis of the *AKAP12α*, *β*, and *γ* promoters in JMML. (A) Schematic representation of the *AKAP12* locus showing the promoter regions of *AKAP12α*-, *β*- and *γ*-isoforms with their respective CpG islands. All isoforms share a common N-terminus encoded by 2 exons (C2 and C3). CpG islands are depicted as gray boxes (<300 bp, light gray). The MassARRAY amplicons (gray lines) and their positions relative to their respective TSS are illustrated underneath the CpG islands: *α*1: −868to −438 bp from TSS, *α*2: −462 to −131 bp from TSS, *β*1: −193 to +267 bp from TSS, *γ*1: −243 to +229 bp from TSS. Methylation heat maps are shown for each amplicon. Each row represents a sample and each column a CpG unit. Percentage of DNA methylation ranges from 0% (light green) to 100% (dark blue). Gray indicates unavailable data. HG: healthy granulocytes. (B) The mean DNA methylation levels for each amplicon are displayed as dot plots. The methylation levels between JMML samples (circles) and healthy granulocytes (diamonds) were compared using an unpaired t test with Welch's correction. (C) Leukemic cells isolated from bone marrow (BM) and peripheral blood (PB) were obtained from three JMML patients. DNA methylation values measured for the AKAP12 locus (amplicons *α*1, *α*2, *β* and *γ*) show a very good correlation between the two tissues (r = 0.996). (D) The methylation profile of amplicon *α*1 at CpG-unit resolution is shown for JMML and HG. JMML cases were grouped according to their methylation into 25% quartiles. The lower quartile (LQ) contains hypomethylated JMML samples (dashed black line) and the upper quartile (UQ) represents the hypermethylated ones (black line). The average methylation profile of HG (n = 23) is depicted as a light gray line. (E) Methylation levels for CpG units 12, 13, and 14 in AKAP12 amplicon *α*1 are depicted for HG and all JMML samples, as well as for hypomethylated (LQ) and hypermethylated (HQ) JMML samples. JMML samples show a statistically significant increase in methylation as compared to HG (*P* < 0.0001).

be observed in this region and these methylation differences might reflect in part the phenotypic diversity among the samples. Overall, the described region shows a highly significant methylation increase as compared to normal controls (*P* < 0.0001). Applying our criteria for hypermethylation to this subregion of the *AKAP12α* promoter (CpGs 12–14), 49% of the JMML samples would be categorized as showing significant hypermethylation.

This aberrantly methylated region upstream of the AKAP12α TSS has been shown to be part of a sequence stretch that is highly conserved in mammals.[25] We used an *in silico* prediction tool to search for potential transcription factor binding sites (TFSEARCH; version 1.3) in this region. We found several putative transcription factor (TF) binding sites at and proximal to the hypermethylated region (Fig. 1D). The *in silico* analysis predicted a GATA2/GATA1 binding site that precisely overlaps with the observed hypermethylation hotspot in the AKAP12α promoter region. This is of special interest, since both GATA2 expression and binding are tightly regulated epigenetically during early haematopoietic commitment.[26,27] Our motif search further identified an additional E-box predicted to bind the homeobox protein Nkx-2 that is located immediately 5′ of the differentially methylated DNA stretch. An upstream stimulatory factor (USF) binding site was predicted 50 bp downstream of the hypermethylated region. Finally, a serum response factor (SRF) binding site was predicted which is located 502 bp upstream of the transcription start site (TSS). Together, these findings suggest that this differentially methylated region harbors TF binding sites that may be disrupted by DNA hypermethylation. This, in turn, might ultimately result in aberrant silencing of AKAP12α in JMML.

DNA methylation of the AKAP12α promoter correlates with AKAP12α transcription

In order to test if AKAP12α promoter hypermethylation leads to AKAP12α silencing, we investigated AKAP12α mRNA expression levels in JMML. Quantitative RT-PCR of 19 JMML cases and 5 control samples (granulocytes of healthy donors) revealed a 13-fold decreased AKAP12α expression in JMML ($P < 0.0001$; Fig. 2A).

To further investigate the effects of AKAP12α promoter methylation and the impact of GATA binding on promoter activity, we carried out luciferase reporter assays using the pCpGL-basic reporter vector. The genomic region spanning the AKAP12α amplicons α1 and α2 (−794 bp to +92 bp around the AKAP12α TSS) was cloned into the CpG-free vector. In addition, a construct harboring a deletion of the predicted GATA binding motif was generated using site-directed mutagenesis (from now on referred to as AKAP12α_ΔGATA). The luciferase reporter vector containing the AKAP12α insert was either methylated *in vitro* or left unmethylated. Each construct was transiently transfected into K562 cells, and luciferase activity was measured. The unmethylated AKAP12α_ΔGATA construct reproducibly showed a modest reduction of promoter activity (76% activity as compared to the wild type construct; $P = 0.0439$) in the luciferase assay (Fig. 2B). More importantly, both methylated constructs showed a significant decrease of promoter activity as compared to their unmethylated counterparts ($P < 0.0001$ for AKAP12 wild type; $P = 0.0013$ for AKAP12α_ΔGATA). Again, this effect was more pronounced in the AKAP12α_ΔGATA construct which showed only 14% promoter activity while the methylated wild type construct still had 30% promoter activity as compared to the unmethylated wild type construct (Fig. 2B). In summary, these findings suggest that GATA binding plays, at least in part, a role in the activation of the AKAP12α promoter. This is further supported by our observation that deletion of the GATA motif has no effect on promoter activity in HEK293T cells, which do not express GATA transcription factors (data not shown). At the same time, our data clearly indicate that additional transcription factor binding events, which are also methylation dependent, crucially contribute to AKAP12α expression.

Next, we tested whether demethylation of the AKAP12α promoter leads to re-expression of AKAP12α. Therefore, we chose two myeloid leukemia cell lines, Kasumi-1 and HL-60, which harbor RAS mutations and thus show a constitutively active MAPK-signaling pathway (Table S4).[33] MassARRAY analysis showed high levels of DNA methylation (94%) at the AKAP12α promoter in both cell lines under steady state conditions (Fig. 3A-B). In line with these findings, qRT-PCR revealed low mRNA expression levels of AKAP12α in Kasumi-1 and HL-60 at baseline. A recent publication has demonstrated that depletion of HDAC7 leads to re-expression of AKAP12 in endothelial cells.[34] Therefore, we asked the question whether HDAC-inhibition alone or in combination with DNMT-inhibition leads to re-expression of AKAP12α.

Both cell lines were treated for 72 h either with the DNMT-inhibitor DAC, the pan-HDAC-inhibitor SB939, or a combination of both inhibitors. DNA methylation levels of the AKAP12α promoter, as well as the AKAP12α mRNA expression levels were quantified every 24 h during the treatment period (day 1–3) and 3 d and 11 d after end of drug treatment (day 6 and day 14). Untreated cells were cultured in parallel as a control. DAC treatment led to a time-dependent decrease of DNA methylation in the AKAP12α promoter region. At the end of the 3-day treatment period, we observed an absolute methylation difference, as compared to baseline of 42% and 29% in Kasumi-1 and HL-60, respectively (Fig. 3A-B). This effect was accompanied by a 2.3- to 3.5-fold increase in AKAP12α expression at the end of the treatment period in HL-60 and Kasumi-1 cells, respectively (Fig. 3C-D). HDAC inhibition did not affect DNA methylation levels at the AKAP12α promoter as a single agent nor in combination with DAC. In contrast, HDAC inhibition resulted in a modest transient increase of AKAP12α expression levels in both cell lines and the combination of DAC + SB939 resulted in a slightly stronger reactivation of AKAP12α expression than either agent alone (Fig. 3C-D). Interestingly, the kinetics of AKAP12α reactivation were different in both cell lines, anyhow, the reactivation of AKAP12α was only transient under all conditions tested: AKAP12α gene expression decreased following drug withdrawal and this decrease seemed to be paralleled by increasing levels of AKAP12α promoter methylation (Fig. 3A-D).

Together, these findings demonstrate that high levels of AKAP12α promoter DNA methylation indicate transcriptional repression of AKAP12α and that pharmacological demethylation may lead to re-expression of AKAP12α. These data are well in line with published genome-wide data that show a global inverse correlation of DNA methylation dynamics with gene expression changes in the context of normal haematopoietic differentiation.[27] Since
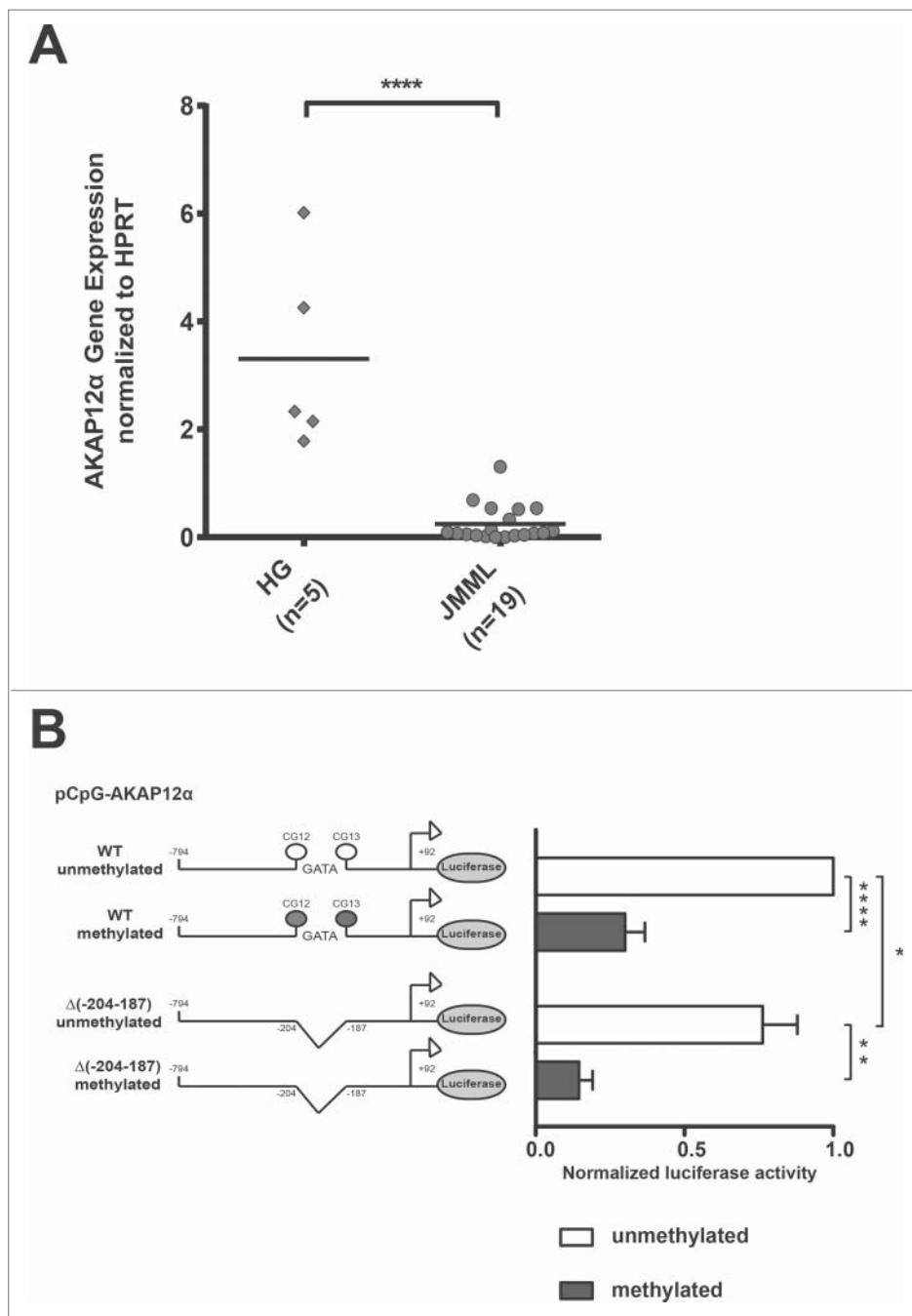
Figure 2. *AKAP12α* expression and promoter methylation. (A) *AKAP12α* mRNA expression levels measured by qRT-PCR in five healthy granulocyte samples and 19 JMML patient samples are shown. Horizontal lines represent mean values. The *AKAP12α* mRNA expression was significantly lower in JMML patients as compared to HG (*P* = 0.0002; Mann-Whitney U test). (B) Schematic representation of the AKAP12α promoter sequence with and without GATA-motif cloned into the CpG-free (pCpGL) luciferase reporter vector. K562 cells were transfected either with an unmethylated or with an *in vitro* methylated pCpGL vector construct. Luciferase activity was measured and normalized to the unmethylated Renilla-luciferase construct. The results are relative to the activity of the unmethylated *AKAP12α* promoter. The bar chart shows the mean luciferase activity ± SD (n = 4) of the methylated promoter construct and the unmethylated construct. Statistical significance was analyzed by a Student's t-test.

hyperactive RAS signaling is a hallmark of JMML, re-expression of the negative RAS signaling regulator *AKAP12* could be one potential mechanism that might explain the treatment responses frequently observed in patients upon demethylating therapy.[1,35] In addition, HDAC-inhibition was also able to re-express *AKAP12a*, although this effect even under ongoing treatment was only transient if no DNMT-inhibitor was given concomitantly. This transient effect of HDAC-inhibition on *AKAP12α* expression is in slight contrast to the data published by Turtoi et al., but this discrepancy might be explained by the fact that Turtoi et al. used an siRNA knock-down approach to abrogate HDAC7 expression, which not only inhibits the function of the enzyme but also depletes the entire protein.[34] In addition, our data set is generated in haematopoietic cells while the paper of Turtoi et al. use vascular endothelial cells for their experiments. It is clear that different cell types might show
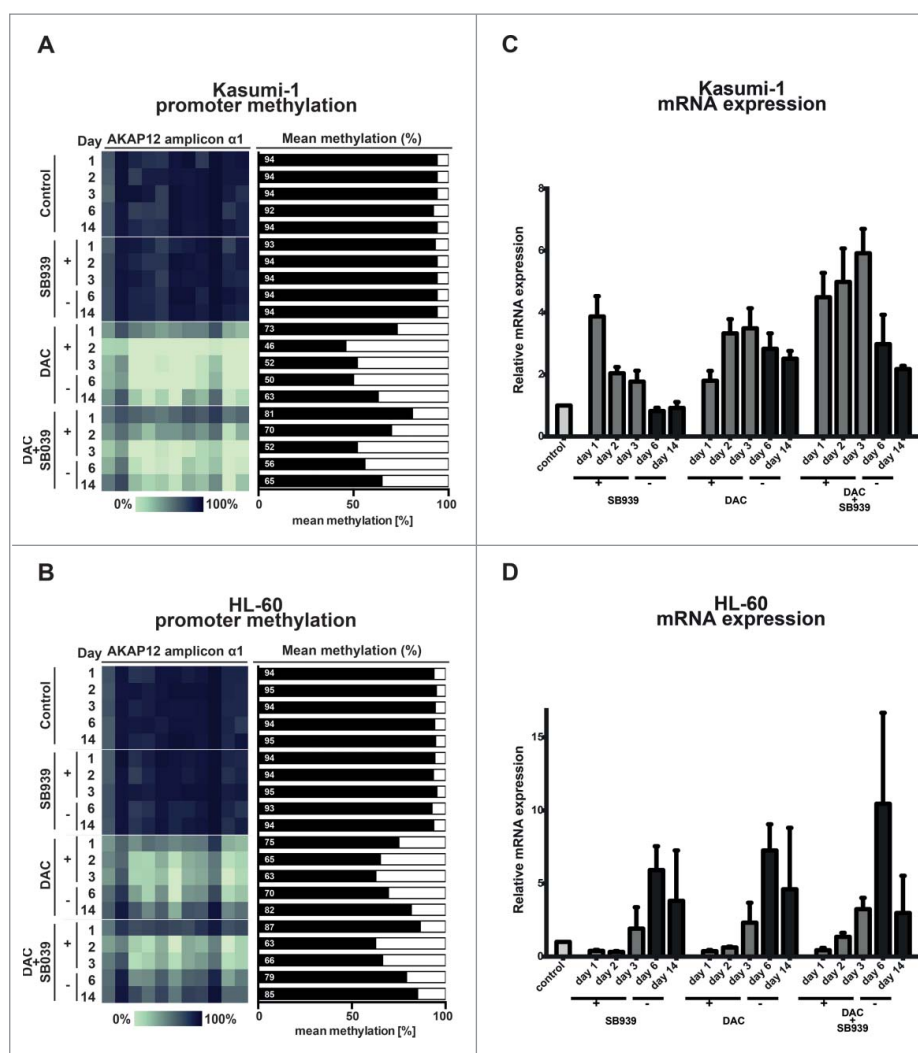
Figure 3. Epigenetic silencing of *AKAP12α* in *RAS*-mutated AML cell lines can be reverted by DAC treatment. Time course of *AKAP12α1* promoter methylation in Kasumi-1 (A), and HL-60 cells (B) upon treatment with either SB939, DAC or a combination of both drugs and subsequent drug withdrawal. Cells were treated with the respective drug(s) for 72 h (day 1–3) and then observed for 11 d upon drug withdrawal (day 6–14). Time course of *AKAP12α* mRNA expression in Kasumi-1 (C), and HL-60 cells (D) upon drug treatment and subsequent drug withdrawal. Untreated control cells (Ctrl) are shown as light gray bars, time-points under drug treatment are shown in dark gray, and time-points upon drug withdrawal are depicted as black bars.

distinct regulatory mechanisms as indicated already by the differential response observed between Kasumi-1 and HL-60.

AKAP12α promoter methylation correlates with clinical prognostic factors and is elevated in relapsed JMML cases

We correlated *AKAP12α* DNA methylation levels of 81 JMML patients with their clinical and hematological characteristics (Table 1). This revealed significant correlation with a number of parameters: Higher levels of *AKAP12α* promoter methylation were associated with platelet counts $< 70 \times 10^9$/l ($P = 0.02$), bone marrow blasts $\geq 5\%$ ($P = 0.03$), and aberrant karyotype ($P = 0.03$). The mutational status was also associated with significant differences in *AKAP12α* promoter methylation ($P = 0.04$). In a posthoc test the presence of a *PTPN11* mutation was associated with significantly higher methylation levels as compared to *CBL*-mutated

($P = 0.007$), and *KRAS*-mutated ($P = 0.017$) cases and showed a tendency for higher methylation as compared to samples with no known mutation ($P = 0.048$).

Interestingly, the strongest correlation of *AKAP12α* DNA methylation levels was observed with two well-established prognostic parameters, namely increased age at diagnosis ($P < 0.001$), and elevated fetal hemoglobin levels (HbF; $P < 0.001$).[2,36] In line with this, patients who would experience a relapse after HSCT showed a higher methylation level at diagnosis as compared to those who did not relapse ($P = 0.03$). No differences were found with respect to overall survival and event-free survival.

In order to investigate whether the levels of *AKAP12α* promoter methylation are not only associated with the risk of relapse but also increase during the course of disease, we analyzed DNA samples from four JMML patients of whom we had material, both at the time of diagnosis and at relapse after one or more transplants. Samples taken at the time of relapse displayed elevated *AKAP12α* promoter CpG

Table 1 Correlation of AKAP12 promoter methylation with clinical parameters in JMML patients (N = 81).

|	Correlation (Spearman)	P	Categorized (Median)	P	n
N	81		0.328		
Age [years]	0.598	<0.001			
<2 yrs			0.292		48
>= 2 yrs			0.424	<0.001	33
Sex					
Male			0.327		53
Female			0.333	n.s.	28
Leukocytes [10^9/l]	−0.101	n.s.			81
Missing					0
<33			0.329		47
>=33			0.325	n.s.	34
Platelets [10^9/l]	−0.214	0.06			80
Missing					1
<70			0.403		39
>=70			0.301	0.02	41
Hemoglobin [g/dl]	0.047	n.s.			78
Missing					3
Myeloblasts (BM) [%]	0.211	n.s.			77
Missing					4
<5%			0.301		29
>=5%			0.350	0.03	48
Monocytes (PB) [%]	−0.098	n.s.			81
Monocytes (BM) [%]	0.131	n.s.			77
Missing					4
Spleen size at diagnosis [cm below the costal margin]	0.217	0.06			79
Missing					2
Hemoglobin F (age-adjusted)					
Normal			0.284		19
Elevated			0.406	<0.001	35
Missing					27
Karyotype					
Normal			0.314		52
Aberrant			0.376	0.03	26
Missing					3
Mutation#					
NF1			0.386		10
PTPN11			0.424		28
KRAS			0.299		12
NRAS			0.312		9
CBL			0.296		10
No mutation			0.322	0.04	9
Missing[*]					3
Survival					
Alive			0.327		49
Dead			0.329	n.s.	32
Alive after HSCT					
Yes			0.332		44
No			0.337	n.s.	25
No HSCT					12
Relapse after HSCT					
Yes			0.434		19
No			0.319	0.03	50
No HSCT					12

[*]D 375, D 600: not complete; D 120: Noonan
#Post-Hoc Tests:
Differences in comparison PTPN11 vs.
● CBL (P = 0.007)
● KRAS (P = 0.017).
● Tendency: PTPN11 vs. "no mutation" (P = 0.048)

methylation levels as compared to the diagnostic samples (Fig. 4). In the patient who experienced a second relapse, the *AKAP12α* promoter methylation seemed to increase during the course of disease with the highest level of *AKAP12α* promoter methylation detected at the time of the second relapse.

In conclusion, we demonstrate that the *AKAP12α* promoter shows high DNA methylation levels in JMML, which go along with reduced *AKAP12α* mRNA expression levels. Our data further suggest that differential binding of myeloid transcription factors might contribute to *AKAP12α* silencing. Importantly, *AKAP12α* promoter methylation levels are associated with known prognostic factors such as age at diagnosis and elevated HbF levels, which suggests involvement of *AKAP12α* in disease biology. Alternatively, since recent literature described several differentially methylated loci in JMML, which all correlated with clinical outcome, it might be tempting to speculate that a global epigenetic
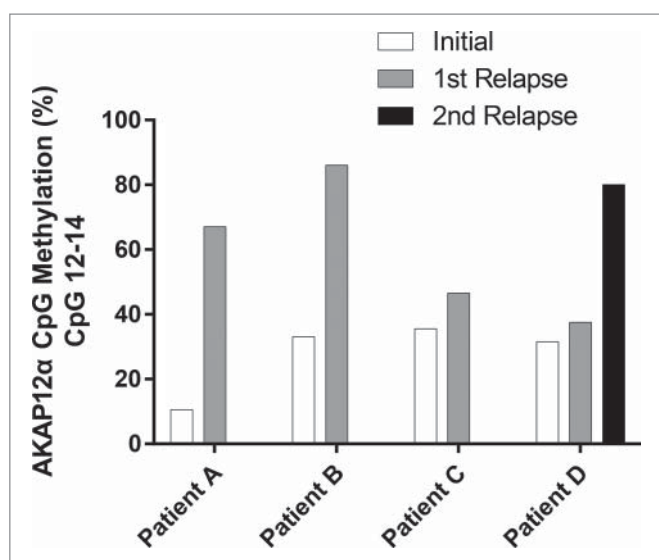
Figure 4. Longitudinal comparison of *AKAP12α* DNA methylation levels at diagnosis and at relapse. The mean *AKAP12α* promoter methylation level measured at CpGs 12–14 is depicted for JMML patients (n=4) who experienced relapse after treatment.

re-patterning occurs during the onset of JMML pathogenesis.[7,8] Further studies will be needed to elucidate the exact molecular mechanisms leading to *AKAP12α* transcriptional repression and the impact of aberrant signaling in establishing aberrant methylation patterns within the *AKAP12α* promoter region.

Material and methods

Patient samples

Mononuclear cells and granulocytes were isolated via density gradient centrifugation from peripheral blood or bone marrow of 81 unselected German JMML patients. Informed consent was obtained from the parents or legal guardians in accordance with the Declaration of Helsinki and approval was granted from the institutional review committees of each participating center. Patients had been registered in the European Working Group of MDS in Childhood (EWOG-MDS) studies EWOG-MDS 98 and EWOG-MDS 2006 (www.clinicaltrials.gov identifiers NCT00047268 and NCT00662090). Peripheral blood granulocytes (n = 23) from healthy donors were used as controls. CD34+ cells (n = 10) from bone marrow of healthy donors were obtained from Lonza (Basel, Switzerland). Genomic DNA was isolated using either the Puregene (Qiagen, Hilden, Germany) or the Transfast (Peqlab, Erlangen, Germany) isolation kit. Trizol reagent (Invitrogen, Karlsruhe, Germany) was used to isolate total RNA. For a subset of JMML samples (n = 23) DNA and RNA was extracted simultaneously using the AllPrep kit (Qiagen, Hilden, Germany).

Cell lines

The human myeloid leukemia cell lines Kasumi-1, HL-60, and K562 were grown in RPMI 1640 medium (PAA Laboratories,

Cölbe, Germany) supplemented with 10% FCS, 1% sodium-pyruvate, and 1% penicillin/streptomycin (Sigma, St. Louis, MO, USA). Cells were kept at 37°C in an atmosphere containing 5% CO_2. DNA and RNA were isolated using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) and Trizol reagent, respectively.

5-aza-2′-deoxycytidine (DAC) and SB939 treatment of cell lines

Cell lines (Kasumi-1 & HL-60) were seeded at a density of 0.8×10^6 cells/ml. Cell lines were treated with the DNMT inhibitor 5-aza-2′-deoxycytidine (DAC) (Sigma-Aldrich, St. Louis, MO, USA), and the histone deacetylase inhibitor SB939 (Cayman Chemical, Ann Arbor, MI, USA) either alone or in combination for 72 h. Cell culture medium and inhibitors were renewed every 24 h. After completion of the 72 h treatment, cells were grown for additional 72 h in cell culture medium without addition of inhibitors. Samples for isolation of genomic DNA and total RNA were taken at 24, 48, 72, and 144 h from treated and control cells.

Functional studies of the AKAP12α promoter

Functional promoter CpG methylation analysis was carried out using a CpG-free vector backbone (pCpGL-basic) provided by Dr. Michael Rehli (Department of Internal Medicine III, University Hospital, Regensburg, Germany). Table S1 depicts the *AKAP12α* promoter construct (−794 bp to +92 bp relative to TSS) covering the promoter CpG-island used for luciferase reporter assay. The construct was amplified with Phusion High-Fidelity DNA polymerase (Fermentas, St. Leon-Rot, Germany), and purified using the Qiaquick PCR purification kit (Qiagen, Hilden, Germany). Subsequently, the PCR-product was cloned into the pCpGL-basic vector using the *Bgl*II and *Hin*dIII restriction sites. To test the impact of GATA binding on *AKAP12α* promoter activity, a 17-nt fragment containing a GATA motif was deleted using QuikChange II XL site-directed mutagenesis kit (Agilent Technologies) according to the manufacturer's instructions. Briefly, two oligonucleotide primers complementary to the vector were extended using *PfuUltra* HF DNA polymerase. Following temperature cycling, the reaction mixture was placed on ice for 2 min, and then treated with *Dpn*I (1h, 37°C) to digest the parental DNA template and to select for deletion-containing synthesized DNA. The mutated plasmid was then ethanol precipitated and transformed into PIR electrocompetent cells. The deletion was verified by Sanger sequencing.

Primer sequences for site-directed mutagenesis:

GATA-CG-Δ(26–42)-FW cctgacatcacttgttctgggcctgcgtggc

GATA-CG-Δ(26–42)-RV gccacgcaggcccagaacaagtgatgtcagg

In vitro methylation of plasmid DNA was carried out as described.[37] Briefly, the plasmid was incubated with *M.Sss*I (2.5 U/μg DNA) in the presence of 160 μM SAM for 4 h at 37°C. Another 160 μM SAM was added after 2 h to boost the reaction. A control plasmid was treated equally but without the addition of methyltransferases or SAM. All plasmids were treated with proteinase K to remove bound proteins. The

completeness of methylation was tested via digestion of the plasmid with the methylation sensitive restriction enzyme *Hpa*II and subsequent agarose gel separation.

Transient transfection assays

Transient transfection of K562 cells was performed using the Amaxa Cell Line Nucleofector Kit V, according to the manufacturer's instructions. Briefly, for each transfection experiment, 1×10^6 cells/sample were used, pelleted by centrifugation (200 g, 10 min, RT) and resuspended in 100 μL Nucleofector solution. One microgram of Firefly luciferase reporter vector (pCpGL-basic) and 3 μg of Renilla luciferase control vector (pRL-Actin) were added and nucleofection was performed using "T-016." Pre-warmed cell culture medium (RPMI1640 + 10% FCS; 500 μl) was added to the cuvette and the cells were subsequently plated into a 96-well plate in quintuplicates (technical replicates) and cultured at 37°C in 5% CO_2. Luciferase activity was measured 24 h later. Firefly luciferase activity was normalized to Renilla luciferase activity, and the average of technical replicates was calculated. Each experiment was performed in biological quadruplicates.

Bisulfite treatment and quantitative DNA methylation analysis

Genomic DNA was bisulfite treated using the EZ DNA methylation kit (Zymo Research, Irvine, CA). Bisulfite-converted DNA was PCR amplified using Hotstar Taq (Qiagen, Hilden, Germany). Amplified regions ranged 400–500 bp and were designed close to the transcription start sites of the three *AKAP12* isoforms (α, β, and γ) (Table S2). The MassARRAY EpiTYPER assay (Sequenom, San Diego, CA) was carried out as described before.[38,39] Briefly, the PCR amplified regions were *in vitro* transcribed, RNaseA cleaved, desalted, and subjected to matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS).

Reverse transcription and quantitative real-time PCR

Total RNA (1μg) was reverse transcribed using SuperScript II and oligo-dT primer (Invitrogen, Karlsruhe, Germany). Quantitative PCR was performed on a LightCycler 480 (Roche Diagnostics, Mannheim, Germany) using QuantiTect SYBR Green mix (Sigma, St. Louis, MO). Conditions for qRT-PCR and primer sequences are summarized in Table S3. Measurements were carried out in triplicates and relative expression levels were calculated using the ΔΔCT method. The reference genes *HPRT, TBP*, and *ACTB* were used for normalization.

Statistical analyses

To determine the significance of promoter CpG methylation levels between JMML and healthy controls, an unpaired t-test with Welch's correction was performed. *P*-values below 0.05 were assumed to be statistically significant. Expression values (mRNA) were compared using the non-parametric Kruskal-Wallis test with adjacent *post-hoc* Mann-Whitney U test. For

the mutational analysis we first used the Kruskal-Wallis test and then the Mann-Whitney U test (*post-hoc*). All statistics was performed using GraphPad Prism software version 5.04 for Windows (GraphPad Software, La Jolla California USA, http:www.graphpad.com).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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