

## ORIGINAL ARTICLE

## Altered miRNA and gene expression in acute myeloid leukemia with complex karyotype identify networks of prognostic relevance

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Recently, the p53-miR-34a network has been identified to have an important role in tumorigenesis. As in acute myeloid leukemia with complex karyotype (CK-AML) *TP53* alterations are the most common known molecular lesion, we further analyzed the p53-miR-34a axis in a large cohort of CK-AML with known *TP53* status (*TP53*<sup>altered</sup>, *n* = 57; *TP53*<sup>unaltered</sup>, *n* = 31; altered indicates loss and/or mutation of *TP53*). Profiling microRNA (miRNA) expression delineated *TP53* alteration-associated miRNA profiles, and identified miR-34a and miR-100 as the most significantly down- and upregulated miRNA, respectively. Moreover, we found a distinct miR-34a expression-linked gene expression profile enriched for genes belonging to p53-associated pathways, and implicated in cell cycle progression or apoptosis. Clinically, low miR-34a expression and *TP53* alterations predicted for chemotherapy resistance and inferior outcome. Notably, in *TP53*<sup>unaltered</sup> CK-AML, high miR-34a expression predicted for inferior overall survival (OS), whereas in *TP53*<sup>biallelic altered</sup> CK-AML, high miR-34a expression pointed to better OS. Thus, detailed molecular profiling links impaired p53 to decreased miR-34a expression, but also identifies p53-independent miR-34a induction mechanisms as shown in *TP53*<sup>biallelic altered</sup> cell lines treated with 15-deoxy- $\Delta^{12,14}$ -prostaglandin. An improved understanding of this mechanism might provide novel therapeutic options to restore miR-34a function and thereby induce cell cycle arrest and apoptosis in *TP53*<sup>altered</sup> CK-AML.

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## INTRODUCTION

Acute myeloid leukemia (AML) is a genetically heterogeneous clonal stem cell disorder characterized by the accumulation of genetic alterations leading to aberrant proliferation and differentiation.<sup>1,2</sup> In addition to chromosomal abnormalities, several genetic lesions of pathogenic and prognostic relevance could be identified, such as gene mutations and/or gene expression signatures. Moreover, microRNAs (miRNAs) have recently been implicated in the pathogenesis of AML;<sup>3</sup> miRNAs consisting of ~22-nucleotides regulate post-transcriptional expression of protein-coding mRNAs in proliferating cells.<sup>4</sup> Malignant cells, in general, as well as AML blasts show an aberrant miRNA expression pattern,<sup>5</sup> and given that miRNAs are located in genomic regions frequently affected by translocations, deletions and/or amplifications in cancer, including leukemia, supports their role in tumorigenesis. Furthermore, functional studies confirmed the important pathogenic role of miRNA deregulation in hematological malignancies, including AML.<sup>3</sup>

Similar to global gene expression profiling (GEP), recent studies using miRNA expression profiling identified cytogenetic subgroup-associated miRNA signatures in AML. Specific signatures were delineated for core-binding factor leukemias [t(8;21)(q22;q22), inv(16)(p13.1;q22)/t(16;16)(p13.1;q22)], acute promyelocytic leukemia [t(15;17)(q22;q12)], 11q23 rearranged AML, as well as for cytogenetically normal AML.<sup>3</sup> In addition, miRNA signatures

are also correlated with molecular markers of prognostic impact, such as mutations in *NPM1*, *FLT3* and *CEBPA*. However, recent studies have shown that not only miRNA expression signatures, but also expression values of single miRNAs can be of prognostic relevance, for example, the expression of miR-191 and miR-199a, which were associated with adverse overall survival (OS) in intermediate- and poor-risk AML.<sup>6</sup> Similarly, in cytogenetically normal AML, higher expression of miR-181a was correlated with longer OS and a higher complete remission (CR) rate.<sup>7</sup>

With regard to the biological impact of miRNA deregulation in cancer, recently the inactivation of members of the miR-34 family, which can induce cell cycle arrest, senescence and apoptosis, has been shown to provide a selective advantage for cancer cells.<sup>8</sup> Although especially inactivating mutations of *TP53* appear to significantly contribute to decreased miR-34 expression in tumors, there have also been reports demonstrating the miR-34-encoding genes themselves to be targets of genomic mutations or epigenetic silencing. Furthermore, located on 1p36, a chromosomal band proposed to harbor a tumor suppressor gene due to homozygous deletions seen in neuroblastoma and in other tumor types, miR-34a was revealed as a candidate tumor suppressor based on its tumor suppressive properties.<sup>9</sup> In accordance, in chronic lymphocytic leukemia, 17p deletions and/or *TP53* mutations have been associated with decreased expression of the direct p53 target miR-34a, and thus with resistance to

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chemotherapy. Furthermore, in this leukemia, miR-34a expression itself could be associated with chemotherapy-refractory disease irrespective of *TP53* alteration status.<sup>10</sup>

In AML with complex karyotype (CK-AML),<sup>11</sup> little is known about miRNAs despite the fact that frequent *TP53* alterations are associated with dismal outcome due to a high rate of chemotherapy resistance.<sup>12</sup> Therefore, to gain additional insight into the molecular pathogenesis of CK-AML, including the role of miRNAs, we performed an integrative analysis using miRNA profiling and global GEP in 88 molecularly characterized CK-AML with known *TP53* alteration status. Findings were evaluated both with regard to their biological relevance *in vitro* and a potential clinical impact in primary leukemia.

## PATIENTS AND METHODS

### Patients

DNA and RNA were extracted from frozen peripheral blood and/or bone marrow samples from 88 adult CK-AML patients. The definition of CK-AML followed recommended criteria.<sup>1,11</sup> The diagnosis of AML was based on French-American-British Cooperative Group criteria<sup>13</sup> and on the WHO criteria for cases diagnosed after 2004;<sup>14</sup> 85/88 (97%) patients were treated on consecutive multicenter treatment trials of the AML Study Group (AMLSG), applying age-adjusted intensive chemotherapy: AMLHD98A ( $n=21$ ; NCT00146120) and AMLSG07-04 ( $n=30$ ; NCT00151242) for younger patients (16 to 60 years); AMLHD98B ( $n=6$ ) and AMLSG06-04 ( $n=28$ ; NCT00151255) for elderly patients ( $>60$  years).<sup>15,16</sup> All studies were approved by local ethics committees and all patients gave informed consent for treatment, cryopreservation of samples and molecular analyses according to the Declaration of Helsinki.

### Cytogenetics and molecular genetics

On the basis of banding analysis, 63 (72%) of the 88 CK-AML patients exhibited a monosomal karyotype (CK+/MK+) as previously defined.<sup>17</sup> The most frequent monosomy was monosomy 7 in 23 patients (37% of the CK+/MK+ subgroup). The molecular *TP53* status assessed as previously described<sup>18</sup> was as follows: *TP53* mutations in 51/88 (58%), genomic loss of *TP53* in 36/88 (41%) CK-AML, with a total of 57/88 (65%) cases showing *TP53* alterations (biallelic, 75%; monoallelic, 25%; see Supplementary Table 1 for detailed clinical and cytogenetic information).

### miRNA expression profiling in CK-AML

Expression of miRNAs was analyzed using the Agilent miRNA Microarray System (miRBase V12.0) according to the manufacturer's protocol (Agilent Technologies Inc., Santa Clara, CA, USA). In brief, total RNA isolated from mononuclear AML cells (*TP53*<sup>unaltered</sup>,  $n=8$ ; *TP53*<sup>altered</sup>,  $n=9$ ) with the *mirVana* miRNA Isolation Kit (Applied Biosystems/Ambion, Austin, TX, USA) was directly labeled. The microarray data were extracted and processed using the Feature Extraction Software, and data analysis was performed with the GeneSpring GX software using the standard normalization and transformation settings (Agilent Technologies Inc.). A miRNA was considered differentially expressed when the expression difference was at least 1.5-fold, with a  $P$ -value of  $<0.05$  between groups (unpaired Mann-Whitney test). The complete filtered miRNA microarray data set is provided as Supplementary Table S2.

### Validation and evaluation of miRNA findings

Quantitative reverse-transcription PCR (qRT-PCR) of miR-34a and miR-100 using the TaqMan MicroRNA Reverse Transcription Kit and MicroRNA Assays (Applied Biosystems Inc., Foster City, CA, USA) was performed in 88 CK-AML cases as previously described.<sup>19</sup> Expression of *RNU6B* was measured as endogenous control for normalization of data.

### Identification of miR-34a- and *TP53* status-associated gene expression profiles

On the basis of our previously published GEP data with available miR-34a expression ( $n=20$ ) and *TP53* mutation status ( $n=32$ ),<sup>20,21</sup> we performed supervised analyses, Class Comparison and Pathway Class Comparison analyses, using BRB-Array Tools Version 3.6.1 (available at <http://linus.nci.nih.gov/BRB-ArrayTools.html>) and R Version 2.6.0 (available at

[www.r-project.org](http://www.r-project.org)) as previously reported.<sup>22</sup> In addition, gene signatures were further evaluated using the Molecular Signatures Database (MSigDB, <http://www.broadinstitute.org/gsea/msigdb>).<sup>23</sup>

To further investigate and validate the miR-34a-associated cDNA microarray-based gene expression patterns, we analyzed distinct subgroups defined by *TP53* alteration and miR-34a expression status using Human Genome GeneChip U133plus2.0 microarrays according to standard protocols reported elsewhere.<sup>24</sup> Here, exemplary samples from *TP53*<sup>unaltered</sup> ( $n=6$ ) and *TP53*<sup>biallelic altered</sup> ( $n=6$ ) CK-AML characterized by either high (CK+/miR-34a<sup>high expression</sup>, above median miR-34a expression of the entire cohort) or low (CK+/miR-34a<sup>low expression</sup>, below median miR-34a expression of the entire cohort) miR-34a expression ( $n=3$  in each group) were analyzed. Data normalization and analyses were carried out as described above (GEO accession number: GSE39730).

### Cell lines and cell culture

Leukemia cell lines (HEL, HL-60 and K-562) were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ) and cultured according to their guidelines ([www.dsmz.de/human\\_and\\_animal\\_cell\\_lines/](http://www.dsmz.de/human_and_animal_cell_lines/)) as previously reported.<sup>25</sup> Cell lines were treated in triplicate with 15-deoxy- $\Delta^{12,14}$ -prostaglandin (PGJ<sub>2</sub> dissolved in methyl acetate; Sigma Aldrich, St Louis, MO, USA) at a final concentration of 10  $\mu$ M for 24 h. For the control experiments, cell lines were treated with an equivalent volume methyl acetate (Sigma Aldrich) for 24 h. From treated cell lines, RNA was isolated and miR-34a expression measured by qRT-PCR as outlined above.

### Statistical analyses

Pairwise comparisons between patient characteristics were performed by Mann-Whitney test for continuous variables and by Fisher's exact test for categorical variables. Clinical endpoints were CR after induction therapy, relapse-free survival (RFS), event-free survival (EFS) and OS, as recommended.<sup>1</sup> A conditional logistic regression model incorporating stratification according to treatment intensity was used to analyze associations between baseline characteristics and achievement of CR. A Cox model with stratification to account for treatment intensity was used to identify prognostic variables; in addition to *TP53* alteration, age, logarithm of white blood cell count, lactate dehydrogenase serum level, monosomy 7 and miR-34a expression were added as explanatory variables in all regression analyses. To provide quantitative information on the relevance of results, 95% confidence intervals (95% CIs) of hazards ratios (HRs) were computed. Statistical analyses were performed using GraphPad Prism 4 (GraphPad Software, La Jolla, CA, USA) and PASW Statistics 18 (IBM Corporation, Armonk, NY, USA).  $P$ -values  $<0.05$  were considered to indicate statistical significance.

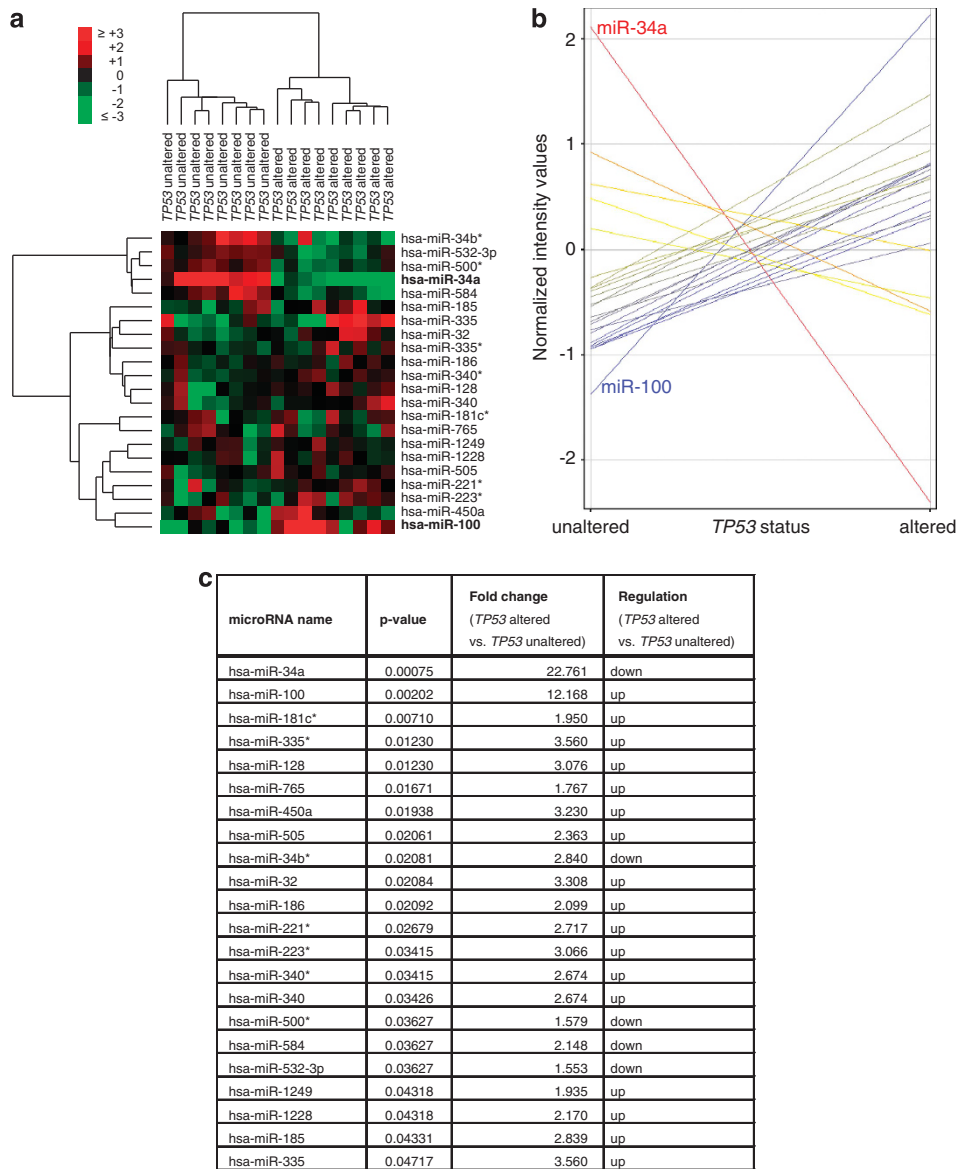
## RESULTS

### *TP53* status-associated miRNA profiles

To address the potential impact of the p53-miR-34a network in CK-AML, we performed miRNA expression profiling in a cohort of 17 CK-AML cases (*TP53*<sup>altered</sup>,  $n=9$ ; *TP53*<sup>unaltered</sup>,  $n=8$ ). Supervised analysis identified 22 differentially expressed miRNAs ( $P<0.05$ ) being correlated with the *TP53* alteration status, of which miR-34a and miR-100 were the most significantly down- and upregulated miRNAs, respectively (Figure 1). Although miR-34a was on average 22.8-fold downregulated in *TP53*<sup>altered</sup> AML cases, miR-100 showed 12.2-fold higher expression levels in *TP53*<sup>altered</sup> compared with *TP53*<sup>unaltered</sup> AML cases, an inverse correlation not previously reported.

### Evaluation of miR-34a and miR-100 expression in CK-AML

We subsequently investigated miR-34a and miR-100 using qRT-PCR in all 88 CK-AML cases to further evaluate the interconnection between the two miRNAs and with regard to the *TP53* status. For miR-34a, we observed a significant correlation of expression levels with *TP53* alteration (Figure 2a). However, for miR-100 there was no significant correlation with *TP53* alteration (Figure 2b), but there was a significant inverse correlation between miR-34a and miR-100 expression (Figure 2c). Thus, our observations demonstrate that miR-34a and *TP53* alteration status are not exclusively linked, as we found *TP53*<sup>unaltered</sup> cases with low



**Figure 1.** *TP53* status-associated miRNA profiles. (a) miRNAs differentially expressed between *TP53*<sup>unaltered</sup> and *TP53*<sup>altered</sup> cases ( $P < 0.05$ , unpaired Mann–Whitney test). MiRNAs ( $n = 22$ , rows) and CK-AML patients (*TP53*<sup>unaltered</sup>,  $n = 8$ ; *TP53*<sup>altered</sup>,  $n = 9$ ; columns) were hierarchically clustered. Mean-centered  $\log_2$  expression values are depicted by a pseudocolor scale, as indicated. (b) Profile plot view of the respective 22 miRNAs. Ascending lines depict miRNAs with higher expression and descending lines miRNAs with lower expression in *TP53*<sup>altered</sup> cases compared with *TP53*<sup>unaltered</sup> cases. The slope of the line correlates with the fold change difference of the respective miRNA between the two groups. (c) Table indicating the 22 differentially expressed miRNAs sorted by  $P$ -value. Fold change between the two groups and type of regulation (up or down) are indicated (*TP53*<sup>altered</sup> vs *TP53*<sup>unaltered</sup>).

miR-34a expression and *TP53*<sup>altered</sup> cases with high miR-34a expression (Figure 2d), indicating that additional determinants of miR-34a expression in CK-AML remain to be identified.

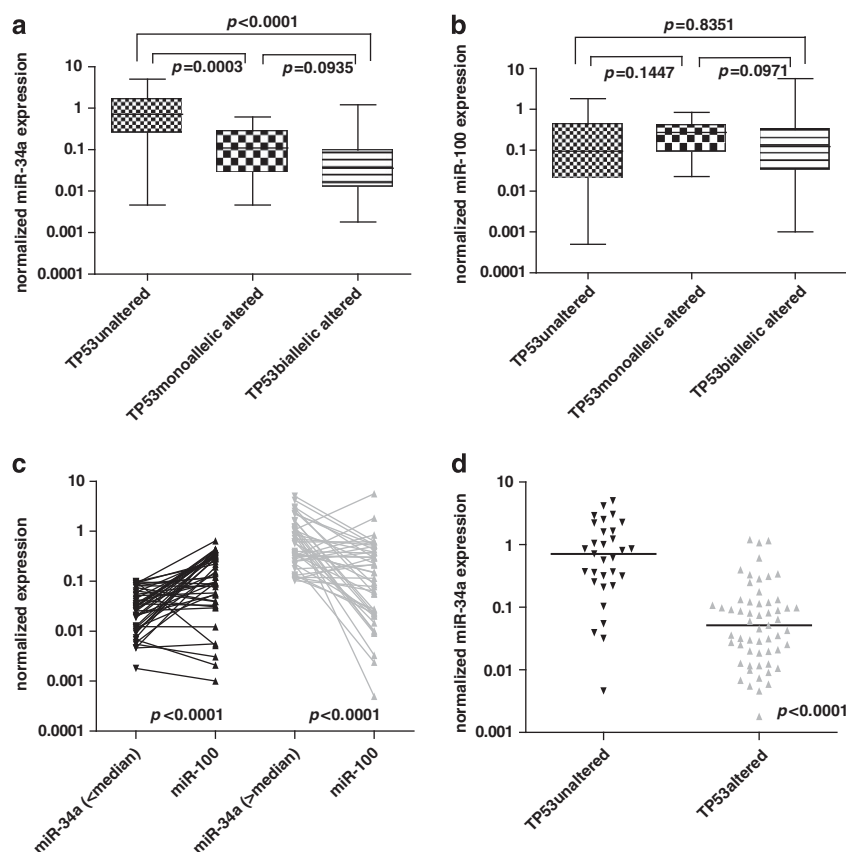
Delineation of miR-34a-associated GEP

To further characterize the molecular anatomy of CK-AML, we reanalyzed our previously published GEP data for cases with newly available *TP53* alteration status and miR-34a expression information and identified a distinct miR-34a expression-associated gene expression pattern.

Supervised Class Comparison analysis revealed 1219 clones to be significantly differentially expressed among groups (miR-34a expression below median of the entire cohort, CK<sup>+</sup>/miR-34a<sup>low</sup> expression ( $n = 10$ ) versus miR-34a expression above median of the entire cohort, CK<sup>+</sup>/miR-34a<sup>high</sup> expression ( $n = 10$ ))

at a nominal  $P$ -value  $< 0.05$  (Supplementary Table 3). One of the most differentially expressed genes was *TP53*, with a significantly lower expression in CK<sup>+</sup>/miR-34a<sup>low</sup> expression ( $P = 0.004$ ). Pathway Class Comparison analysis showed the miR-34a signature to be enriched for 66/287 investigated Biocarta gene sets ( $P < 0.05$ ; Supplementary Table 4). These included pathways implicated in cell cycle arrest, apoptosis or tumor suppression (for example, cell cycle G2/M checkpoint, cell cycle G1/S checkpoint, regulation of cell cycle progression by Plk3, or tumor suppressor Arf pathway).

To address the interconnection between miR-34a and p53, and because p53 is having a central role in most of these pathways, we looked for a *TP53* alteration-associated gene expression pattern. Thereby, supervised Class Comparison analysis revealed 1631 clones to be significantly differentially expressed among groups (*TP53*<sup>unaltered</sup> ( $n = 22$ ) versus *TP53*<sup>altered</sup> ( $n = 10$ )) at a nominal  $P$ -value  $< 0.05$  (Supplementary Table 5). In addition to *TP53*, we



**Figure 2.** Expression of miR-34a and miR-100 in CK-AML. Correlation of miR-34a expression (**a**) and miR-100 expression (**b**) with *TP53* alteration (two-group comparisons were calculated by Mann–Whitney test). Data are shown for the genotypes *TP53*<sup>unaltered</sup>, *TP53*<sup>monoallelic altered</sup> and *TP53*<sup>biallelic altered</sup>. (**c**) Inverse correlation of miR-34a and miR-100 expression in CK-AML (Wilcoxon matched-pair test). (**d**) Subgroup-specific miR-34a expression in *TP53*<sup>unaltered</sup> and *TP53*<sup>altered</sup> CK-AML delineating cases with low miR-34 expression among *TP53*<sup>unaltered</sup> CK-AML, and cases with high miR-34a expression among *TP53*<sup>altered</sup> CK-AML (Mann–Whitney test).

observed that other genes located in 17p13 also showed decreased expression, including, for example, *SEN3*, *ZBTB4* and *DVL2*, consistent with a gene dosage effect.<sup>26</sup> Pathway Class Comparison analysis showed the *TP53* signature to be enriched for 35/287 investigated Biocarta gene sets ( $P < 0.05$ ; Supplementary Table 6). These included several pathways known to be regulated by p53 (for example, the AKT–mTOR or the hTERT (telomerase reverse transcriptase in human) pathway).

Comparing the miR-34a- and the *TP53* alteration-associated pathways, an overlap of distinct pathways could be observed, such as the RHOA (Ras homolog gene family, member A) signaling pathway, the vascular endothelial growth factor pathway, the STAT3 (signal transducer and activator of transcription 3) pathway or the AKT signaling pathway; but moreover, we also identified pathways that were mutually exclusive, such as the CXCR4 signaling pathway or the phosphatase and tensin homolog pathway being enriched only in the *TP53* signature.

#### Correlation of miR-34a expression with clinical characteristics, response to therapy and survival

Correlation with clinical characteristics and outcome was restricted to patients enrolled in consecutive multicenter treatment trials of the AMLSG ( $n = 85$ ; median age, 57 years (range 19–77)), and was performed based on: (i) data dichotomized at the median miR-34a expression and (ii) data grouped according to the quartiles of miR-34a expression.

CK-AML with low miR-34a expression (below median expression of the entire cohort, CK<sup>+</sup>/miR-34a<sup>low expression</sup>) were older (median

age, 59 versus 52 years,  $P = 0.006$ ) and showed, in trend, higher lactate dehydrogenase serum levels (median U/l, 591 versus 415,  $P = 0.05$ ) (Table 1). Subgroup analysis (miR-34a expression 1st quartile, CK<sup>+</sup>/miR-34a<sup>1st quartile</sup>, versus 2nd–4th quartiles, CK<sup>+</sup>/miR-34a<sup>2nd–4th quartiles</sup>, of the entire cohort) revealed higher white blood cell count ( $P = 0.02$ ) for CK<sup>+</sup>/miR-34a<sup>1st quartile</sup> (Table 1).

MiR-34a expression was associated with resistance to chemotherapy. Response to induction therapy for CK<sup>+</sup>/miR-34a<sup>1st quartile</sup> and CK<sup>+</sup>/miR-34a<sup>2nd–4th quartiles</sup> was as follows: CR 14 (3/21) versus 42% (26/62;  $P = 0.03$ ), refractory disease 57 (12/21) versus 34% (21/62;  $P = 0.07$ ) and early/hypoplastic death 29 (6/21) versus 24% (15/62;  $P = 0.77$ ), respectively (Table 1). Other variables predicting for poor response to induction therapy were age (CR 18 (6/34) versus 47% (23/49) for CK-AML > 60 years and CK-AML < 60 years, respectively;  $P = 0.009$ ) and *TP53* alteration (CR 25 (13/52) versus 52% (16/31) for *TP53*<sup>altered</sup> and *TP53*<sup>unaltered</sup> CK-AML, respectively;  $P = 0.02$ ). In multivariable analysis, only age retained its significance for CR achievement (Table 2).

MiR-34a expression was associated with inferior survival. The 3-year estimated survival rates for CK<sup>+</sup>/miR-34a<sup>low expression</sup> and CK<sup>+</sup>/miR-34a<sup>high expression</sup> patients were as follows: OS, 4 versus 20% (log-rank,  $P = 0.006$ ); EFS, 0 versus 7% ( $P = 0.003$ ); and RFS, 8 versus 33% ( $P = 0.13$ ), respectively, (Table 1 and Figure 3). Other variables predicting for inferior OS in univariable analysis were *TP53* alteration ( $P = 0.0007$ ), monosomy 7 ( $P = 0.049$ ) and in trend age (> 60 years;  $P = 0.051$ ). Multivariable analysis revealed miR-34a-expression (HR, 1.47; 95% CI, 1.06–2.03;  $P = 0.02$ ), *TP53* alteration (HR, 2.93; 95% CI, 1.43–6.02;  $P = 0.003$ ), logarithm of



**Table 1.** Pretreatment characteristics and outcome according to miR-34a expression

	miR-34a expression			miR-34a expression		
	Low	High	P-value	1st quartile	2nd–4th quartile	P-value
<i>Clinical data</i>	n = 44	n = 44		n = 22	n = 66	
Gender (male/female)	19 (43%)/25 (57%)	21 (48%)/23 (52%)	0.83	14 (64%)/8 (36%)	26 (39%)/40 (61%)	0.08
Age (years, median)	59	52	0.006	63	56	0.05
<i>AML history</i>						
De novo	38 (86%)	33 (75%)	0.28	20 (91%)	51 (77%)	0.22
Secondary/therapy-related	6 (14%)	11 (25%)	0.28	2 (9%)	15 (23%)	0.22
WBC (10 <sup>6</sup> /l, median)	11.4	14.1	0.79	21.6	9	0.02
Platelet count (10 <sup>6</sup> /l, median)	50	42	0.71	51	43	0.73
Hemoglobin (g/l, median)	8.9	9.0	0.95	8.7	9	0.53
BM blast count (% , median)	79	75	0.61	89	70	0.06
PB blast count (% , median)	48	47	0.74	65	42	0.04
LDH serum level (U/l, median)	591	415	0.05	776	445	0.06
<i>Molecular genetics</i>	n = 44	n = 44		n = 22	n = 66	
TP53 alteration	40 (91%)	17 (39%)	<.0001	21 (95%)	36 (55%)	0.0003
<i>Response to induction therapy</i>	n = 40	n = 43		n = 21	n = 62	
Complete remission	11 (27%)	18 (42%)	0.25	3 (14%)	26 (42%)	0.03
Refractory disease	19 (48%)	14 (33%)	0.18	12 (57%)	21 (34%)	0.07
Early death	10 (25%)	11 (25%)	1.00	6 (29%)	15 (24%)	0.77
<i>Outcome</i>	n = 41	n = 44		n = 22	n = 63	
OS						
(Months, median)	3.93	7.17	0.006	3.27	7.00	0.0002
(3-year survival rate, %)	4	20		0	16	
EFS						
(Months, median)	0.88	1.20	0.003	0.73	1.13	0.002
(3-year survival rate, %)	0	7		0	5	
RFS						
(Months, median)	6.28	12.33	0.13	5.33	9.5	0.05
(3-year survival rate, %)	8	33		0	27	

Abbreviations: Low, miR-34a expression below median of the entire cohort; High, miR-34a expression above median of the entire cohort; 1st quartile, lowest miR-34a expression of the entire cohort; WBC, white blood cell count; BM, bone marrow; PB, peripheral blood; LDH, lactate dehydrogenase; TP53 alteration, loss and/or mutation of TP53; OS, overall survival; EFS, event-free survival; RFS, relapse-free survival.

white blood cell count (HR, 1.71; 95% CI, 1.02–2.89;  $P=0.04$ ) and age (HR, 1.03; 95% CI, 1.01–1.04,  $P=0.008$ ), but not lactate dehydrogenase serum level or monosomy 7 as significant variables for OS (Table 2).

Owing to the wide range of miR-34a expression in TP53<sup>unaltered</sup> ( $n=31$ ) and TP53<sup>biallelic altered</sup> ( $n=41$ ) CK-AML, we performed explorative subgroup analyses, revealing that among TP53<sup>biallelic altered</sup> CK-AML, those with high miR-34a expression (4th quartile ( $n=11$ )) compared with 1st–3rd quartiles ( $n=30$ ) of the TP53<sup>biallelic altered</sup> cohort had significantly better OS ( $P=0.0225$ , Figure 4a); but contrariwise, among TP53<sup>unaltered</sup> CK-AML, those with high miR-34a expression (4th quartile of the TP53<sup>unaltered</sup> cohort,  $n=8$ ) had significantly worse OS ( $P=0.0104$ ; Figure 4b). There were no significant differences in pre-treatment patient characteristics between the respective groups.

#### miR-34a-associated gene expression profiles within distinct TP53 genotype subgroups

Further molecular characterization using GEP identified distinct miR-34a-associated gene expression patterns within the TP53<sup>unaltered</sup> and TP53<sup>biallelic altered</sup> CK-AML subgroups, respectively.

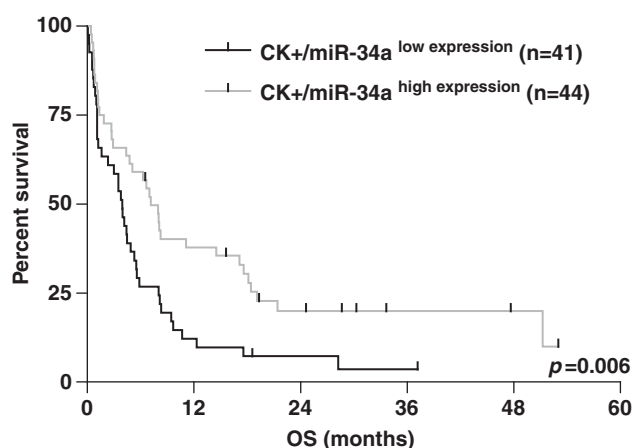
In the TP53<sup>unaltered</sup> CK-AML subgroup, supervised Class Comparison analysis revealed 981 probe sets to be significantly differentially expressed at a nominal  $P$ -value  $<0.05$  (Supplementary Table 7), not comprising TP53. However, the Pathway Class Comparison analysis showed the miR-34a-associated signature to be enriched for 52/290 investigated Biocarta gene sets ( $P<0.01$ ; Supplementary Table 8). These included several pathways having a role in onco-/leukemogenesis or tumor suppression, like the RAS, PI3K-AKT-mTOR, or the phosphatase and tensin homolog pathway. Furthermore, we found genes of RHO cell motility and the CXCR4 signaling pathway significantly enriched (Supplementary Table 8).

In the TP53<sup>biallelic altered</sup> CK-AML subgroup, Class Comparison analysis revealed 1197 probe sets to be significantly differentially expressed at a nominal  $P$ -value  $<0.05$  (Supplementary Table 9). The Pathway Class Comparison analysis showed the miR-34a-associated signature to be enriched for 59/290 investigated Biocarta gene sets ( $P<0.01$ ; Supplementary Table 10). These included the pathways mentioned above, but moreover, additional pathways implicated in cell cycle control (cell cycle: G2/M checkpoint, regulation of cell cycle progression by Plk3), DNA damage (ATM signaling) and/or apoptosis (induction of apoptosis through DR3 and DR4/5 death receptors).

**Table 2.** Multivariable analyses of outcome

	Response			OS			RFS		
	OR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value
CK-AML									
TP53 alteration	0.29	0.08–1.14	0.08	2.93	1.43–6.02	0.003	2.68	0.83–8.62	0.10
miR-34a expression	0.78	0.42–1.44	0.42	1.47	1.06–2.03	0.02	1.90	1.14–3.18	0.01
Age	0.96	0.92–0.99	0.03	1.03	1.01–1.04	0.008	1.01	0.98–1.04	0.54
Logarithm of WBC	0.38	0.13–1.11	0.08	1.71	1.02–2.89	0.04	2.58	0.82–8.06	0.10
LDH serum level	2.02	0.33–12.20	0.45	1.25	0.54–2.88	0.60	1.23	0.21–7.29	0.82
Monosomy 7 <sup>a</sup>	1.18	0.36–3.83	0.78	1.69	0.98–2.93	0.06	4.44	1.38–14.29	0.01

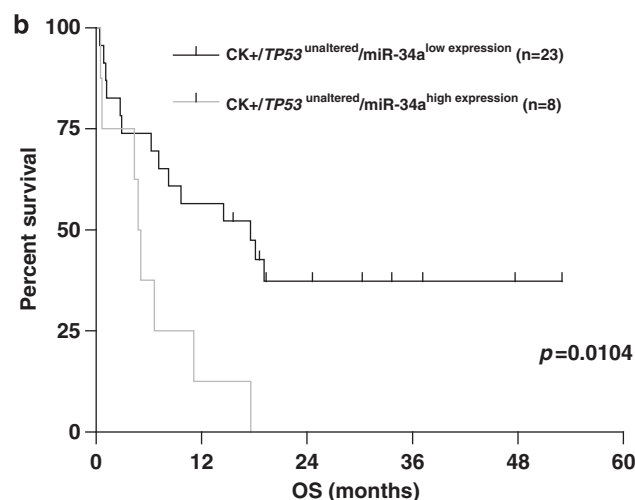
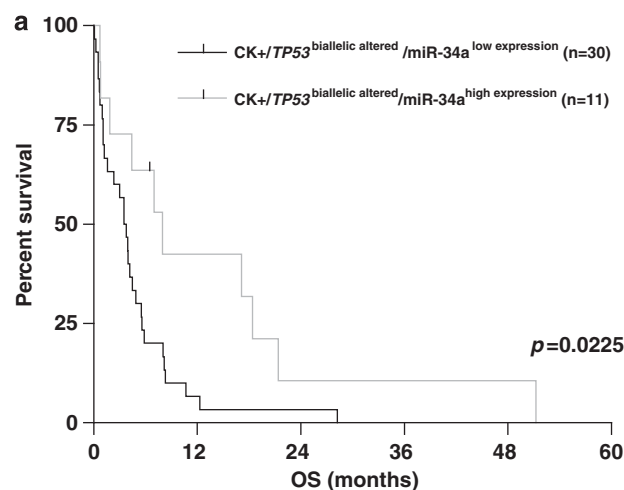
Abbreviations: Response, CR achievement after induction chemotherapy; CI, confidence interval; OS, overall survival; RFS, relapse-free survival; OR, odds ratio; HR, hazards ratio; WBC, white blood cell count; LDH, lactate dehydrogenase. <sup>a</sup>Determined by chromosome banding analysis.



**Figure 3.** Kaplan–Meier survival estimates according to miR-34a expression. Data are shown for the genotypes CK<sup>+</sup>/miR-34a<sup>low</sup> expression and CK<sup>+</sup>/miR-34a<sup>high</sup> expression for overall survival (OS, months; log-rank test).

#### TP53-independent miR-34a upregulation via peroxisomal proliferator-activated receptor- $\gamma$ agonist treatment

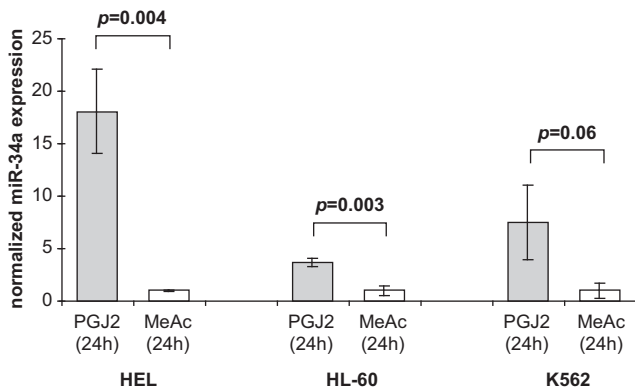
To further evaluate the biological relevance of the miR-34a-associated gene expression patterns, we used the Connectivity Map, a repository of gene expression profiles from human cell lines treated with a large number of small bioactive molecules,<sup>27</sup> to identify modulators that might reverse the respective transcriptional changes. Specifically, we focused on the gene signature discriminating low and high miR-34a-expressing TP53<sup>biallelic altered</sup> CK-AML subgroups (1st–3rd quartiles of the TP53<sup>biallelic altered</sup> cohort ( $n = 30$ )) to query the Connectivity Map for compounds for which the activity is inversely linked to this signature, thereby possessing the potential ability to induce TP53-independent miR-34a upregulation. Among the perturbagens with negative enrichment scores (indicating compounds that may repress the biological state encoded in the low miR-34a expression signature), we observed PGJ<sub>2</sub>, a natural peroxisomal proliferator-activated receptor- $\gamma$  ligand, to be one of the most prominent drugs. To test our hypothesis that this compound might invert the low miR-34a-associated expression signature via the induction of miR-34a expression in a TP53-independent way, we treated three myeloid cell lines with known biallelic TP53 alteration (HEL, HL-60 and K-562) with PGJ<sub>2</sub>. Although the TP53<sup>biallelic altered</sup> cell lines showed very low miR-34a expression levels similar to the TP53<sup>biallelic altered</sup> CK-AML subgroup (data not shown), treatment with PGJ<sub>2</sub> for 24 h induced a 3.7 to 18.1-fold upregulation of miR-34a expression in the respective cell lines (Figure 5).



**Figure 4.** Kaplan–Meier survival estimates for overall survival (OS) for CK-AML according to molecular genetic findings. OS based on miR-34a expression of the respective cohort for the genotypes TP53<sup>biallelic altered</sup> (a) and for TP53<sup>unaltered</sup> (b).

#### Identification of potential miR-34a target genes

To identify putative miR-34a-regulated target genes in CK-AML, we performed an integrative analysis comparing predicted miR-34a targets and our TP53 alteration-associated gene expression signatures (see above), as it was recently shown that mammalian miRNAs predominantly act to decrease target mRNA levels.<sup>28</sup> Using different prediction algorithms (MicroCosm Targets



**Figure 5.** PGJ<sub>2</sub> induced *TP53*-independent expression of miR-34a. Treatment of *TP53*<sup>biallelic altered</sup> cell lines with PGJ<sub>2</sub> for 24 h results in a significant upregulation of miR-34a expression in HEL and HL-60, and also in trend in K-562 (see indicated *P*-values; normalized miR-34a expression depicts the fold-change in PGJ<sub>2</sub>-treated cells compared with the respective controls that have been set to 1.0).

(formerly miRBase Targets),<sup>29</sup> miRWalk,<sup>30</sup> DIANA-microT v3.0,<sup>31</sup> TargetScan<sup>32</sup> and PicTar<sup>33</sup>), we identified 2019 potential miR-34a target genes predicted by at least one of the algorithms (range: 235–1221 predicted targets). Intersection of this theoretical direct miR-34a target gene list with genes upregulated in CK<sup>+</sup>/*TP53*<sup>altered</sup> cases, which display low miR-34a expression and thus an increased abundance of target mRNA was anticipated, revealed potential miR-34a-regulated candidates (Supplementary Table 11). Although this approach identified known miR-34a target genes such as the transmembrane glycoprotein *CD44*,<sup>34</sup> cyclin-dependent kinase *CDK6*,<sup>35</sup> *ACSL1* (acyl-CoA synthetase long-chain family member 1)<sup>36</sup> and the transcription factor *E2F3*,<sup>37</sup> our integrative analysis also pointed to novel candidate genes of potential oncogenic relevance, such as *BCL6*, *CCNE2* (Cyclin E2, related to the known miR-34a target *CCND1*), the colony stimulating factor 1 receptor gene (*CSF1R*) and *ICOSLG* (inducible T-cell co-stimulator ligand, previously associated with AML<sup>38</sup>). Additional genes of potential pathogenic relevance include *ALDOA* (aldolase A), a leukemia-associated antigen expressed in chronic myeloid leukemia,<sup>39</sup> and *TPD52* encoding the tumor protein D52.

## DISCUSSION

In this study, we delineated the miRNA and mRNA profiles associated with genetically defined CK-AML subtypes. We show here that *TP53*<sup>altered</sup> CK-AML harbor a specific miRNA profile, which comprises, among others, miR-34a and miR-100 as the most significantly down- and upregulated miRNAs, respectively. Notably, other known p53-induced miRNAs (for example, miR-145, miR-107, miR-192, miR-215, and miR-16) were not found significantly altered, most likely due to cell type dependent p53-associated gene expression patterns. Moreover, CK<sup>+</sup>/*TP53*<sup>altered</sup> and CK<sup>+</sup>/miR-34a<sup>low expression</sup> are characterized by similar gene expression profiles enriched for genes belonging to pathways known to be regulated by p53 and/or implicated in cell cycle progression or apoptosis. These findings fit well with a p53-dependent miR-34a expression reported in many other tumors, which seems to be independent of the tumor type.<sup>8,10</sup> Although p53 protects cellular integrity and p53 activation induces cell cycle arrest, apoptosis, or senescence following DNA damage, these effects are in part mediated through p53-dependent induction of miR-34a, which has been shown to be a direct p53 target.<sup>8</sup> Accordingly, ectopic miR-34 expression also

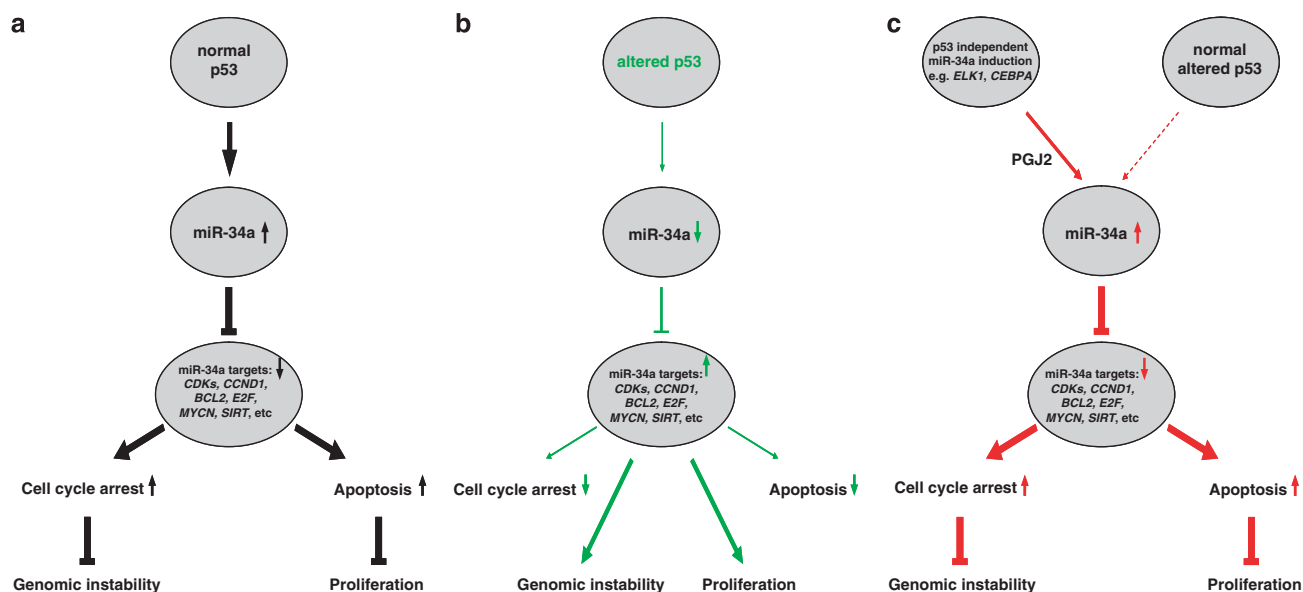
induces apoptosis, cell cycle arrest or senescence, and loss of miR-34 expression has been linked to chemotherapy resistance because of impaired apoptosis induction by p53-activating agents.<sup>8,10</sup>

Consistent with findings from chronic lymphocytic leukemia, in our study, miR-34a expression was also associated with resistance to chemotherapy. Furthermore, miR-34a expression also predicted for inferior survival. As miR-34a is a direct target of p53, it was not surprising that we observed similar clinical characteristics and endpoints for CR, EFS, OS and RFS in *TP53*<sup>altered</sup> CK-AML. In multivariable analysis, only miR-34a expression retained its significance for OS and RFS. Thus, these data further demonstrate the important role of the p53-miR-34a network in CK-AML that warrants further investigation in larger patient cohorts.

The correlation of high miR-100 expression with *TP53* alteration seen in our microarray analyses has not been reported previously. Although the analysis of our entire CK-AML patient cohort by qRT-PCR showed miR-100 expression only in trend correlated with *TP53* alteration, we could confirm a significant inverse correlation between miR-34a and miR-100 expression. MiR-100 has not been extensively studied, and little is known about miR-100 function in leukemia. Nevertheless, miR-100 expression was recently found to be associated with resistance to chemotherapy in pediatric acute lymphoblastic leukemia,<sup>40</sup> and it was shown to have a role in controlling proliferation because of its function as an endogenous repressor of the serine/threonine protein kinase mammalian target of rapamycin (mTOR).<sup>41</sup> Furthermore, a recent study in AML showed that miR-100 can regulate G1/S transition and S-phase entry, and block the terminal differentiation of cells.<sup>42</sup> These observations, in addition to the deregulation seen in our study, point to an important role of miR-100 in AML pathogenesis.<sup>42</sup> Thus, the inverse correlation between miR-100 and miR-34a expression may contribute to the chemoresistance and the enrichment of mTOR pathway members in the *TP53*<sup>unaltered</sup>-associated CK-AML signature. Although this speculation of course warrants further investigation, in view of recent reports on the role of miR-100 in cancer,<sup>40–42</sup> our observations strongly suggest a respective role in CK-AML.

Unexpectedly, among *TP53*<sup>biallelic altered</sup> CK-AML, not all cases exhibited low miR-34a expression and explorative subgroup analyses revealed that those with high miR-34a expression (4th quartile) had significantly better OS (Figure 4a). High miR-34a expression in *TP53*<sup>biallelic altered</sup> CK-AML was not related to the type of biallelic *TP53* alteration (hemizygous, homozygous mutations or location of mutation). However, these cases harbored a distinct gene expression profile different from that of low miR-34a-expressing *TP53*<sup>biallelic altered</sup> CK-AMLs. Although this signature was enriched for genes belonging to pathways implicated in cell cycle control, DNA damage and/or apoptosis, our data suggest different pathomechanisms leading to a p53-independent miR-34a upregulation in these cases (Figure 6c). Reflecting biological mechanisms, we could use this signature to identify a compound with ability to induce miR-34a expression in a *TP53*<sup>biallelic altered</sup> background. Treatment of *TP53*<sup>biallelic altered</sup> cell lines with PGJ<sub>2</sub> delineated to be negatively linked to the low miR-34a expressing *TP53*<sup>biallelic altered</sup> CK-AML signature resulted in a marked upregulation of miR-34a expression, thereby offering the possibility to, in part, restore defective p53 signaling.

In accordance, recent findings further underline the hypothesis that miR-34a expression can also be regulated in a p53-independent manner. During oncogene-induced senescence, a mechanism preventing tumorigenesis, miR-34a can be upregulated by ELK1, an E-twenty six family transcription factor, and can act as a tumor suppressor by targeting the proto-oncogene *MYC*.<sup>43</sup> Another transcription factor regulating miR-34a expression is the CCAAT-enhancer binding protein alpha (CEBPA). During granulopoiesis, CEBPA upregulates miR-34a that targets *E2F3*, thereby blocking myeloid cell proliferation.<sup>44</sup> Mutations in *CEBPA*



**Figure 6.** Modeling the p53-miR-34a network and its potential impairment in CK-AML. (a) Intact p53-induced upregulation of miR-34a leads to cell cycle arrest and apoptosis through silencing of miR-34a target genes. (b) Impaired p53 leads to weak or no miR-34 induction resulting in impaired cell cycle arrest and apoptosis, and thereby in degree to genomic instability and proliferation. (c) p53-independent miR-34a induction also leads to cell cycle arrest and apoptosis through silencing of miR-34a target genes.

are found in approximately 10% of AML<sup>1</sup> and expression profiling revealed significantly lower miR-34a expression in *CEBPA*-mutated AML compared with *CEBPA* wild-type AML.<sup>45</sup>

Similarly, the inverse prognostic impact of miR-34a expression in *TP53*<sup>unaltered</sup> CK-AML (high miR-34a expression (4th quartile) was correlated with significantly worse OS ( $P = 0.0104$ )) (Figure 4b) might also be related to p53-independent miR-34a induction. In agreement with this idea, the gene expression signature discriminating the high miR-34a expression cohort within the *TP53*<sup>unaltered</sup> cases did not contain *TP53* or correlate with p53 pathway genes, but we found deregulated *CXCR4* and *RHOA* signaling in these cases that might lead to upregulation of miR-34a via the activation of ERK signaling.<sup>46</sup> Alternatively, p53 could be active in all *TP53*<sup>unaltered</sup> patients, and in cases with very low expression of miR-34a, other mechanisms could prevent p53 from inducing miR-34a expression. Although only one case exhibited a deletion of the miR-34a locus 1p36, which resulted in the lowest expression of miR-34a observed in this group, the other *TP53*<sup>unaltered</sup> cases showed no 1p36 alteration (see Rücker *et al.*<sup>12</sup> and unpublished data). Although the miR-34a gene has been reported to be epigenetically inactivated at high frequency in different solid tumors due to aberrant CpG methylation of its promoter,<sup>8</sup> no silencing of miR-34a has been reported in AML yet.<sup>47</sup> However, only 20 cases of undisclosed cytogenetics were analyzed in this study. Thus, epigenetic inactivation of the *miR-34a* gene could nevertheless contribute to atypical low expression of miR-34a in *TP53*<sup>unaltered</sup> patients, even though a probably rare event in AML.

With regard to the downstream effects of miR-34a in CK-AML, our integrative miRNA and mRNA expression data analysis proved to be useful, as it identified several known miR-34a target genes as well as new candidates with potential oncogenic relevance. For example, *TPD52* has recently been reported to be downregulated after induction of miR-34a expression and could be validated as a direct miR-34a target.<sup>48</sup> *TPD52* is also found overexpressed in many cancer types, and overexpression of its murine ortholog mD52 in cell lines resulted in enhanced proliferation and anchorage-independent cell growth,<sup>49</sup> thereby also suggesting a potential role of *TPD52* in deregulated p53 signaling in CK-AML. Thus, our study provides a solid basis for further investigation of the complex networks involved in CK-AML.

In summary, we could demonstrate the prognostic relevance of miR-34a expression in CK-AML. As in other cancers, *TP53* alterations are associated with repressed miR-34a expression in CK-AML. However, our detailed, multidimensional analysis could further characterize complex molecular networks and delineate subgroups of *TP53*<sup>unaltered</sup> CK-AML with poor outcome despite the presence of miR-34a. In addition, we could demonstrate that in *TP53*<sup>altered</sup> cases, miR-34a might be expressed independent of p53 by mechanisms also associated with better response to chemotherapy and improved outcome. Although these mechanisms need to be further explored, they ultimately might be targeted therapeutically and could open new strategies to deal with CK-AML.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Leukemia website (<http://www.nature.com/leu>)