

## Expression of the p14<sup>ARF</sup> tumor suppressor predicts survival in acute myeloid leukemia

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**Cell cycle aberrations are associated with therapy outcome in many types of cancer. We analyzed mRNA expression levels of 18 cell cycle-related genes in bone marrow samples from 78 acute myeloid leukemia (AML) patients and six controls using high-throughput quantitative RT-PCR. Samples of AML patients contained significantly increased mRNA expression levels of the mdm2 and c-myc oncogenes. Also, the average expression levels of p14<sup>ARF</sup> and p16<sup>INK4A</sup> were higher in patient samples compared to controls. Leukemic blasts and control bone marrow samples did not differ significantly in the expression levels of proliferation-associated genes such as cyclin A2 and pcna. When single genes were analyzed for prognostic significance in Kaplan–Meier and Cox regression analyses, a low p14<sup>ARF</sup> level emerged as a strong and independent predictor for poor survival ( $P=0.04$  and  $0.029$ ). Subsequently, p14<sup>ARF</sup> mRNA levels were analyzed in a second, independent patient population ( $n=57$ ). Again, low p14<sup>ARF</sup> levels were associated with a worse outcome. Finally, immunohistochemistry analysis of AML tissue arrays confirmed the widespread expression of c-myc and p14<sup>ARF</sup> in AML on the protein level. Taken together, the expression of the p53 regulators mdm2 and p14<sup>ARF</sup> are altered in AML, and low p14<sup>ARF</sup> levels indicate a poor prognosis.**

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### Introduction

Recent advances in gene expression analysis allow systematic evaluation of multiple genes and patient samples in the search for novel therapeutic targets and prognosis determinants. Quantitative RT-PCR by the 5' nuclease technology has evolved into a reliable and specific method that allows accurate analyses of gene expression in multiple clinical specimens at the same time.<sup>1</sup>

In the current study, we analyzed G1/S checkpoint alterations in acute myeloid leukemia (AML). The G1/S checkpoint integrates several intracellular signaling pathways, and alterations of the G1/S transition play a role in the pathogenesis of most if not all cancers.<sup>2</sup> In addition, important mechanisms and genes that control G1/S transition have been identified in recent years.<sup>3</sup> AML is characterized by a massive infiltration of the bone marrow with a uniform blast population. The leukemic blasts show disturbed differentiation capacity and altered control of proliferation and survival pathways. However, little is known about the molecular mechanisms that govern the

disturbance of blast cell number. Only sporadic cases of AML have been described with inactivating mutations of p53 or with the inhibition of Rb function.

We hypothesized that quantitative assessment of the expression levels of cell cycle genes in primary patient samples might reveal meaningful data about the activation status and the prognostic importance of cell cycle-associated pathways in AML.

Our study indicates that expression levels of the p53 regulators mdm2 and p14<sup>ARF</sup> are increased in AML patients' blasts. The p14<sup>ARF</sup> tumor suppressor is an important predictor for patients' prognosis. These findings raise the possibility that alterations of the p53 pathway play an important prognostic role in AML.

### Material and methods

#### Patient samples

AML patients ( $n=78$ ) were enrolled into the treatment optimization trial of the AMLCG study group in Germany.<sup>4</sup> Patient characteristics can be seen in Table 1. The patients were treated with one course of induction chemotherapy containing cytarabine, daunorubicine and thioguanin. Patients younger than 60 years and all patients with blast persistence received a second course of induction with high-dose cytarabine and mitoxantrone.<sup>4</sup> The six control samples were obtained from patients with nonhematological diseases whose bone marrow aspirates were obtained for diagnostic reasons. The second AML patient group ( $n=57$ ) was treated by a different protocol in different study hospitals. The patients received four cycles of intensive chemotherapy with cytarabine, idarubicin and etoposide used for induction therapy followed by consolidation therapy that either included high-dose cytarabine or autologous stem cell transplant. Written consent was obtained from all patients. All bone marrow samples from AML patients were obtained at the time of diagnosis. Blasts were enriched from bone marrow samples by density centrifugation of heparinized bone marrow samples at the time of diagnosis and frozen at  $-80^{\circ}\text{C}$  until the experiments were performed.

#### Analysis of gene expression by real-time quantitative RT-PCR

RNA was isolated from frozen blasts using standard procedures. Total RNA ( $1\mu\text{g}$ ) was transcribed into cDNA using random hexamers and Superscript II reverse transcriptase (Gibco life technology). The cDNA was diluted to  $200\mu\text{l}$  with ddH<sub>2</sub>O, and  $2.5\mu\text{l}$  were used for each PCR reaction. The quantification of

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**Table 1** Patients' characteristics

Patients	N = 78			
Sex	36 male/42 female			
Age	24–70 years (mean 52)			
FAB (known n = 77)				
M0	4/77		5.1%	
M1	15/77		19.2%	
M2	21/77		26.9%	
M3	4/77		5.1%	
M4	22/77		28.2%	
M5	9/77		11.5%	
M6	2/77		2.6%	
Complex karyotype (known n = 59)	6/59		11.9%	
Result induction therapy (known n = 59)				
CR	43/59		72.9%	
Death with AML	6/59		10.1%	
Persistent AML	7/59		11.9%	
Hypoplasia	3/59		5.1%	
	Number (n)	Median	Mean ± s.d.	Range
Leukocytes (× /μl)	78	36 200	83 347 ± 163 244	1100–1 017 000
Platelet count (× /μl)	78	47 000	70 534 ± 64 133	1700–325 000
Blasts in BM (%)	72	89.5	78.7 ± 19.7	20–100
LDH (U/ml)	68	443	651 ± 500	88–2949

Samples were chosen for quantitative RT-PCR analyses based on the high percentage of leukemic blasts in the bone marrow.

mRNA levels was carried out using a real-time fluorescence detection method. The cDNA was amplified by PCR in an ABI prism 7700 sequence detector (PE Biosystems, Foster City, CA, USA). All primer and probe combinations were positioned to span an exon–exon junction (sequences will be provided on request). C/EBP $\alpha$  expression was analyzed using SYBR-green for detection purposes.<sup>5</sup> When genomic DNA was used as a template, no bands were seen after PCR amplification. The probes were labeled at the 5' ends with VIC (GAPDH probe) or with FAM (all others) and at the 3' ends with TAMRA, which served as a quencher. The 5'–3' nuclease activity of the *Taq* polymerase cleaved the probe and released the fluorescent dyes (VIC or FAM), which were detected by the laser detector. After the detection threshold was reached, the fluorescence signal was proportional to the amount of PCR product generated. Initial template concentration can be calculated from the cycle number when the amount of PCR product passes a threshold set in the exponential phase of the PCR reaction. The samples were analyzed simultaneously to reduce the variability: each 96-well plate carried 12 standard samples (serial dilutions of U937 cDNA), the six control samples and the 78 patient samples. The relative gene expression levels were calculated using standard curves generated by the serial dilutions of U937 cDNA. All samples were independently analyzed at least twice for each gene. GAPDH expression levels were used for standardization purposes. Expression levels of the TATA-binding protein (TBP) served as an additional control for the cDNA quality.<sup>6</sup>

#### Tissue array construction and immunohistochemistry analyses

Tissue array construction of formalin-fixed and paraffin-embedded trephine bone marrow biopsies of 85 patients diagnosed with primary, untreated AML was performed as described previously.<sup>7,8</sup> A diagnostic Giemsa-stained section

served as control to enable the definition of areas with the highest amount of blast cells. Two punches were arrayed per patient to analyze intratumoral heterogeneity of c-myc and p14<sup>ARF</sup> expression.

Tissue sections were mounted on SuperFrost/Plus slides and dewaxed in xylene. For p14<sup>ARF</sup> and c-myc, the sections were microwaved in 10 mM citrate buffer pH 6.0 (10 min, 120°C). After washing in PBS, sections were incubated with the primary antibodies (c-myc, Santa Cruz Biotech, 9E10, 1:150; p14<sup>ARF</sup> Santa Cruz Biotech, FL-132, 1:20). The D-APAAP method was used for detection. C-myc and p14<sup>ARF</sup> expression were analyzed using a semiquantitative scoring system ranging from 0 to + + + with respect to the intranuclear staining intensities.

#### Statistical analyses

Statistical data analyses were performed using SPSS 10.0. for Windows. When two groups were compared, we used the Mann–Whitney *U*-test for quantitative variables and  $\chi^2$  test for qualitative variables. Means of several groups were compared with the Kruskal–Wallis test. For survival analyses, patients were followed up until relapse, death or last contact and patients undergoing allogeneic bone marrow transplantation were censored at this time. The analyses for patient survival were performed with early deaths (<85 days) being excluded, since these deaths are often therapy and/or infection related. Kaplan–Meier plots were compared using the log-rank test. All *P*-values indicate two-sided comparisons and a *P*<0.05 was considered significant. Average linkage clustering was performed using centered correlations to calculate the distances of genes and samples, after all gene expression data had been log transformed, normalized and median centered in relation to the variation of expression over all samples and genes. The software from M Eisen was used for this purpose.<sup>9</sup>

## Results

### *Transcriptional profiling by high-throughput quantitative RT-PCR in AML*

Transcriptional profiling of genes associated with G1/S transition was performed in bone marrow samples of AML patients at the time of diagnosis. Samples were selected for transcriptional profiling for their high percentage of leukemic blasts in the bone marrow (median 90%). After RNA isolation and reverse transcription, a real-time RT-PCR-based approach was used for transcriptional profiling of genes associated with G1/S cell cycle transition.

Bone marrow specimens from 78 patients and six controls (nonhematological diseases) were analyzed on 96-well plates along with a standard curve consisting of duplicates of six serial dilutions of cDNA of the U937 cell line. Thus, all samples were analyzed simultaneously, and all analyses were performed at least twice. In total, 20 genes were analyzed; besides known housekeeping- and cell cycle-associated genes, we also chose the mRNA of the telomerase catalytic subunit (hTERT) that correlates with telomerase activity. The transcription factor *c/EBP $\alpha$*  has recently been linked to the differentiation block in AML and cell cycle regulation.<sup>10,11</sup> Altogether, more than 4400 independent quantitative PCR reactions were performed. Expression data were standardized using GAPDH expression levels. Comparison analyses with standardization by the housekeeping gene TBP revealed a close correlation between the resulting data sets (data not shown).

### *Cluster analyses of expression data*

To analyze the association between gene expression profiles and sample groups, we performed hierarchical clustering (Figure 1). Among the bone marrow samples, the six controls clustered close to each other. Owing to limited amounts of cDNA, four out of the 18 genes could not be analyzed in two controls (gray boxes). Certain types of differentiation (indicated by FAB subtypes) clustered together. Cluster 1 contained most of the FAB M1 phenotype leukemias (11/15, 73%), whereas eight of nine (89%) FAB M5 leukemias grouped in cluster 2. Half of all FAB M2 leukemias (10/20, 50%) were found in cluster 3. These data indicated that leukemias resembling certain differentiation phenotypes associated with specific cell cycle gene expression patterns. In addition, more closely related leukemias were usually found in the same cluster. For example, almost three-quarters of the samples in cluster 2 either showed myelomonoblastic (FAB-M4) or monoblastic (FAB-M5) morphology. Karyotype analysis was available for 59 patients. None of the clusters was associated with specific karyotypic changes (data not shown).

### *Differences in gene expression between patients and controls*

Next, we analyzed differences in gene expression between controls and patients. Samples derived from normal bone marrow and AML blasts did not show significant differences in the expression levels of genes associated with proliferative activity. Indeed, mRNA levels of the proliferation-associated genes cyclin A2 and *pcna* were essentially unchanged or even higher in control samples compared to AML samples (Figure 2). The *c-myc* as well as the *mdm2* proto-oncogenes were higher

expressed in AML samples in comparison to normal controls on the mRNA level ( $P=0.023$  and  $0.012$ , Mann–Whitney test). Also, increased expression of the tumor suppressors p14<sup>ARF</sup> and p16<sup>INK4A</sup> was found in the patient group compared to the controls ( $P=0.007$  and  $0.01$ ). Expression levels of p14<sup>ARF</sup> and p16<sup>INK4A</sup> correlated weakly but statistically significant ( $r=+0.396$ ,  $P<0.001$ ).

Tissue arrays were constructed from bone marrow biopsies of patients diagnosed at the University of Münster with a first diagnosis of AML. These independent AML samples were analyzed by immunohistochemistry (IHC) to verify the expression of *c-myc* and p14<sup>ARF</sup> in AML blast cells on the protein level (Figure 3a). These analyses demonstrated that both genes were robustly expressed at the protein level in many cases of AML. Expression of *c-myc* was detectable in 76 out of 85 AML patients (Figure 3b). The staining for *c-myc* was detected predominantly in the nucleus but some staining was also present in the cytoplasm of some blast cells. Staining of p14<sup>ARF</sup> was almost exclusively found in the nucleus and most AML patient samples expressed p14<sup>ARF</sup> (Figure 3c).

### *Prognostic role of cell cycle genes for survival of AML patients*

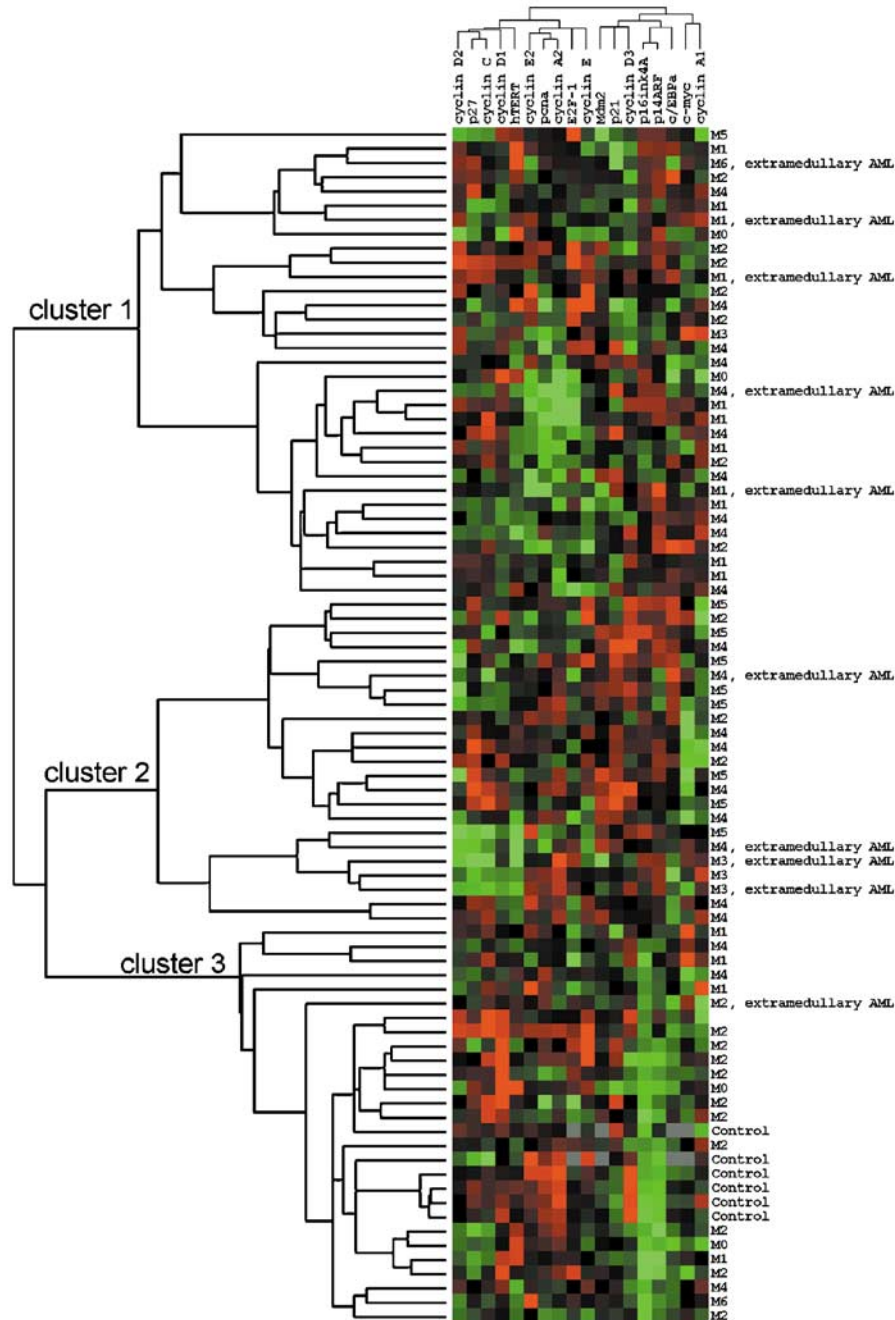
To identify genes, whose expression levels were associated with differences in survival, the mRNA samples were divided for each gene into two groups with one showing 'high' and the other one 'low' expression. The median of all patients' samples ( $n=78$ ) was chosen as the cutoff, so half of all samples were regarded as 'low' and the other half as 'high'. Cox regression analysis was performed to identify genes that were associated with relapse-free survival. The following parameters were included into this analysis: FAB type, gender, FLT3-mutation status, leukocyte count, LDH level, age and the different cell cycle genes. In this analysis, low p14<sup>ARF</sup> levels emerged as an independent determinant of poor prognosis ( $P=0.029$ ). In contrast, proliferation-associated genes as indicated by the expression of *pcna* or E2F-1 were not associated with patients' prognosis.

Kaplan–Meier plots indicated that patients with low p14<sup>ARF</sup> had a mean relapse-free survival time of 294 days after the first complete remission was reached (Figure 4a). In contrast, patients with high levels of p14<sup>ARF</sup> showed a mean remission duration of 563 days ( $P=0.04$ ). Also, when early deaths were excluded, patient survival was significantly longer in patients with high p14<sup>ARF</sup> levels compared to patients with low p14<sup>ARF</sup> levels (mean 696 vs 402 days,  $P=0.04$ ) (Figure 4b). While differences in p14<sup>ARF</sup> expression were clearly related to patients' relapse-free survival, no significant effects of p14<sup>ARF</sup> expression on the rate of remission induction were found (data not shown).

The second gene identified to be of prognostic importance, *c-myc*, was associated with a poor prognosis when it was expressed at high levels (data not shown). However, in another set of patient samples, this finding could not be confirmed (see below). Also, p16<sup>INK4A</sup> expression levels that correlated with p14<sup>ARF</sup> expression levels were not associated with patients' survival.

### *P14<sup>ARF</sup> determines prognosis in a second set of AML patients*

To confirm the prognostic role of p14<sup>ARF</sup> mRNA expression in AML independently, we analyzed a second set of AML patients' samples ( $n=57$ ). These patients were treated within the



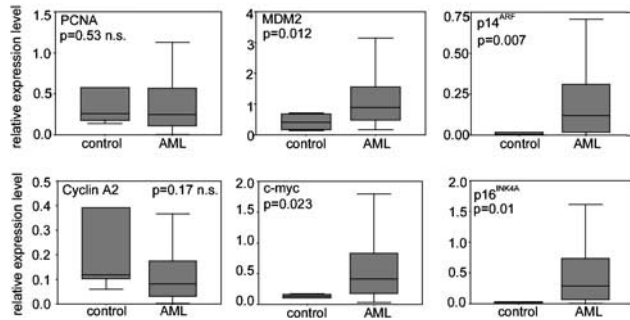
**Figure 1** Cluster analysis of cell cycle gene expression data. For cluster analysis, gene expression data were standardized using the mean expression values of all cell cycle genes ( $n = 18$ ). Data were log transformed and normalized for genes and samples before uncentered hierarchical clustering was performed for genes and samples (see Materials and methods). Green boxes indicate lower expression than the mean expression and red boxes depict higher expression. The gray boxes present in some of the control samples indicate that limited amounts of cDNA precluded analysis. Cluster trees are shown for samples and genes.

Southern German AML study group (SHG). RNA was obtained at the time of diagnosis. The cutoff values determined for the first data set were chosen to distinguish between low and high expressing samples in the second data set. Survival analyses were performed for p14<sup>ARF</sup> expression and for c-myc expression. No significant differences in survival were obtained for low or high c-myc expressing samples (data not shown). Overall, 56% of the patient samples expressed high levels of p14<sup>ARF</sup> and 44% expressed low levels. For survival analyses, early deaths and patients lost to follow-up were excluded. Among the remaining patients, blast cells from 16 patients were low expressing (16/38,

42%) and 22 were high expressing (22/38, 58%). Patients with samples expressing low levels of p14<sup>ARF</sup> showed significantly worse survival (mean 577 days) compared to patients with high p14<sup>ARF</sup> levels (mean 1077 days) (Figure 5). This difference was statistically significant ( $P = 0.026$ ).

### Discussion

The main findings of our study are: First, AML blasts and normal bone marrow cells do not differ in overall expression of



**Figure 2** Gene expression differences between AML patient samples and control bone marrow. Expression levels of cell cycle-related genes in bone marrow samples from AML patients ( $n=78$ ) and normal controls ( $n=6$ ) are shown in the different boxplots. GAPDH expression levels were used for standardization. Statistical significance of the differences was calculated using the Mann–Whitney  $U$ -test.

proliferation-associated cell cycle genes. Second, several genes associated with the c-myc–p14<sup>ARF</sup>–p53 pathway were significantly induced in AML samples, indicating pathway activation. Finally, the p14<sup>ARF</sup> mRNA expression level emerged as an important predictor for patients' prognosis.

Expression analysis by quantitative RT-PCR revealed that mdm2, p14<sup>ARF</sup>, p16<sup>INK4A</sup> and c-myc are expressed at high levels in AML blasts. Three of these genes are closely connected within one pathway: c-myc leads to p14<sup>ARF</sup> induction, which in turn regulates mdm2–p53 interaction and activity. Both c-myc as well as mdm2 are oncogenic, and overexpression of mdm2 is a common mechanism of p53 inactivation.<sup>12</sup> Mutations of the p53 tumor suppressor itself are relatively rare in *de novo* AML.<sup>13</sup> Thus, p53 activity could be abrogated in AML by mdm2 overexpression. Expression of the p14<sup>ARF</sup> tumor suppressor gene was increased in many AML samples. The high expression of p14<sup>ARF</sup> in AML might at least partially be a result of the c-myc overexpression.<sup>14</sup>

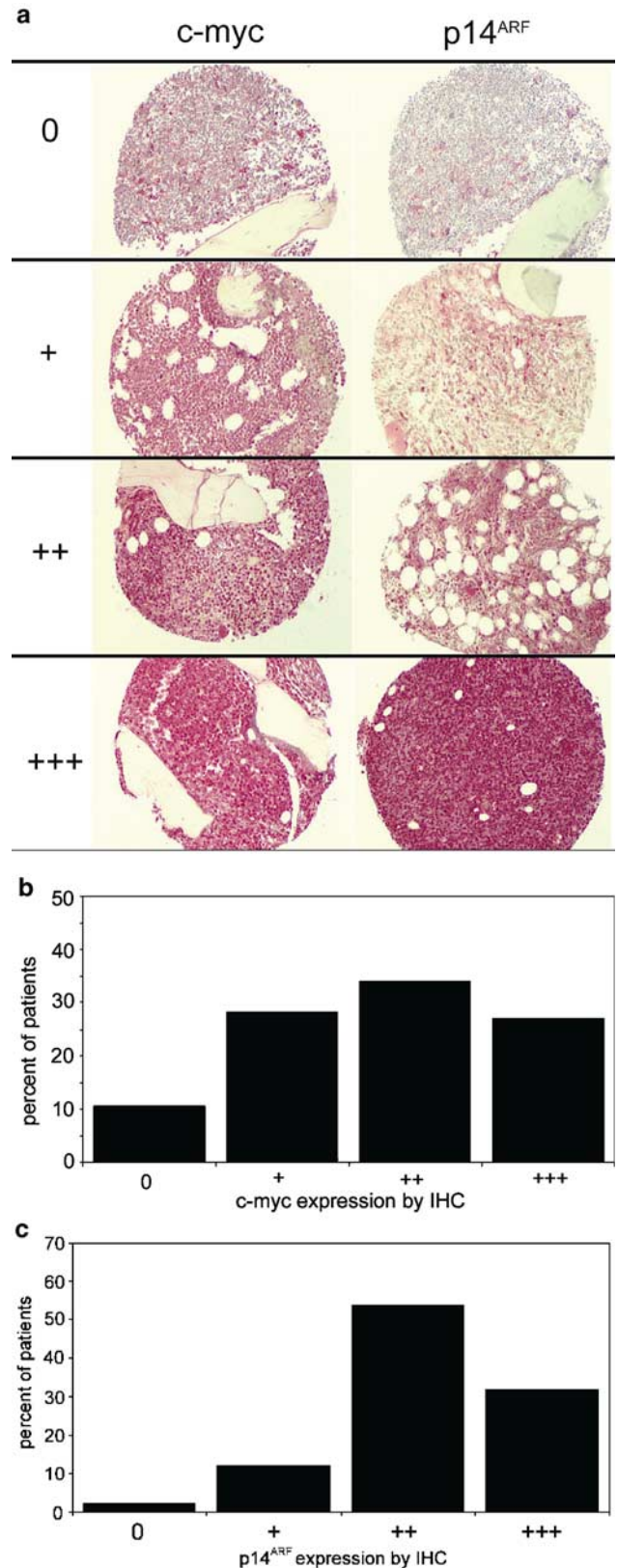
The p16<sup>INK4A</sup> gene was also expressed at higher levels in leukemic samples compared to normal controls. A weak correlation was found between p14<sup>ARF</sup> and p16<sup>INK4A</sup> expression. This coregulation of p14<sup>ARF</sup> and p16<sup>INK4A</sup> is likely to depend on their genomic proximity and their sharing of exons 2 and 3 on the mRNA level.<sup>15</sup> In karyotype analyses, no gross changes on chromosome 9p were found indicating that p14<sup>ARF</sup> expression differences were caused by other mechanisms. In addition, besides the reported low expression of p14<sup>ARF</sup> in t(8;21)-positive samples,<sup>16</sup> no association between karyotype changes and p14<sup>ARF</sup> expression levels was found (data not shown).

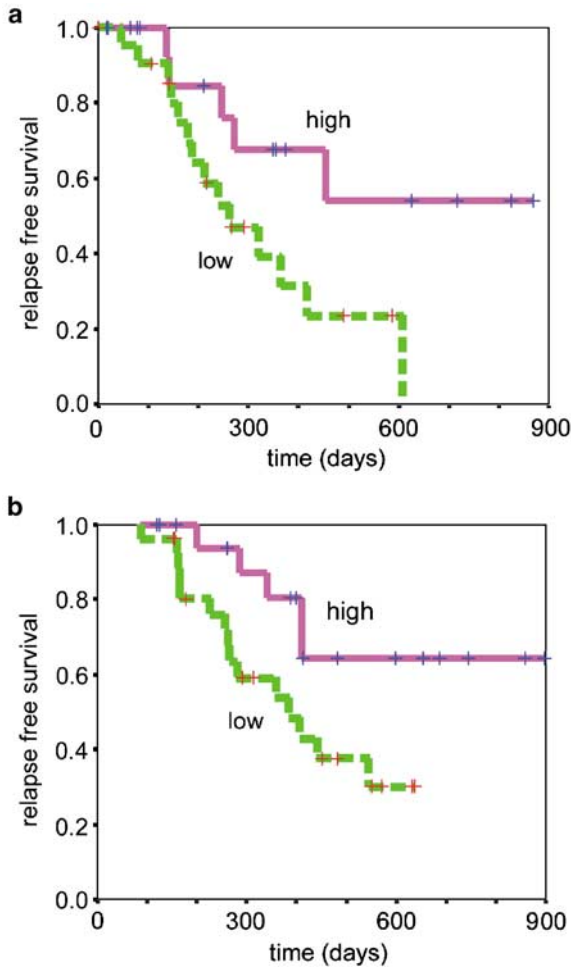
Cluster analysis of our data set revealed that samples of similar differentiation and morphology (FAB subtypes) clustered together. In addition, patients in cluster 2 failed induction therapy significantly more often than patients in the other clusters.

To analyze the prognostic significance of the analyzed cell cycle regulators, samples were grouped in 'high' and 'low' expressers based on the median expression level of all patients.

**Figure 3** Tissue array analysis of c-myc and p14<sup>ARF</sup> expression by IHC. Overall, 112 bone marrow biopsies including samples from 85 AML patients at the time of diagnosis were arranged as a single paraffin block before sections were cut. Each bone marrow biopsy was represented two times on each slide. Expression levels of c-myc and p14<sup>ARF</sup> were analyzed by IHC. Examples of samples regarded as 0, +, ++ and +++ are shown. (a) Indicates the number of AML specimens expressing the different amounts of c-myc protein. (b) Statistical analysis of p14<sup>ARF</sup> expression in AML samples by IHC.

The decreased p14<sup>ARF</sup> expression emerged as an independent prediction for shortened relapse-free survival in a multivariate analysis. In Kaplan–Meier plots, patients with low p14<sup>ARF</sup> expression showed significantly worse survival.

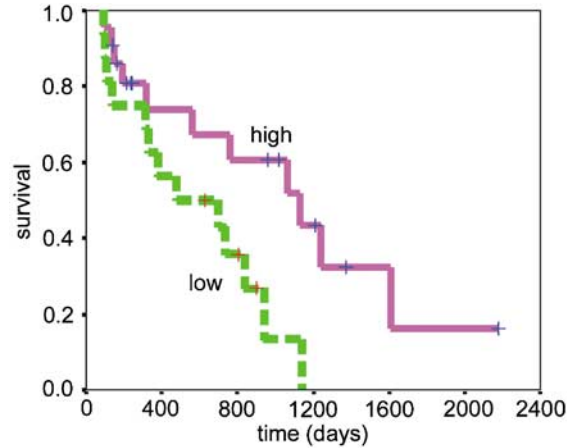




**Figure 4** p14<sup>ARF</sup> expression and survival in AML patients. (a) AML patients were divided in two groups with ‘high’ or ‘low’ p14<sup>ARF</sup> expression levels depending on the median expression of all patients. Only patients who reached a complete remission after one or two courses of induction therapy were included into the relapse-free survival analyses. Statistical significance was calculated using the log-rank test ( $P=0.04$ ). (b) Survival analysis for patients expressing low or high levels of p14<sup>ARF</sup>. Only patients surviving longer than 85 days were included into the analysis to exclude patients who died because of early infections and bleeding ( $P=0.04$ ).

From a statistical point of view, the multiple comparisons that were made to analyze the relevance of the cell cycle genes for survival enhanced the possibility that differences were found by chance. Independent verification was required. Therefore, we analyzed a second set of patient samples using the same cutoff value as for the first patient group. Low p14<sup>ARF</sup> expression was again associated with a poor prognosis. This result is even more striking given that patients of the two study groups were treated by different chemotherapy regimens and initial sample preparations were performed in different laboratories. This independent confirmation of these results stresses the relevance of our findings and indicates that p14<sup>ARF</sup> might be a suitable prognostic marker for clinical use.

While our study established the prognostic role of p14<sup>ARF</sup> mRNA levels in AML, the prognosis prediction capacity of p14<sup>ARF</sup> protein is unclear. In our tissue array, the incomplete follow-up information precluded survival analyses. Since p14<sup>ARF</sup> regulates p53, the association of low p14<sup>ARF</sup> levels with reduced survival is not surprising. The loss of ARF is known to



**Figure 5** p14<sup>ARF</sup> expression in a second patient set and survival analysis. Samples from AML patients treated within a second study group were analyzed for p14<sup>ARF</sup> mRNA levels. Subsequent to standardization with GAPDH expression levels, samples were regarded as p14<sup>ARF</sup> high or low expressing based on the cutoff value determined for the first patient group. Kaplan–Meier plots were drawn and survival was calculated for patients surviving longer than 85 days. The differences in survival were statistically significant ( $P=0.026$ ).

induce chemoresistance and to inhibit radiation-associated apoptosis.<sup>17</sup> The patients analyzed in our study underwent intensive chemotherapy following the diagnosis of AML. In these patients, p14<sup>ARF</sup> expression levels were not associated with the induction of a complete remission (data not shown). However, subsequent relapses were much more frequent in p14<sup>ARF</sup> low expressing patient samples. This might indicate that low p14<sup>ARF</sup> expression relates to a higher likelihood for the development of chemotherapy resistance.

So far, the prognostic relevance of the c-myc–ARF pathway was unknown for AML patients. Deletions of the ARF/INK4A locus have recently been reported in a small percentage of AML patients (5%), and deletions indicated a poor prognosis.<sup>18</sup> However, neither the strong prognostic role of p14<sup>ARF</sup> for all patients nor which one of the tumor suppressors at the ARF/ink4A locus might be relevant has been described.

In a collaborative effort, we have recently shown that AML1-ETO-positive leukemias express significantly less p14<sup>ARF</sup> than other AML blasts.<sup>18</sup> Also, AML1 induced p14<sup>ARF</sup> and this phenomenon was associated with senescence in hematopoietic cells. These results were the first indication for a direct involvement of p14<sup>ARF</sup> in AML pathogenesis. In combination with our current results, these findings underscore the potential role of this pathway in leukemogenesis.

AML1-ETO directly repressed p14<sup>ARF</sup>, while the presence of AML1-ETO itself indicated a favorable prognosis. In our patient population, only six of the samples were AML1-ETO positive, therefore the overall effects of AML1-ETO on patient outcome were small. It is well established that prognostic favorable translocations such as t(8;21) and t(15;17) over-ride adverse prognostic parameters such as a simultaneously present complex karyotype.<sup>19</sup> It is possible that a similar mechanism exists with regard to p14<sup>ARF</sup> expression. The reasons for low p14<sup>ARF</sup> expression obviously differ between t(8;21)-positive and -negative leukemias. This might also explain the distinct prognostic implications of p14<sup>ARF</sup> expression in different types of AML. As a consequence, the analysis of p14<sup>ARF</sup> levels might be especially valuable in patients with a normal karyotype, since the prognosis for this patient group is most difficult to predict.

The important role of c-myc for pathogenesis and chemoresistance of several cancers is well known.<sup>20,21</sup> However, to our knowledge the high levels of c-myc expression in AML demonstrated in our study have not been reported previously. One reason that this area has not been intensively studied might be that translocations involving c-myc and overexpression of c-myc by extrachromosomal copies (double minute) in AML are rare.<sup>22</sup>

In summary, low p14<sup>ARF</sup> expression indicates a poor prognosis in AML. Novel approaches to improve the still unsatisfactory outcome of AML therapy may focus on altering the p14<sup>ARF</sup>-c-myc pathway as an addition to conventional chemotherapy.

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