Activation of the RAS Pathway Is Predictive for a Chemosensitive Phenotype of Acute Myelogenous Leukemia Blasts

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Abstract

Purpose: Activation of the RAS pathway plays a major role in cancer cells. In acute myeloid leukemia (AML), mutations of the RAS genes cause an intrinsic activation of this pathway. Until now, clinical studies could not find clear association of RAS mutations with the clinical outcome after AML therapy. This could be due to alternative initiating events for activation of the RAS pathway like constitutive tyrosine kinase activation or mutations in Ras-regulating genes.

Experimental Design: In total, 191 AML patients (126 as training population and 65 as test population) were studied for Ras activity with a glutathione S-transferase pull-down assay using Raf binding of activated Ras.

Results: AML samples showed a wide range of Ras activity values, which was in contrast to normal bone marrow donors who showed no or very limited Ras activity. Using a Ras binding score based on semiquantitative Western blotting, we defined patients with strong Ras activity and compared Ras activity with RAS mutation. Surprisingly, only a minority of RAS mutated AML samples (22.2%) showed strong Ras activity, whereas 25 patients presented strong Ras activity in the absence of RAS mutations. Clinical outcome did not show differences according to RAS mutations. In contrast, Ras activity predicted for a high response rate (P < 0.05) and proved to be an independent factor for overall survival rate (P < 0.05) in younger AML patients receiving high-dose 1-β-d-arabinofuranosylcytosine as induction therapy.

Conclusion: The data highlight the role for alternative pathways of Ras activation without RAS mutations. Intrinsically activated Ras seems to increase sensitivity of the AML blast to high-dose 1-β-d-arabinofuranosylcytosine therapy.

Normal function of small G-proteins of the RAS family is a prerequisite of cellular homeostasis. Therefore, activation of Ras proteins is tightly regulated with factors able to activate Ras (guanine-nucleotide exchange factors) or factors able to counteract Ras activation (GTPase activating proteins; for recent review, see ref. 1).

Activating point mutations of RAS genes have been generally accepted as oncogenic events in the tumorigenesis of a variety of malignancies. The mechanisms by which mutant Ras is transforming affected cells have been extensively studied. In general, RAS mutations lead to a resistance of the Ras proteins to signaling provided by GTPase activating proteins thus remaining in the active GTP-bound state (2, 3). This constitutive activity is furthermore translated via a cascade of key signaling events that contribute to the regulation of proliferation, apoptosis, and differentiation. One example for such a signaling cascade is RAS-GTP recruitment of Raf proteins to the plasma membrane which is activating phosphorylation of mitogen-activated protein kinase (MAPK) kinase and consequently extracellular signal–regulated kinases (ERK). Extracellular signal–regulated kinase activity has been linked to proliferative stimulation and activation of antiapoptotic pathways (4).

In acute myelogenous leukemia (AML) activating point mutations could be shown to affect almost exclusively N-RAS and K-RAS at codons 12, 13, and 61 (5–7). These alterations were detected with varying frequencies (25–40%) and resemble one of the most common genetic alterations detected in AML.

The consequences of Ras activity in AML blasts are difficult to predict and may depend on additional genetic alterations. Thus, it is well established that Ras activity may account for an increased proliferation rate and decreased apoptosis rate in experimental systems and there is ample evidence that downstream effectors of Ras, like Erk and Akt readily transmit this signaling. However, under certain conditions, Ras activity may even give rise to increased senescence and proapoptotic signaling (8, 9). Recently, elegant studies using conditional expression systems of K-Ras clearly showed a growth factor independent proliferative advantage in transformed hematopoietic cells with subsequent development of a myeloproliferative disease in mice (10, 11).
Given the potential effect of activating point mutations on cellular physiology, one might expect in AML patients an association between RAS mutations and an aggressive course of the disease. However, there may be interactions between genetic alterations (e.g., RAS mutations) and therapeutic modalities. Thus, RAS mutations harbor poor prognosis in lung cancer without adjuvant chemotherapy, whereas in lung cancer or AML, RAS mutations may be associated with better outcome after chemotherapy (6, 12), which may be caused by a differential sensitivity of RAS mutated cells towards 1-β-arabinofuranosylcytosine (ara-C) containing chemotherapy (13). However, other studies revealed conflicting data and did not show an independent effect of RAS mutations on therapy outcome (5, 7, 14).

One potential reason for this inconsistency may be that AML samples with wild-type RAS have acquired activation of Ras by constitutive signals arising from gain-of-function alterations in upstream tyrosine kinases. To date, several models of constitutive Ras activation in AML cell lines have been proposed. It is possible that aberrantly expressed cytokines can activate Ras. Examples for an autocrine stimulatory effect on Ras in AML include constitutive expression of basic fibroblast growth factor and vascular endothelial growth factor (15, 16). Additionally, signaling molecules that interact with Ras activation may interfere with normal GTPase activity of the cell, as it is the case for neurofibromin (17), or activate Ras by aberrantly amplifying signals from other growth factor receptors associated molecules, like the PTPN11-encoded phosphatase SHP-2 (18). Finally, FLT-3 internal tandem duplications (ITD), the most common type of genetic alteration in standard risk AML patients, was shown to exert transforming potential via the Ras pathway (19).

Considering this, the state of activity of Ras may shed more light on clinical consequences of the Ras pathway compared with mutational analysis of the RAS gene. Therefore, we initiated a study highlighting the importance of Ras activity in a large homogeneously treated AML patient population.

Materials and Methods

Patient blast samples/cell lines. In total, 191 previously untreated AML patients were studied. Patients with the diagnosis AML French-American-British subtype M3 were excluded and treated in a separate trial. Blast samples were obtained from each patient at the time point of diagnosis. Mononuclear cells were prepared by a standardized ficoll procedure and cryopreserved in vials containing 5 to 20 × 10⁶ cells.

Additionally, nine bone marrow samples of healthy donors were acquired at the time point of donation. Mononuclear cells were prepared and cryopreserved exactly as it is described for AML blast samples. All patients and healthy donors gave written informed consent for the use of their samples.

HeLa cells served as control in the precipitation assay for Ras activity. Cells were obtained by the German Collection of Microorganisms and Cell Cultures (Bremsweich, Germany). The cell line was grown in RPMI 1640 supplemented with 1% penicillin, streptomycin, 10% FCS, and 1% nonessential amino acids under standard conditions. Twelve hours before stimulation, experiment cells were grown in starving medium (containing 0.5% FCS). Thereafter, cells were stimulated with epidermal growth factor (50 ng/mL) for 5 minutes.

Treatment characteristics. The study of the SHG06 study group is using two different induction treatment schedules (14). Younger patients (≤60 years) received a double-induction treatment, of which the second regimen contained intermediate dose ara-C (10 g/m²). Post-remission therapy is priority based and risk adapted, including chemotherapy for AML, good risk patients, allogeneic transplantation with related donors, autologous or chemotherapy for patients with high risk, and allogeneic transplantation with the option of unrelated donors, autologous, or chemotherapy for patients with high risk. Patients ages >60 years were treated with low-dose ara-C. Post-remission therapy in older patients was individualized with the option of intermediate dose ara-C application. Cytogenetic stratification was done only in patients ages >60 years, because older patients were not treated in a risk-adapted fashion.

Cytogenetic risk groups were defined as follows: high risk: -5/del(5q), -7/del(7q), hypodiploid karyotypes (besides 45,X—Y or —X), inv(3q), t(11q), +11, +13, +21, +22, t(6;9); t(9;22); t(11;1); t(3;3), multiple aberrations; intermediate risk: patients without low-risk or high-risk constellation; low risk: t(8;21) and t(8;21) combined with other aberrations. AML patients with inv16 were considered intermediate risk.

Complete remission (CR) was defined as the presence of <5% of blast cells in a standardized bone marrow puncture after the second course of induction therapy. Only patients with fully regenerated blood counts were considered in CR. The study was approved by the ethics committee of the University of Dresden (review no. EK210396).

Precipitation of activated Ras (glutathione S-transferase pull-down assay). Ras precipitation probes were analyzed in two different cohorts from two independent investigators (S.S. and A.F.). The training set included 126 patient samples; the test set included 65 samples (n = 191). For the first cohort of patients (n = 126), genomic DNA was available for PCR-based techniques. Therefore, all analyses regarding Ras mutations refer to 126 patients.

Samples to be analyzed were chosen by the percentage of blasts determined at the time point of diagnosis. Thus, we decided to analyze exclusively blast samples with a blast percentage >50% at the time point of diagnosis (either pB or bone marrow). After identification of these samples, vials were thawed. To exclude samples with low viability, samples were stained with trypan blue. Specimens with a viability <50% were excluded from the analysis. Both stimulated HeLa cells (2 × 10⁷) and blast samples (1–2 × 10⁷) with a sufficient blast count and viability were washed twice in ice-cold PBS. After centrifugation at 1,800 rpm at 4°C, cells were immediately referred to ice-cold MLB lysis buffer (600–800 μL; 25 mmol/L HEPES, 150 mmol/L NaCl, 10% glycerol, 1% Igepal, 0.25% SDS, 10 mmol/L MgCl₂, 1 mmol/L EDTA, 1 mmol/L sodiumorthovanadate, 25 mmol/L sodium fluoride, 10 μg/mL leupeptin, and 10 μg/mL aprotinin). The solution was vigorously shaken at 4°C for 30 minutes. Thereafter, cellular debris was pelleted by centrifugation with 14,000 rpm at 4°C and 500 μL of the samples were immediately frozen at −80°C. Proteins were quantified using the bicinchoninic acid protein assay (Pierce, Rockford, IL). Protein (30 μg) was resolved by 12% SDS-PAGE, transferred to Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham Pharmacia Biotech, Freiburg, Germany) and immunoblotted with the primary antibody detecting human actin (Sigma-Aldrich, München, Germany; 1:1,000 at 4°C, overnight). The antigen was detected with a horseradish peroxidase–coated secondary mouse antibody (1:2,500 at room temperature, 1 hour) and enhanced chemiluminescence reagent (Amersham Pharmacia Biotech). Blots were controlled by immunostaining of actin (see Fig. 1). Only probes revealing non-disintegrated actin immunosignals were chosen for further evaluation by the RAF-based precipitation method.

Thereafter, protein samples were thawed and sample volumes were adjusted to 1 mL containing 500 μg of protein. Next, samples were precleared with 20 μL glutathionagarose (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 minutes at 4°C. After centrifugation at 14,000 rpm at 4°C supernatants were used for glutathione S-transferase (GST) pull-down with 10 μL of a GST-tagged fusion protein corresponding to residues 1-116 of the human Ras binding domain of Raf-1 (Upstate Biotechnology, Waltham, MA). Samples were incubated at 4°C for 30 minutes. After centrifugation and three times washing in MLB buffer,
the pellets were dissolved in Western probe buffer and heated at 95°C for 5 minutes. Supernatants were used for Western blotting using primary antibodies detecting precipitated RAS (Upstate Biotechnology; 1:1,000, at 4°C, overnight). The antigen was detected with a horseradish peroxidase–coated secondary mouse antibody (1:2,500, room temperature, 1 hour) and enhanced chemiluminescence reagent (Amersham Pharmacia Biotech). Moreover, the same blots were immunostained with primary antibodies detecting GST (Santa Cruz Biotechnology) to assure equal loading of the precipitate. Again, the antigen was detected with a horseradish peroxidase–coated secondary mouse antibody (1:2,500, room temperature, 1 hour) and enhanced chemiluminescence reagent (Amersham Pharmacia Biotech).

Immunoblotted signals were scanned with a Biometra device and analyzed with ScanPack3 software (Biometra, Göttingen, Germany). Relative amounts of activated Ras were calculated using a ratio of the obtained absorbance values for Ras*/Ras antigen (Amersham Pharmacia Biotech). Furthermore, the same blots were used for Western blotting (see also Materials and Methods); underlined cases, Ras activity positive samples. %Blasts as the percentage of blast cells (in bone marrow) at the time point of diagnosis.

**Detection of N-RAS/K-RAS point mutations.** Genomic DNA was extracted from 5 × 10⁶ cells using either phenol/chloroform extraction after proteinase K digestion or a silica based procedure (Qiagen DNA Blood Kit, Qiagen, Hilden, Germany) according to the manufacturer’s protocols.

N-RAS mutations were determined as previously described using a denaturing heteroduplex-high-pressure liquid chromatography–based method (20). In brief, primer pairs were as follows: N12/13 forward, 5'-GACTCGTGTTGCTTGGAACCT-3' and reverse, 5'-TGCGATGGTGGTGAACCT-3'; N61F forward, 5'-CAATGGTGATTCACTTGACAC-3' and reverse, 5'-AAAGATCGTCCCTTTAGAGAATAAT-3'. PCR conditions were for exon 1, Hot Star Taq (Qiagen, Valencia, CA), 0.625 unit primers (12.5 pmol), N12/13 forward, N12/13 reverse. Denaturing, 95°C for 15 minutes and 94°C for 30 seconds; annealing, 55.5°C for 1 minute; extension, 72°C for 1 minute for 35 cycles; final cycle at 72°C for 10 minutes and for exon 2, Hot Star Taq (Qiagen, Valencia, CA), 0.625 unit primers (12.5 pmol), N61F forward, N61R reverse. Denaturing, 95°C for 15 minutes; 94°C for 30 seconds; annealing, 55.5°C for 1 minute; extension, 72°C for 1 minute for 35 cycles; final cycle at 72°C for 10 minutes. Heteroduplexes were generated by means of a thermal cycler as follows: 95°C for 5 minutes; 95°C, reducing at 1°C per 22 seconds, for 70 cycles. Then, 10 L heteroduplexed PCR product per well were loaded from 96-well plates and analyzed by heteroduplex high-pressure liquid chromatography under the following conditions: flow, 0.9 mL/min, 47% to 52% buffer (B) in 0.1 minute, to 60% B in 4 minutes at 61°C.

K-RAS (codon 12) mutations were detected with a RFLP PCR method essentially as it was described previously (21). The sequences of primers were P1 5'-CTGTGGTAGTGCGCCT-3', P2 5'-GTCAGAGAAGACCTTTATCGTT-3', and P3 5'-TGACCTAAGTATGTCATAT-3'. The primers for the first PCR were P1 (sense) and P2 (antisense) that flanked codon 12 of the K-RAS gene. After the first PCR amplification, the PCR product was digested with 1 μL (10 units/mL) of MstI at 37°C for 1 hour. Subsequently, 5 μL of the reaction mixture were subjected to a second round of PCR and amplified for 30 cycles under the same conditions using primers, sense P1 and antisense P3. The second PCR products were also digested with 1 μL (10 units/mL) of MstI for 1 hour. Mutational screening was done by agarose gel electrophoresis.

All aberrant PCR fragments corresponding to a potential mutation in N-RAS as detected by heteroduplex high-pressure liquid chromatography or K-RAS as detected by RFLP-PCR were subjected to direct sequencing.

Detection of FLT-3 ITD mutations was exclusively done as previously reported (22). **Statistical analysis.** Differences in age, leukocyte, and bone marrow blast distributions were calculated using the Mann-Whitney test. Pearson’s χ² test was used to identify differences in the distribution of cytogenetic risk groups and RAS activity and mutation frequencies in
the respective patient category. Distribution inhomogeneities in FAB groups were tested with a logistic regression analysis. Furthermore, logistic regression analysis using multiple variables was done to identify the effect of single variables on CR. Cox regression analysis was used to identify independent variables associated with overall survival and disease-free survival. Estimates of overall survival and disease-free survival were calculated by the method of Kaplan and Meyer. Rates of overall survival were compared using the log rank test. All Ps are two sided and a significance level of 0.05 was used. All analyses were done using SPSS software.

**Results**

**RAS mutations in AML.** Previous studies in AML patients reported that RAS mutations are mainly found in N-RAS 12, N-RAS 13, N-RAS 61, and K-RAS 12 with only incidental cases with K-RAS 13 and K-RAS 61 mutations. Therefore, we focused on the investigation of N-RAS 12, N-RAS 13, N-RAS 61, and K-RAS 12. In total, 27 of 126 (21.4%) patients studied had mutations in N-RAS and/or K-RAS genes. In detail, we detected 12 mutations at the N-RAS 12 locus, two mutations at N-RAS 13, four mutations at N-RAS 61, and 12 mutations at K-RAS 12. Among these mutations, three patients were affected by a double mutation at N-RAS 12 + N-RAS 13 (one patient), N-RAS 12 + K-RAS 12 (one patient), and one mutation at N-RAS 61 + K-RAS 12 (one patient). All identified mutations led to an amino acid change in the corresponding Ras protein.

Clinical characteristics in RAS mutated versus nonmutated AML patients are shown in Table 1. There were no significant differences in age, FAB, or cytogenetic risk groups regarding the investigated RAS mutation distribution. In contrast, we observed significantly more patients with prior MDS and secondary AML that were shown to have mutations in N-RAS/ K-RAS, respectively (Table 1). As it was previously published, there is a negative correlation between the occurrence of RAS mutations and the presence of FLT-3 ITD mutations in the investigated patient samples.

**Ras activation and clinical characteristics in acute myeloid leukemia patients.** In the first step, Ras activity of nine healthy bone marrow donors was determined by the RAF-GST pull-down assay. As it is shown in Fig. 1A, Ras activity of healthy bone marrow donor samples was very low or undetectable (Fig. 1A). The ratio Ras*/Ras varied substantially between AML samples indicating that Ras activity is not simply affected by the amount of leukemic cells in the investigated patients.

Next, we investigated the relationship between RAS mutational status of the AML samples and Ras activity status (Table 3). Using the described threshold for Ras activity definition, most patients were defined as RAS mutation and Ras activity negative. Surprisingly, 77.7% of patients with a RAS mutation were classified as Ras activity negative cases. In contrast, 25% of patients without RAS mutations were shown to have strong Ras activity.

**Table 1. RAS mutations and clinical characteristics in AML**

<table>
<thead>
<tr>
<th></th>
<th>No RAS mutation (n = 99)</th>
<th>RAS mutation (n = 27)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age ≤ 60, n (%)</td>
<td>54 (73.9)</td>
<td>19 (26.0)</td>
</tr>
<tr>
<td>Age &gt; 60, n (%)</td>
<td>45 (84.9)</td>
<td>8 (15.1)</td>
</tr>
<tr>
<td>Leukocyte, median (range)</td>
<td>39.0 (0.7-334)</td>
<td>35.6 (8.6-292)</td>
</tr>
<tr>
<td>Bone marrow blasts, median (range)</td>
<td>68.0 (8.5-94.9)</td>
<td>55.5 (6.5-94.5)</td>
</tr>
<tr>
<td>Status at diagnosis, n (%)</td>
<td>De novo AML</td>
<td>90 (90.9)</td>
</tr>
<tr>
<td>Prior MDS</td>
<td>8 (8.1)</td>
<td>6 (22.2)*</td>
</tr>
<tr>
<td>t-AML</td>
<td>1 (1.0)</td>
<td>3 (11.1)</td>
</tr>
<tr>
<td>French-American-British classification, n (%)</td>
<td>M0</td>
<td>4 (4)</td>
</tr>
<tr>
<td>M1</td>
<td>23 (23.2)</td>
<td>3 (11.1)</td>
</tr>
<tr>
<td>M2</td>
<td>27 (27.3)</td>
<td>8 (29.6)</td>
</tr>
<tr>
<td>M4</td>
<td>16 (16.2)</td>
<td>4 (14.8)</td>
</tr>
<tr>
<td>M4eo</td>
<td>4 (4.0)</td>
<td>2 (7.4)</td>
</tr>
<tr>
<td>M5a</td>
<td>15 (15.2)</td>
<td>5 (18.5)</td>
</tr>
<tr>
<td>M5b</td>
<td>7 (7.1)</td>
<td>3 (11.1)</td>
</tr>
<tr>
<td>M6</td>
<td>2 (2.0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>M7</td>
<td>1 (1.0)</td>
<td>1 (3.7)</td>
</tr>
</tbody>
</table>

| Cytogenetic risk, n (%) | Good risk | 2 (2.0) | 0 (0) |
| Standard risk | 41 (41.1) | 12 (44.4) |
| High risk | 11 (11.1) | 7 (25.9) |
| Unknown | 45 (45.4) | 8 (29.6) |
| FLT-3 ITD positive, n (%) | 25 (25.3) | 2 (7.4)* |

**Table 1.** RAS mutations and clinical characteristics in AML

Note: t-AML is secondary AML after previous cytotostatic treatment. Cytogenetic stratification was done only in patients ages ≤ 60 years (patients ages 60 years, unknown).

*P < 0.05 (logistic regression analysis).

P < 0.05 (logistic regression analysis).

P = 0.06 (χ², two sided).

**T**reatment characteristics of RAS-mutated and Ras-activated acute myeloid leukemia patients. RAS mutations were not associated with treatment response criteria (see Table 4). In contrast, univariate analysis showed that patients with strong Ras activity were more likely to achieve CR compared with their Ras activity–negative counterparts (CR in Ras activity–positive versus Ras activity–negative patients, 64.5% versus 46.3%). This association was strongest in younger patients receiving high-dose ara-C during the induction therapy (89.5% versus 55.6%, P < 0.05), whereas older patients with strong Ras activity and low-dose ara-C treatment were not affected by the Ras activity score (data not shown).

Moreover, strong Ras activity accounted for an increased overall survival rate again mainly in patients under the age of 61. Disease-free survival and probability of relapse were not affected by the Ras activity score in the initially investigated 126 patients (data not shown).

To further substantiate data on clinical outcome according to the Ras activity score, another 65 patients were investigated as
Discussion

Activating point mutations of the RAS genes are among the most common observed genetic alterations in AML patients. It could be shown that these mutations account for phenotypic changes in transformed hematopoietic cells like hyperproliferation, hypersensitivity to growth factor stimulation, and IL-3–independent colony-forming unit activity (10, 11). Recently, it was found that other genetic or epigenetic alterations may regulate Ras activity in AML blasts (17, 18). The current study therefore investigated whether Ras activity is restricted to patients carrying RAS mutations and determined the clinical effect of Ras activity in a large AML study population.

Table 4. Ras mutations and clinical outcome

<table>
<thead>
<tr>
<th>Patients</th>
<th>No RAS mutation</th>
<th>RAS mutation</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>All (n = 126)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CR (%)</td>
<td>51.5</td>
<td>48.1</td>
<td>NS</td>
</tr>
<tr>
<td>Median OS (mo)</td>
<td>10.4 (7.7-13.1)</td>
<td>9.7 (4.6-14.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Median DFS (mo)</td>
<td>7.6 (4.3-10.9)</td>
<td>15.7 (3.6-27.9)</td>
<td>NS</td>
</tr>
<tr>
<td>≤60 y (n = 73)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CR</td>
<td>66.7</td>
<td>57.9</td>
<td>NS</td>
</tr>
<tr>
<td>Median OS (mo)</td>
<td>15.8 (8.7-22.9)</td>
<td>10.4 (3.0-17.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Median DFS (mo)</td>
<td>13.3 (0-33.9)</td>
<td>10.4 (0-28.4)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Abbreviation: NS, not significant.
RAS mutations were not associated with strong Ras activity because only 22.2% of RAS samples with mutation showed at the same time activation. Not surprisingly, we found that strong Ras activity may arise even in patients without activating point mutations. As there are multiple pathways that positively interact with Ras activity, it is very difficult to ascertain which pathway is activated in the individual patient. However, the clinically most important interaction could be Ras activation by ITD of FLT-3 which has been shown by Mizuki et al. (19). Our data also show inverse relation between the occurrence of FLT3-ITD and RAS mutations. However, the data presented here do not support the idea that an isolated FLT-3 ITD may lead to a strong Ras activity in AML because only a minority of FLT-3–positive samples was shown with a strong Ras activity. These data are in accordance with Mizuki et al. who showed only moderate Ras activation after transfection of a constitutively active FLT-3. Moreover, the finding that Ras activity may overcome worse prognosis in younger patients even in the presence of FLT-3 mutations is arguing for an FLT-3–independent cellular mechanism affecting the sensitivity of cells towards high-dose ara-C.

Birkenkamp et al. investigated 22 AML samples for the presence of Ras activation and mutation and found four RAS-mutated patient samples with a varying degree of Ras activation (23). The small number of patient samples investigated did not allow for further conclusions. However, considering our results, it must be asked whether the AML blast has the opportunity to counteract Ras activity that is associated with RAS mutation. Molecules that potentially interfere with the activation of the Ras pathway include members of the Sprouty gene family and the Raf kinase inhibitor RKIP which have been shown to effectively abrogate Ras/Raf/MAPK kinase signaling (24, 25). However, clinical data in AML patients are missing and further studies have to show an effect of those molecules.

Our results indicate that AML patients with high Ras activity show high sensitivity against cytotoxic treatment compared with the Ras activity–negative counterparts. However, this is true only for AML patients ages <61 years. The occurrence of AML in older patients is associated with the presence of bad risk cytogenetic alterations, p53 mutations, and epigenetic alterations like P-gp/MDR1 expression that may interfere with a successful treatment approach (7) in the presence of Ras.

### Table 5.
Multivariate analysis of the effect of Ras activity score on CR rate and OS in AML patients ages ≤60 years (data from 111 patients were compared)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard ratio for CR (95% confidence ratio)</th>
<th>P</th>
<th>Hazard ratio for death (95% confidence interval)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLT-3 ITD</td>
<td>—</td>
<td>NS</td>
<td>2.2 (1.3-3.7)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Ras activity score (high)</td>
<td>4.1 (1.3-12.9)</td>
<td>&lt;0.05</td>
<td>0.45 (0.25-0.84)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Cytogen risk</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Good risk</td>
<td>—</td>
<td>NS</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>High risk</td>
<td>0.3 (0.1-0.8)</td>
<td>&lt;0.05</td>
<td>2.3 (1.3-3.7)</td>
<td>&lt;0.01</td>
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<tr>
<td>Disease status</td>
<td>—</td>
<td>NS</td>
<td></td>
<td>NS</td>
</tr>
</tbody>
</table>

NOTE: One patient was lost for the analysis due to missing FLT-3 ITD values. Hazard ratio rates are not given if no statistical significance was determined. Cytogenetic risk model was tested after grouping for the SHG96 study low-risk and high-risk classification and comparison against standard-risk group patients.

Abbreviation: NS, not significant.
activity in AML blasts. Because we observed even younger patients with a bad cytogenetic risk (e.g., monosomy; ref. 7) and Ras activity that readily achieved remission in the current treatment protocol (data not shown), another explanation for the observed differences in treatment outcome seems likely. In the AML-SHG-96 study, younger AML patients received far higher doses of the cytotoxic compound ara-C compared with older patients for induction treatment. Efficacy of ara-C treatment in AML may depend on properties of ara-C like the expression of ara-C transporters and the activity and expression of important enzymes for ara-C phosphorylation/depolyphorylation (26). Another critical property of ara-C efficacy is the proliferative activity in AML blasts. Thus, younger AML patients with high Ras activity may show increased susceptibility towards the application of high-dose ara-C treatment. Given the potential dependence of ara-C efficacy on an activated Ras pathway, even a subgroup of older patients may benefit from a strategy using high-dose ara-C (27). In contrast, low-dose ara-C as applied in our treatment protocol may not be sufficient to exert its positive effects on cell cycle inhibition and apoptosis in older patients. These notions are supported by previous reports from Koo et al. (13). The authors found increased sensitivity of ara-C in RAS-transformed human kidney epithelial cells. RAS-transformed cells failed to arrest the cell cycle in response to ara-C thereby giving rise to an increased rate of ‘‘intra S-phase apoptosis.’’ In this light, the data presented here raise the question whether modulation of growth promoting pathways will be of clinical importance in AML. Results from clinical trials investigating the role of farnesyltransferase inhibitors in AML indicate a potential role for Ras activity in AML blasts. Given the potential dependence of ara-C efficacy on an activated Ras pathway, even a subgroup of older patients may benefit from a strategy using high-dose ara-C (27). In contrast, low-dose ara-C as applied in our treatment protocol may not be sufficient to exert its positive effects on cell cycle inhibition and apoptosis in older patients. These notions are supported by previous reports from Koo et al. (13). The authors found increased sensitivity of ara-C in RAS-transformed human kidney epithelial cells. RAS-transformed cells failed to arrest the cell cycle in response to ara-C thereby giving rise to an increased rate of ‘‘intra S-phase apoptosis.’’ In this light, the data presented here raise the question whether modulation of growth promoting pathways will be of clinical importance in AML. Results from clinical trials investigating the role of farnesyltransferase inhibitors in AML indicate a potential role for Ras activity in drug sensitivity towards farnesyltransferase inhibitors. In contrast, silencing of the Ras pathway does not seem beneficial for a subsequent chemotherapy using cell cycle active compounds like ara-C. There are now preclinical data showing that inhibition of growth promoting pathways like FLT-3 ITD or MAPK kinase activity may reduce sensitivity of a subsequent treatment with ara-C (29, 30). Alternatively, only recently, it was published that strong Ras activation may be associated with reduced proliferation and enhanced matura-

differentiation and differentiation of leukemic cells (31). Thus, Ras activity in the here-investigated population may also account for a commitment to granulocytic differentiation that is more sensitive to ara-C therapy as highly resistant myeloid precursors.

The data presented here indicate that Ras activity resembles a sensitizing mechanism of blasts to the application of ara-C. Whether these properties are restricted only to patients presenting with an activated Ras pathway at diagnosis or whether these properties can be induced by exogenous stimulation of AML blast and consequently lead to an increased response rate awaits further clinical investigation.

Acknowledgments

We thank Silke Soucek for help with all statistical analyses and the following physicians of the German SHG AML96 study group who entered their patients into the trial: D. Huhn and O. Kniege (Universitätsklinikum Charité, Berlin); E. Späth-Schwabke and S. Hesse-Amojo (Krankenhaus Spandau, Berlin); O. Rick and W. Siegert (Charité Campus Mitte, Berlin); R. Kolloch and U. Krümpelmann (Krankenanstalten Gleed, Bielefeld); K-H. Pflüger and T. Wolff (Evangel. Diakonissenanstalt, Bremen); H.H. Heidtmann (St. Joseph-Hospital, Bremen); F. Marquard (Allgemeines Krankenhaus, Celle); F. Fiedler and R. Herbst (Krankenhaus Küchwald, Chemnitz); M. Gramatzki and G. Helm (Universitätsklinikum, Erlangen); J.-G. Saal (Malteser Krankenhaus, Flensburg); H.-G. Hoffkes and M. Arland (Städtisches Klinikum, Fulda); E. Fajihauer (St. Elisabeth-Krankenhaus, Halle); N. Schmitz (Allgemeines Krankenhaus, St. Georg, Hamburg); H. Schmidt and K. Buhrmann (Kreiskrankenhaus, Hameln); H. Dürk (St. Marien-Hospital, Hamm); M. Burk (Klinikum Stadt, Hanau); A-D. Ho (Universitätsklinikum, Heidelberg); A. Bartholomäus (St. Bernward Krankenhaus, Hildesheim); A.A. Fauser (Klinik f. Hämatologie/Onkologie und KMT, Idrar-Oberrhein); H. Link and F-G. Hagmann (Westfälische Klinikum, Kaiserslautern); G. Kochling (Kreiskrankenhaus, Leer); K-P. Schalk (St. Vincent-Krankenhaus, Limburg/Lahn); S. Fetscher (Städtisches Krankenhaus Süd, Lübeck); T. Wagner (Universitätsklinikum, Lübeck); A. Neubauer (Universitätsklinikum, Marburg); H. Bodenstein, J. Tischler (Klinikum Minden, Minden); H. Pohlmann and N. Brack (Städtisches Krankenhaus München-Harlaching, München); H. Wandt, K. Schäfer-Eckart, and T. Denzel (Städtisches Klinikum, Nürnberg); B. Seebinger (Klinikum Offenburg, Offenburg); F. Hirsch (Kreiskrankenhaus, Offenburg); T. Geer and H. Heijmeyer (Diakonie-Krankenhaus, Schwabach-Hall); J. Labenz (Ev. Jung-Stilling-Krankenhaus, Siegen); J. Keesberger (Diakonissen-Krankenhaus, Stuttgart); W. E. Aulitzky and L. Leimer (Robert-Bosch-Krankenhaus, Stuttgart); M.R. Clemens and R. Mahlberg (Mutter-Kind-Klinik, Trier); R. Schwedtfeiger (Deutsche Fachklinik für Diagnostik, Wiesbaden); R. Engberding and R. Winter (Stadtkrankenhaus, Wolfsburg); M. Sandmann (Klinikum St. Antonius, Wuppertal); M. Wilhelm, F. Weissinger, and H. Rucke-Lanz (Universitätsklinikum, Würzburg).

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