Mutational analysis of K-ras codon 12 in blood samples of patients with acute myeloid leukemia

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\textbf{A B S T R A C T}

Mutations in K-ras are frequent in acute myeloid leukemia (AML). The association of these mutations to clinical features and their prognostic value are unclear. We used quantitative PCR with peptide nucleic acid mediated PCR clamping to specifically analyze 257 blood samples of 31 AML patients for K-ras codon 12 alterations. A total of 20 samples of nine patients harbored a K-ras mutation. The most frequent mutation was the GTT variant which causes an amino acid exchange from glycine to valine. Correlation with clinical data suggests K-ras mutations to be associated with higher age and a better response to anti-leukemic chemotherapy.

\section{1. Introduction}

Acute myeloid leukemia (AML) is a neoplastic disease of the hematopoietic cells which leads to an accumulation of immature myeloid cells in bone marrow and blood. A number of activating and loss-of-function mutations have been described in AML [1]. They include alterations in the three closely related Ras genes – N-ras, K-ras and, less frequently, H-ras – which are involved in the regulation of cell proliferation and differentiation [2]. Naturally occurring alterations in the Ras genes are predominantly found in codon 12 and with lower frequencies in codon 13 or 61 [2,3]. Mutations of these sites lead to a constant activity of the Ras protein which can cause uncontrolled cell proliferation and escape from apoptosis [2]. As a post-initiation event, a Ras mutation in an AML subclone can lead to higher malignancy and outgrowth of this clone. In some cases, different Ras mutations can be present in separate subclones [4,5]. The clinical relevance of Ras mutations in AML remains controversial. While some studies suppose Ras-mutations to be correlated with a better outcome [6,7], others found altered Ras genes to be associated with a lower remission rate or shorter survival [8,9] or could not verify Ras mutations to be of clinical significance [10–15]. Many publications do not differentiate between the different Ras genes; others are limited to N-ras mutations. These observations indicate the possibility of another Ras gene than N-ras to be of clinical importance. So far, especially the role of K-ras mutations in AML is unclear.

Alterations in the K-ras genes have been detected in clinical samples using a number of different methods including sequencing [16,17], single strand confirmation polymorphism (SSCP) [15], restriction fragment length polymorphism (RFLP) [11], mutant-allele-specific amplification [18], and allele-specific oligonucleotide hybridization [7]. Recently, a real-time PCR assay with mutant specific hybridization probes combined with peptide nucleic acid (PNA)-mediated PCR clamping of the wild type alleles was successfully used to identify K-ras codon 12 point mutations in plasma and tumor tissue samples of patients with pancreatic cancer [19,20]. This reliable and fast method allows quantitative detection of altered alleles with a very high sensitivity (1:100,000, mutated to wild type alleles) and genotyping of the mutation by post-PCR melting curve analysis.

The aim of our study was to establish a solid method for the detection of K-ras codon 12 mutations in blood samples of AML patients using this method. Correlation with clinical data was done to evaluate these K-ras alterations as a marker for patient or disease subgroups and prognostic indicator.
2. Material and methods

2.1. Samples

This study included 31 patients with AML that were treated at the Department of Medical Oncology and Hematology, Charité, Campus Virchow-Klinikum, Berlin, Germany. Our trial was exempt from approval by our ethics committee. All patients were included in other ethics committee-approved studies that even allowed additional research using blood samples. After informed consent was obtained, blood samples (9 ml) of 31 AML patients were collected in EDTA containing tubes. The first sample from each patient was taken before starting anti-leukemic chemotherapy. Follow-up samples were taken when available during the follow-up period with a minimum interval of 7 days between two samples from the same patient. Additionally, three blood samples from a control group of three healthy individuals were obtained. For white cell enrichment, the buffy coat of the blood samples was isolated and then washed with 9 ml erythrocyte lysis buffer. For white cell enrichment, the buffy coat of the blood samples from a control group of three healthy individuals was obtained. For white cell enrichment, the buffy coat of the blood samples was isolated and then washed with 9 ml erythrocyte lysis buffer (NH₄Cl 8.29 g/l in H₂O) and centrifuged (1500 rpm, 10 min) three times. Genomic DNA was isolated using the QiAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instruction. Extracted DNA was resuspended in a final volume of 20 μl.

2.2. Detection of K-ras codon 12 mutations

K-ras mutation analysis was done using real-time PCR with PNA-mediated PCR clamping and subsequent melting curve analysis as described previously [19]. In this assay, a pair of mutant-specific hybridization probes is used to measure the amount of the target sequence during amplification. These are fluorescent-labelled DNA oligonucleotides which are designed to bind to adjacent target sequences within the amplified target sequence, which brings them in close proximity. Only then can a fluorescence resonance energy transfer (FRET) between the two fluorophores take place [21]. The emitted fluorescence intensity is monitored during the PCR.

Because of the higher thermal stability of complementary PNA-DNA hybrids compared to DNA dimers [22,23], binding of these hybridization probes to K-ras wild type alleles is suppressed in the presence of specific PNA. As a consequence, the detected fluorescence signal specifically represents the amplified mutant DNA. With this method, point mutated alleles can be detected in the presence of an excess of wild type alleles with a sensitivity of up to 1 × 10⁻⁵ [19]. Hybridization probes were designed for the frequent K-ras codon 12 (wild type sequence GGT, coding for glycine) mutations GTT (valine), and were successfully tested to also detect other gene alterations at codon 12 [19]. For further analysis of samples with a non-GTT mutation, similar hybridization probes for K-ras codon 12 GAT (aspartate) and TGT (cysteine) mutations were created.

Rapid genotyping of the mutations is performed by melting curve analysis of the PCR products. At a steadily increasing temperature, fluorescence intensity is monitored, and the temperature with the most rapid decrease in fluorescence (melting temperature) is determined. Changes in the melting temperature result from even single base mismatches between the hybridization probes and the target sequence due to point mutations. DNA from the human colon carcinoma cell line SW480 with a homozygous K-ras codon 12 GTT mutation served as positive control for the valine mutation. Positive controls for the K-ras codon 12 GAT (aspartate) and TGT (cysteine) mutations were established using DNA from the human pancreatic carcinoma cell lines Panc-1 and MiaPaCa2.

For each sample, the PCR was first done without PNA to verify integrity of the template. Then, mutation analysis was performed in a PCR with PNA using the valine sensor hybridization probes.

The PCR reaction mixture contained 10 mM Tris–HCl pH 8.3, 50 mM KCl, 3.75 mM MgCl₂, 125 mM of each deoxynucleotide triphosphate (Invitrogen, Carlsbad, USA), 1 mM of primer K-ras F (5’-AGG GCC TGC TGA AAA TGA CTG-3’), 1 mM of primer K-ras R (5’-GTT CCT GCA CCA GTA ATA TGC A-3’), 0.3 mM of the anchor hybridization probe (donor) labelled with fluorescein on its 3’-end (5’-CGT CCA AAT GAT GAT TCT GAA TTA GCT GTA TCG TCA AGG CAC T-F-3’), 0.3 mM of the K-ras codon 12 GTT (valine) mutation sensor hybridization probe (acceptor) labelled with fluorescein dye LightCycler-Red on its 5’ end (5’-LC Red640-TTC CCT ACG CCA ACA GCT CCA A-P-3’), 2.5 mM PNA (5’-CCT AGG CCA GCT CC-3’) if applicable, applicable and 1.25 U of platinum Taq DNA polymerase (Invitrogen) in a final volume of 20 μl. After denaturation at 95 °C for 3 min, 45 cycles were performed, each consisting of denaturation at 95 °C for 10 s, PNA annealing at 76 °C for 7 s, annealing of primers and hybridization probes at 60 °C for 15 s and elongation at 72 °C for 20 s. Melting curve analysis was performed at steadily increasing temperature from 40 to 85 °C (transition rate 0.3 °C/s). For samples that were identified as K-ras codon 12 non-GTT mutants, the real-time PCR and melting curve analysis was repeated using PCR mixtures with the alternative hybridization probes for the K-ras codon 12 GAT (aspartate) or TGT (cysteine) mutations, respectively (sequences for K-ras codon 12 GAT sensor 5’-LC Red640-TTG CCT ACG CCA TCA GCT CCA A-P-3’, and for K-ras codon 12 TGT sensor 5’-LC-Red705-TTG CCT ACG CCA GCT CCA A-P-3’).

Fluorescence data were analyzed using the LightCycler software (version 3.5, Roche Diagnostics). All oligonucleotides were purchased from TIB Molbiol, Berlin, Germany. PCR was carried out on the LightCycler Instrument (Roche Diagnostics, Mannheim, Germany).

The relative positions of primers, hybridization probes and PNA are shown in Fig. 1 (Genebank accession no. K01519; nucleotide positions 1–164). Validation data of the method, including sensitivity and specificity testing, determination of interassay variability and direct comparison to a previously established method, were published elsewhere [19].

2.3. Statistical analysis

Statistical comparisons between groups (K-ras mutation vs. wild type) were done using Mann–Whitney U-test, t-test, Fisher’s exact test, Monte-Carlo’s test, and Logrank test. Differences were considered significant for p < 0.05. All calculations were performed with SPSS software package version 12.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Basic clinical data

Of the 31 patients in this study, 29 had a newly diagnosed AML while two patients had a relapse of the disease when included.
Median age of the patients with newly diagnosed AML was 50.8 years (range: 25.9–73.0 years). The male–female ratio was 1:1.4 (13 males, 18 females). In six cases, the AML was counted as secondary leukemia as the patients had a history of myelodysplastic syndrome, anti-cancer chemotherapy, exposure to radiation, or a combination of these risk factors prior to initial diagnosis of AML. The frequencies of French–American–British (FAB) subtypes and cytogenetic aberrations are shown in Table 1. After the first cycle of chemotherapy, patients were re-evaluated for clinical and cytological signs of leukemia or complete remission. Twenty-two (70.9%) of the patients were in complete remission after one cycle of chemotherapy. Patients were followed up for a median of 51 weeks (range: 3–118 weeks). During follow-up, nine (29.0%) patients had a relapse of AML after a median relapse free time of 28 weeks (3–62 weeks). Four patients (12.9%) died during follow-up after a median time of 41 weeks (range: 31–55 weeks) after first diagnosis of AML. All deaths occurred after an AML relapse. A synopsis of the patients is presented in Table 2.

A total of 257 blood samples were collected from the AML patients. Of the samples, 48 (18.7%) were collected in a phase of acute leukemia and 209 (81.3%) during a complete clinical remission of the disease.
3.2. K-ras mutation analysis

The DNA extracted from the buffy coats of all blood samples was amplifiable in real-time PCR. Mutation analysis was done in a second real-time PCR containing mutant specific hybridization probes for the K-ras codon 12 GTT (valine) mutation and wild type specific PNA with subsequent melting curve analysis (Fig. 2). Experiments were performed at least twice on each sample. A K-ras mutation

![Fig. 2. K-ras genotyping by melting point analysis of the PCR products. The characteristic melting points of K-ras wild type and K-ras codon 12 GTT mutation (SW480) are indicated by vertical lines, respectively. For the SW480 mutation standard and clinical samples, PNA was used to suppress binding of the fluorescence probes to alleles bearing the K-ras wild type. The clinical sample (5) in the upper picture shows the same melting point as the SW480 mutation standard and can therefore be identified as K-ras codon 12 GTT mutant. The patient sample (6) in the lower diagram shows a lower melting temperature (indicated by a vertical line) than the SW480 mutation standard due to a non-GTT codon 12 mutation of K-ras. The melting curve of the clinical sample (7) in the lower diagram runs along the curve of the no template control; the sample is therefore identified as K-ras wild type. For all clinical samples, K-ras was shown to be amplifiable in a PCR using no PNA (data not shown).]
Fig. 3. Further genotyping of clinical samples with a K-ras codon 12 non-GTT mutation. Clinical samples that showed to bear a non-GTT mutation in K-ras codon 12 in the real-time PCR assay with the valine sensor hybridization probe were further analyzed by repeating the PCR and melting curve analysis using PCR mixtures in which the valine sensor hybridization probe had been replaced by specific hybridization probes for the K-ras codon 12 GAT (aspartate) or TGT (cysteine), respectively. Positive controls for the K-ras codon 12 GAT and TGT mutations were established using DNA from the human pancreatic carcinoma cell lines Panc1 and MiaPaCa2. For the Panc1 and MiaPaCa2 mutation standards and clinical samples, PNA was used to suppress binding of the fluorescence probes to alleles bearing the K-ras wild type. However, no K-ras codon 12 GAT or TGT mutation was found in the patient samples. In the upper picture, the patient sample (4) shows a melting point different from the Panc1 mutation standard and can therefore be identified as K-ras codon 12 non-GAT mutant. In the lower diagram, this patient sample (4) can be identified as K-ras codon 12 non-TGT mutant by a melting temperature different from the MiaPaCa2 standard. Melting points of K-ras wild type, Panc1 and MiaPaCa2 mutation standards and clinical samples are indicated by vertical lines.
was found in 20 of the samples, while 237 samples had the K-ras wild type. K-ras mutations were found both in samples with high white blood (WBC) counts at times of acute leukemia and in samples with very low WBC at times of therapy-induced bone marrow aplasia. The most frequent mutation (14 samples, 70.0%) detected in K-ras codon 12 was a point mutation from the wild type base sequence GGT (encoding glycine) to GTT (valine). The remaining six samples revealing a K-ras mutation showed a different alteration of codon 12. Further testing using the hybridization probes for the K-ras codon 12 mutations GAT and TGT was done. However, none of the samples could be identified as codon 12 GAT or TGT mutant (Fig. 3).

The minimum ratio of alleles with a K-ras codon 12 mutation to wild type alleles was 1:186,250 (eight copies with the K-ras codon 12 GTT variant, \(1.49 \times 10^6\) K-ras wild type copies). In the samples of the control group, only the K-ras wild type was detected.

Nine of the 31 patients (29.0%) had at least one sample with a K-ras mutation. The codon 12 GTT (valine) variant was found in

![Time course bar chart of the patients' K-ras genotypes.](image)

**Fig. 4.** Time course bar chart of the patients’ K-ras genotypes. Each sample is represented by a square element of a bar (light grey, K-ras wild type; dark grey, K-ras mutation; dashed, no sample available). Numbers in elements specify time in weeks after taking the first sample. For samples with K-ras mutation, the base sequence in codon 12 is specified (v, GTT [valine]; x, mutation with a sequence different from GTT). The left bar depicts the initial AML, the right bar depicts an AML relapse (if applicable). Elements left of the respective bold vertical line represent samples before complete remission, elements right of the line represent samples taken in complete clinical remission.
Table 3

<table>
<thead>
<tr>
<th></th>
<th>K-ras wild type (22 patients)</th>
<th>K-ras mutation (9 patients)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (median ± SEMa)</td>
<td>46.4 ± 2.7 years</td>
<td>58.3 ± 3.5 years</td>
<td>0.086</td>
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<tr>
<td>Sex</td>
<td>14 female/8 male</td>
<td>4 female/5 male</td>
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<td>Secondary AMLb</td>
<td>5 (22.7%)</td>
<td>1 (11.1%)</td>
<td>0.642**</td>
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<tr>
<td>FAB subtype</td>
<td></td>
<td></td>
<td>0.516</td>
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<tr>
<td>M0</td>
<td>1 (4.5%)</td>
<td>1 (11.1%)</td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td>3 (13.6%)</td>
<td>2 (22.2%)</td>
<td></td>
</tr>
<tr>
<td>M2</td>
<td>8 (36.4%)</td>
<td>2 (22.2%)</td>
<td></td>
</tr>
<tr>
<td>M3</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>M4</td>
<td>2 (9.1%)</td>
<td>3 (33.3%)</td>
<td></td>
</tr>
<tr>
<td>M5</td>
<td>4 (18.2%)</td>
<td>1 (11.1%)</td>
<td></td>
</tr>
<tr>
<td>M6</td>
<td>2 (9.1%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>M7</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>2 (9.1%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Cytogenetics</td>
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<td></td>
<td>1.000</td>
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<tr>
<td>Normal</td>
<td>12 (54.5%)</td>
<td>4 (44.4%)</td>
<td></td>
</tr>
<tr>
<td>Aberrations</td>
<td>7 (31.8%)</td>
<td>3 (33.3%)</td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>3 (13.6%)</td>
<td>2 (22.2%)</td>
<td></td>
</tr>
<tr>
<td>White blood count (nl) (median ± SEMa)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before CRc</td>
<td>5.6 ± 6.0</td>
<td>6.4 ± 3.8</td>
<td>0.668</td>
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<tr>
<td>In CRc</td>
<td>0.5 ± 0.3</td>
<td>0.6 ± 0.5</td>
<td>0.124</td>
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<tr>
<td>Blasts (nl) in peripheral bloodd (median ± SEMa)</td>
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<td></td>
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<tr>
<td>Blast fraction in bone marrowd (median ± SEMa)</td>
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<tr>
<td>CRc after 1st cycle of induction chemotherapy</td>
<td></td>
<td></td>
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<tr>
<td>Yes</td>
<td>16 (72.7%)</td>
<td>6 (66.7%)</td>
<td>0.324</td>
</tr>
<tr>
<td>No</td>
<td>4 (18.2%)</td>
<td>0</td>
<td></td>
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<tr>
<td>NA</td>
<td>2 (9.1%)</td>
<td>3 (33.3%)</td>
<td></td>
</tr>
<tr>
<td>Relapsed</td>
<td>7 (31.8%)</td>
<td>2 (22.2%)</td>
<td>0.862***</td>
</tr>
<tr>
<td>Relapse free interval in weeks (median ± SEMa)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deathsd</td>
<td>30 ± 7</td>
<td>20 ± 9</td>
<td>0.551***</td>
</tr>
<tr>
<td></td>
<td>3 (13.6%)</td>
<td>1 (11.1%)</td>
<td>0.895***</td>
</tr>
</tbody>
</table>

NA = data not available.
a Standard error of the mean.
b Anamnestic evidence for secondary AML.
c Complete remission.
d At initial diagnosis.
e Within the observation period.
* U-test.
** Fisher’s exact test.
*** Monte-Carlo’s test.
**** Comparison of survival distributions using Logrank test.
***** t-test.

all mutated samples of six of those patients (66.7%). Two cases consistently showed a different K-ras mutation. A change of the mutation was observed in one patient. While a non-GTT mutation was found in K-ras codon 12 after initial AML diagnosis, the codon 12 GTT variant was observed repeatedly during complete remission. A time course synopsis of the K-ras genotype of all patients is shown in Fig. 4.

3.3. Statistical analysis

The median age of the patients with a K-ras mutation was more than ten years above the median age of the patients with wild type K-ras. While no mutation was detected in the youngest six patients of the study, the oldest patient carried the K-ras codon 12 GTT mutation. However, the age difference cannot be considered statistically significant on the basis of the study population (p = 0.086). Patients with a K-ras mutation seem to respond well to chemotherapy as a second therapy cycle was never needed for remission induction in those patients. Again, this observation could not be confirmed statistically. Relapse rates and relapse free survival intervals were comparable between the groups. Furthermore, no statistical correlation was found between the K-ras genotype and secondary development of AML, FAB subtype or cytogenetic findings. The white blood count and proportion of blasts in blood and bone marrow in patients with K-ras wild type and K-ras mutations were comparable. The results of the statistical comparisons are presented in Table 3.

4. Discussion

The aim of our study was to establish a real-time PCR assay with hybridization probes and PNA-mediated PCR clamping for the detection of K-ras codon 12 mutations in blood samples of patients with acute myeloid leukemia. With this method, K-ras mutations have previously been detected in plasma and tissue samples of patients with pancreatic cancer with a sensitivity of 1:100,000 [19]. In the present study, the assay was successfully used to analyze blood samples of AML patients for K-ras mutations. The very high sensitivity of the assay could be confirmed with a detection limit of 8 K-ras copies bearing a mutation in a clinical sample with a $1.86 \times 10^5$-fold excess of the wild type allele. The assay produced reproducible results even for samples with very low white blood counts.

Chen et al. [24] used a comparable assay to screen for K-ras mutations in bile. A similar method was described by Sotlar et al. [25] for the detection of c-kit mutations in children with urticaria pigmentosa. These assays only reached sensitivities of 1:3000 and 1:1000 (mutation to wild type), respectively. The highly differing sensitivities of these closely related methods could partly have been caused by differences in the design of PNAs and hybridization
patients. In studies with low-sensitivity assays, mutations are present only in subclones of the leucemic cells in some instances. We modified the K-ras wild type, suggesting that a K-ras mutation might be an indicator of complete remission during complete remission in some patients in the present study. Flushed out preleukemic bone marrow cells bearing the mutation might have been detected in peripheral blood due to the high sensitivity of the assay.

Interestingly, we found different K-ras mutations before and during complete remission in patient 29. As described by other investigators [5], the two mutations could have co-existed in two different subclones before chemotherapy. In this case, one mutation may have been under the detection limit of the assay or it existed initially only in bone marrow. Another possibility would be a de novo mutation during complete remission.

None of these patients that were positive for K-ras mutation in complete remission had a relapse during follow-up, supporting the evidence that multiple factors are required for the pathogenesis of malignant diseases [33]. Despite this fact, it seems reasonable to screen those patients regularly for a relapse of the leukemia. For a more exact risk evaluation and development of evidence-based after-treatment strategies for those patients, studies with more patients and longer follow-up times are needed.

In this study, we could not find a significant association of the K-ras mutation with age, sex, secondary AML, FAB subtype, cytological aberrations, or blasts in blood or bone marrow. However, the data suggest that K-ras mutations may be more frequent in older patients, but this question must be re-addressed in a larger study population.

The prognostic relevance of mutations in the Ras genes remains to be elucidated. Some authors found Ras mutations to be associated with increased survival [6,7], other studies suggest alterations in Ras genes to be unfavorable [8,34] or could not show an influence on prognosis [11,13–16,28]. Most of these publications, however, focus on N-ras or do not discriminate between the different Ras genes.

Some indication for K-ras to be a positive prognostic marker is given by the data of this study. All patients who were not in complete remission after one cycle of induction chemotherapy carried the K-ras wild type, suggesting that a K-ras mutation might be associated with a good response to anti-leukemic therapy. However, the better response rate compared to patients with the K-ras wild type is not significant in our study due to the low number of patients. The assumption of a better response to therapy is supported by similar observations by other authors, even though all of the studies fail to reach significance level [7,11,13]. Furthermore, the fact that some patients with initial Ras mutations had relapses that were negative for Ras mutations led to speculations that clones with Ras alterations were more sensitive to chemotherapy [7,35]. Larger studies will be needed to give statistical evidence to these observations.

The advantage of the assay we used in this work to detect K-ras mutations is that it can be adapted to detect mutations at other hot-spots of K-ras, e.g. codon 13 or 61, or even other oncogenes, including N-ras or H-ras, by designing specific hybridization probes and PNA. Future studies including more patients and a broader range of mutations will further elucidate the role of Ras mutations in AML.

Conflict of interest

All authors disclose any financial and personal relationships with other people or organizations that could inappropriately influence this work.

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References