Identification of a Set of Seven Genes for the Monitoring of Minimal Residual Disease in Pediatric Acute Myeloid Leukemia

Daniel Steinbach,1 Alexander Schramm,2 Angelika Eggert,2 Masanori Onda,4 Kristin Dawczynski,1 Andreas Rump,3 Ira Pastor,4 Susann Wittig,1 Nadine Pfaffendorf,1 Astrid Voigt,1 Felix Zintl,1 and Bernd Gruhn1

Abstract Background: Monitoring of minimal residual disease (MRD) has become a strong diagnostic tool in acute lymphoblastic leukemia. It is used for risk-adapted therapy and for the recognition of pending relapses. In acute myeloid leukemia (AML), there is still a need for more suitable MRD markers.

Experimental Design: A stepwise approach which combined genome-wide expression profiling, TaqMan low density arrays, and a TaqMan real-time PCR-based screening was used to identify new markers for the monitoring of MRD in AML. Leukemic cells from 52 children with AML and 145 follow-up samples from 25 patients were analyzed.

Results: Seven genes were identified which are vastly overexpressed in many patients with AML compared with healthy bone marrow: CCL23, GAGED2, MSLN, SPAG6, and ST18 as well as the previously described markers WT1 and PRAME. The expression of all genes decreased to normal levels in patients who achieved a continuous complete remission. Elevated levels of at least one gene were found prior to relapse in 7 out of 10 patients who relapsed.

Conclusions: This set of genes should allow a sensitive and specific monitoring of MRD in AML. Notably, some of these markers could also serve as therapeutic targets or might be involved in leukemogenesis. MSLN is already used as a target for immunotherapy in clinical trials in other malignancies.

Major improvements in the treatment of childhood acute myeloid leukemia (AML) have been achieved over the last 30 years. During this period, the survival rates changed from <20% to >50%. This improvement was mainly achieved by an enormous intensification of chemotherapy (1, 2). From early studies, we know that some patients can be cured with much less aggressive therapy. However, for many patients, the outcome is still fatal despite the most intense therapy. The design of risk-adapted therapies represents one of the greatest challenges in the treatment of pediatric AML.

In order to achieve this goal, the identification of residual leukemic cells that can cause a relapse and hence necessitate further treatment of the patient is essential. In acute lymphoblastic leukemia, the monitoring of minimal residual disease (MRD) has become one of the most important diagnostic tools for risk-adapted therapy (3–5). However, in AML, there is still a lack of sensitive and specific markers for the monitoring of MRD (6). In some patients, leukemic cells show specific chromosomal rearrangements such as t(8/21), t(15/17), inv(16), or t(9/11) which are detectable by PCR (7–10). Flow cytometry can be used to detect a combination of antigens present on leukemic cells (11–13). The limitations of this method are antigen switches and a lack of specificity of the antigen combinations for leukemic cells (14, 15).

Measuring the expression of genes, which are overexpressed in leukemic cells compared with healthy bone marrow cells might be more promising for monitoring MRD in AML. The Wilms tumor gene (WT1) has been studied as an MRD marker (16–19). We and others have also suggested the preferentially expressed antigen in melanoma (PRAME) for the detection of MRD (20, 21).

In the present study, we combined the power of microarray technology and real-time PCR to identify genes which are vastly overexpressed in AML compared with healthy blood and bone marrow. Measurement of the genes described here in combination with WT1 and PRAME should allow the monitoring of MRD in a larger proportion of patients and with a better reliability than the measurement of a single gene. If the genes were translated into proteins, they might also be useful markers for a flow cytometry–based monitoring of MRD.

Materials and Methods

Institutional review. This study was approved by the Institutional Review Board of the University of Jena (no. 1545-05/05).
Patients and healthy donors. Pretreatment samples from 52 children with primary AML were analyzed. The main patient characteristics are summarized in Table 1. The initial diagnosis of AML was determined by standard methods (1). We included all patients in whom a clear diagnosis of AML was established and a sufficient amount of cryopreserved leukemic cells was available. The follow-up samples were obtained from bone marrow or blood samples that were taken as part of the routine diagnostics. Written consent was given for the use of all patient samples for this study. The patients were treated according to four consecutive protocols with marked differences in outcome. We therefore did not analyze the prognostic effect of the newly discovered MRD markers.

Ten samples of healthy CD34+ stem cells and 21 samples of healthy bone marrow were obtained from adults who donated for stem cell or bone marrow transplantation. Written consent was given for the use of these samples for this study. Twenty samples of peripheral blood were donated by hospital staff.

Sample collection and processing. All patients were treated and diagnosed at the University Children’s Hospital Jena, Jena, Germany. Ficoll-Hypaque density gradient centrifugation was done in all peripheral blood and bone marrow samples from the patients and the healthy controls. After this procedure, the percentage of leukemic cells in the initial AML samples was >95% as determined by May-Gruenwald-Giemsa stained cytospins.

CD34+ stem cells of healthy donors were isolated using the CliniMACS system (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. The purity of the stem cells was >95% as determined by flow cytometry. All samples were cryopreserved in liquid nitrogen. After thawing the cells, total RNA was measured by photometry. Two micrograms of RNA were transcribed into cDNA using Omniscript (Qiagen). Pools of cDNA contained the same amount of RNA from cryopreserved leukemic cells. The amount of RNA was measured by photometry. Written consent was given for the use of all patient samples for this study. The patients were treated according to four consecutive protocols with marked differences in outcome. We therefore did not analyze the prognostic effect of the newly discovered MRD markers.

Table 1. Initial patient data in 52 children with primary AML in whom pretreatment samples were analyzed

<table>
<thead>
<tr>
<th>Number of patients</th>
<th>52</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age in years (range)</td>
<td>8 (0-19)</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>24/28</td>
</tr>
<tr>
<td>Down syndrome (yes/no)</td>
<td>1/51</td>
</tr>
<tr>
<td>Median WBC in 10^9/L (range)</td>
<td>52 (2-355)</td>
</tr>
<tr>
<td>Auer rods (positive/negative/n.a.)</td>
<td>6/40/6</td>
</tr>
<tr>
<td>Median percentage of leukemic cells</td>
<td></td>
</tr>
<tr>
<td>Bone marrow (range)</td>
<td>86 (44-99)</td>
</tr>
<tr>
<td>Peripheral blood (range)</td>
<td>68 (0-99)</td>
</tr>
<tr>
<td>Inversion inv(16) (positive/negative/n.a.)</td>
<td>6/42/4</td>
</tr>
<tr>
<td>Translocation t(8/21) (positive/negative/n.a.)</td>
<td>6/42/4</td>
</tr>
<tr>
<td>Translocation t(9/11) (positive/negative/n.a.)</td>
<td>5/43/4</td>
</tr>
<tr>
<td>Liver &gt;3 cm below costal margin (yes/no/n.a.)</td>
<td>19/31/2</td>
</tr>
<tr>
<td>Spleen &gt;3 cm below costal margin (yes/no/n.a.)</td>
<td>15/35/2</td>
</tr>
<tr>
<td>FAB type (no. of patients)</td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td>10</td>
</tr>
<tr>
<td>M2</td>
<td>9</td>
</tr>
<tr>
<td>M3</td>
<td>—</td>
</tr>
<tr>
<td>M4</td>
<td>16</td>
</tr>
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<td>15</td>
</tr>
<tr>
<td>M6</td>
<td>—</td>
</tr>
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<td>M7</td>
<td>2</td>
</tr>
</tbody>
</table>

Abbreviation: n.a., not available.

Results

Microarrays. Five microarray experiments were done using the Affymetrix U133A chip. Pooled RNA from 10 individuals was used for each array. We analyzed healthy bone marrow, healthy CD34+ hematopoietic stem cells, myelocytic AML (FAB-type M1 or M2), myelomonocytic AML (FAB-type M4), and monocytic AML (FAB-type M5). The other subtypes of AML are extremely rare in children.

The hypothesis was that for every useful MRD marker, there should be at least 1 in 10 patients expressing the gene at a high level. Therefore, this gene should be detectable on the array. In addition, every useful MRD marker must be expressed at very low levels, or not at all, in healthy bone marrow and therefore should be classified as absent on the respective array.

Hematopoietic stem cells are similar to leukemic cells with respect to maturation stage and proliferation rate. In order to distinguish between healthy stem cells and leukemic cells, the MRD markers must not be expressed in stem cells. Therefore, an MRD marker should also be absent on this array.
About 200 candidate genes were discovered in each AML pool. When comparing two of these lists, the overlap was in the range of 30% to 40%. Only 28 genes were detected in all three pools from AML patients, but were absent in healthy bone marrow and healthy stem cells. One hundred and twenty-four genes were detected in two AML pools, but were absent in both of the healthy controls. Taken together, a set of 152 MRD marker candidates were extracted from the microarrays.

**Validation of candidate genes by real-time PCR in independent patient pools.** The 65 MRD marker candidates with the strongest expression on the microarray were analyzed by TaqMan real-time PCR in two pools of healthy bone marrow and in two pools from AML patients. Again, each of these pools contained samples from 10 individuals. Both AML pools and one of the pools of healthy bone marrow were new and independent from those used for the microarrays. Using this highly sensitive method, none of the genes were found to be completely absent in healthy controls. For the majority of genes, the expression in the AML pools was between 2-fold and 10-fold higher than in healthy bone marrow.

However, we were able to identify 12 genes whose expression was >50 times higher in at least one of the AML pools compared with both pools of healthy bone marrow. The names, symbols, and geneID of the 12 genes are given in Table 2. Evaluation of the remaining 87 MRD marker candidates was achieved by TaqMan low density array analysis. Three more genes where identified, which were >50-fold overexpressed in the AML pools. The names, symbols, and geneID of these three genes are also given in Table 2.

**Analysis of potential MRD markers in individuals.** Expression levels of the 15 genes which are shown in Table 2 were analyzed by TaqMan real-time PCR in individual pretreatment samples from all 52 children with primary AML, 21 samples of healthy bone marrow, and 20 samples of healthy peripheral blood. For each gene, the 90th percentile of its expression in healthy bone marrows was defined as 1 unit. This normalization allowed a direct comparison of the various MRD markers with each other. For eight genes, the highest expression level in the AML samples was >100 times higher than the 90th percentile in healthy bone marrows, i.e., >100 units. The expression of these eight genes is shown in Fig. 1.

Some of these genes were associated with chromosomal rearrangements. Patients with t(8;21) showed a higher expression of PRAME (P = 0.021) and ST18 (P = 0.052). Patients with inv(16) showed a higher expression of MSLN (P = 0.042), a lower expression of CCL23 (P < 0.001), a lower expression of GAGED2 (P = 0.002), a higher expression of ST18 (P = 0.003), and a higher expression of WT1 (P = 0.052).

**Analysis of MRD markers in remission samples.** In 14 patients who had achieved a continuous complete remission, we could retrospectively analyze a total of 40 follow-up samples from bone marrow and 61 follow-up samples from peripheral blood. The follow-up of the patients ranged between 2 and 7 years. The majority of samples were collected while the patients were still under treatment. Expression of all genes, except for CSPG4, decreased to levels comparable to healthy controls (Fig. 2). This result indicates that these tumor markers are leukemia-specific and neither stress-related nor up-regulated in regenerating bone marrow or blood. CSPG4 was not included in further analyses.

**Expression of MRD markers at initial diagnosis and at the time of relapse.** In 12 patients, the expression of the remaining seven potential MRD markers was measured at the time of diagnosis as well as the time of relapse. For each gene, a strong correlation between the expression at the two time points was observed, i.e., the same patients who expressed high levels of a gene at diagnosis also expressed high levels of the respective gene at relapse (Spearman correlation coefficients between 0.62 and 0.94 with corresponding P values between 0.035 and <0.001).

**Analysis of MRD markers prior to relapse.** One of the main clinical applications of MRD markers is to detect pending relapses. We could retrospectively analyze the new MRD markers in 10 patients in whom at least one blood or bone

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**Table 2. Names, symbols, and geneID of 12 genes that were >50-fold overexpressed in AML pools, compared pool healthy bone marrow pools**

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Alternative gene symbols</th>
<th>Gene ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL23</td>
<td>Chemokine (C-C motif) ligand 23</td>
<td>SCYA23, MPIF1, CK-BETA-8, MIP3</td>
<td>6368</td>
</tr>
<tr>
<td>SPAG6</td>
<td>Sperm associated antigen 6</td>
<td>DKFZp4341153, MGC32676, Repro-SA-1</td>
<td>9576</td>
</tr>
<tr>
<td>PRAME</td>
<td>Preferentially expressed antigen in melanoma</td>
<td>MAPE, OIP4</td>
<td>23532</td>
</tr>
<tr>
<td>ST18</td>
<td>Suppression of tumorigenicity 18 (breast carcinoma)</td>
<td>KIAA0535, ZNF387</td>
<td>9705</td>
</tr>
<tr>
<td>WT1</td>
<td>Wilms tumor 1</td>
<td>GUD, WAGR, WT-2, WT33</td>
<td>7490</td>
</tr>
<tr>
<td>MSLN</td>
<td>Mesothelin</td>
<td>MPF, SMR, CAK1</td>
<td>10232</td>
</tr>
<tr>
<td>GAGED2</td>
<td>G antigen, family D, 2</td>
<td>XAGE1</td>
<td>9503</td>
</tr>
<tr>
<td>CSPG4</td>
<td>Chondroitin sulfate proteoglycan 4 (melanoma-associated)</td>
<td>NG2, MCSP, MCSPG, MSK16</td>
<td>1464</td>
</tr>
<tr>
<td>CCNA1</td>
<td>Cyclin A1</td>
<td></td>
<td>8900</td>
</tr>
<tr>
<td>CTGF</td>
<td>Connective tissue growth factor</td>
<td>IGFBP8, CCN2, NOV2</td>
<td>1490</td>
</tr>
<tr>
<td>POU4F1</td>
<td>POU domain, class 4, transcription factor 1</td>
<td>BRN3A, BRN3.0, RDC-1</td>
<td>5457</td>
</tr>
<tr>
<td>TRH</td>
<td>Thyrotropin-releasing hormone</td>
<td></td>
<td>7200</td>
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<tr>
<td>GREM1</td>
<td>Gremlin 1 homologe</td>
<td>CKTSF1B1</td>
<td>26585</td>
</tr>
<tr>
<td>LY64</td>
<td>Lymphocyte antigen 64</td>
<td>RPI05, CD180</td>
<td>4064</td>
</tr>
<tr>
<td>CSRP2</td>
<td>Cysteine- and glycine-rich protein</td>
<td>LMO5, CRP2, SmLIM</td>
<td>1466</td>
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</tbody>
</table>

**Note:** GREM1, LY64, and CSRP2 were identified by TaqMan low density arrays. The other genes were identified by TaqMan real-time PCR for single genes.
marrow sample was available (taken within 1 month prior to relapse). None of these patients showed a typical chromosomal translocation which could be used for the monitoring of MRD. We also analyzed follow-up samples in one patient who did not achieve a remission. Six patients had undergone allogeneic bone marrow transplantation prior to relapse (patients B, C, F, G, I, and J; Fig. 3). Five of these patients (patients D, E, F, G, and J; Fig. 3) had not been included in any of the previous steps of our analysis.

In 7 out of 10 patients who relapsed, at least one MRD marker was overexpressed prior to relapse (patients A-G; Fig. 3). An overexpression was defined as being higher than any of the 41 healthy controls (Fig. 1) and the 101 follow-up samples in patients who achieved a continuous remission (Fig. 2). In patient G, the level of WT1 prior to relapse was 2.8 units. This would not be a significant level for some of the other genes but for WT1, it matched the definition of an overexpression.

In patient A, two markers remained overexpressed throughout the time from diagnosis to relapse. In the other six patients, all markers were temporarily in the reference range but were overexpressed prior to relapse. In patient F, the pathologist described some cells which looked suspicious for leukemia 11 days prior to the diagnosis of the relapse, but could not make a definite diagnosis at that time point. In three patients, the pending relapse could not be detected. They showed overexpressed markers at relapse but not prior to relapse (patients H-J; Fig. 3). In the patient who did not achieve a remission, elevated levels of marker genes were found at any time during follow-up (patient K; Fig. 3).

Expression of MRD markers in peripheral blood versus bone marrow. In our follow-up studies, we analyzed 31 pairs of samples from bone marrow and peripheral blood which were collected from the same patient at the same time point. For all seven genes, we found a strong correlation between the results in peripheral blood and bone marrow (P = 0.009 for CCL23; P < 0.001 for all other genes).

Discussion

Using a stepwise approach which combined genome-wide expression profiling and TaqMan real-time PCR, we could identify five new tumor markers for the monitoring of MRD in AML. In combination with WT1 and PRAME, these markers should allow a more sensitive and specific monitoring of MRD than WT1 or PRAME alone. Combining all seven markers, 28 patients (54%) expressed at least one gene at a level of >100 units. In 18 patients (35%), the level of the strongest marker ranged between 10 and 100 units. In only six patients (11%) were all marker genes expressed at levels of <10 units.

Fig. 1. Expression of 8 potential MRD markers in leukemic cells from 52 children with AML, 21 samples of healthy bone marrow (hBM), and 20 samples of healthy blood (hB). For each gene, 1 unit was defined as the 90th percentile of the expression in healthy bone marrows. P values are given for AML versus hBM (Mann-Whitney test).
Prospective studies are needed to determine what level of overexpression of a gene is necessary for a clinically useful monitoring of MRD. In patients B, E, and G (Fig. 3), the pending relapse was indicated by genes that were expressed with <100 units in the leukemic cells at the time of relapse. The total amount of RNA in leukemic cells is higher than in most healthy cells. Furthermore, the leukemic cells are enriched by Ficoll-Hypaque density gradient centrifugation. Therefore, the proportion of analyzed leukemic RNA will be higher than the proportion of leukemic cells in the respective blood or bone marrow sample. This should improve the sensitivity of detecting leukemic cells (19).

In our follow-up studies, we defined all gene expressions that were <2 units, i.e., two times the 90th percentile of healthy bone marrow, as negative (Figs. 2 and 3). Levels between 2 and 5 units should be interpreted with caution because they may occur in healthy individuals and in continuous complete remission (Figs. 1 and 2). With this definition of a negative sample, the detection of leukemic cells was not limited by the sensitivity of the real-time PCR. For all seven genes, the quantification was reliably possible down to at least one log less with Ct-values ≤35.

The markers analyzed here allowed the detection of pending relapses. If verified in larger studies, this could be particularly useful after allogeneic bone marrow transplantation when full relapses can sometimes be prevented by donor lymphocyte infusion or the withdrawal of immunosuppressive therapy (24). The median time from the elevation of one of the MRD markers until relapse was 22 days. This could be enough time for these actions to be effective or to start a new course of chemotherapy in a patient who was not previously transplanted.

Prospective studies should determine how these markers can be used to analyze the response to remission induction therapy, and help to decide on the intensity of post-remission therapy. Such studies could easily be integrated in ongoing trials which already use WT1 or PRAME as MRD markers.

**Implications other than the monitoring of MRD.** Primarily, we aimed at identifying genes which can be used as MRD markers. However, some of the genes that were discovered may be

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Fig. 2. Expression of potential MRD markers in follow-up samples from 14 patients who achieved a continuous complete remission. Each line represents one patient. For all genes except for CSPG4, the expression in all patients decreased to levels that were in the same range as healthy controls (Fig. 1). For each gene, 1 unit was defined as the 90th percentile of the expression in healthy bone marrows.
equally important as therapeutic targets and might be involved in leukemogenesis. To our knowledge, the expression of MSLN, GAGED2, SPAG6, CCL23, or ST18 in hematologic malignancies have not previously been described.

MSLN is a well characterized target for immunotherapy (25, 26). It is overexpressed in many carcinomas. After successful application in animal models and cell cultures, there are ongoing clinical trials that use an immunotoxin composed of the Fv portion of an anti-MSLN antibody fused to a bacterial toxin (26). Moreover, phase I clinical trials suggested that MSLN might also be a useful antigen for tumor vaccines (27). Western blots also revealed the expression of MSLN in AML, suggesting that it is a possible therapeutic target in this disease as well (data not shown).

Fig. 3. Expression of MRD markers in follow-up samples from 10 patients (A–J) who relapsed and one patient (K) who failed to achieve a remission. Each line represents one gene. X axis, the number of days prior to relapse. For each gene, 1 unit was defined as the 90th percentile of the expression in healthy bone marrows. In patient D, no RNA was available from the time of relapse (marked as "?"”) BM, bone marrow; pB, peripheral blood.

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GAGED2 belongs to the so-called “cancer testis genes.” These genes have an expression pattern that is predominantly restricted to testes in normal tissues, yet they are expressed in many different histologic types of cancer. Because of this pattern of expression, they are intensively studied as targets for cancer immunotherapy (28, 29).

SPAG6 seems to be a new cancer testis gene. It was previously shown to be expressed in the testis but was not found in other healthy tissues (30). Our report is the first to show that SPAG6 is overexpressed in a malignant disease. Notably, SPAG6 has immunogenic properties and autologous antibodies to SPAG6 cause male infertility (30).

Little is known about ST18. Like WT1, it is a zinc-finger protein which has the potential to act as a transcriptional regulator. Very recently, it was shown that ST18 is a tumor suppressor in breast cancer (32). Here it is down-regulated versus low expression; CCL23 might help the leukemic cell clone to overgrow normal hematopoietic cells.

Imaging, Diagnosis, Prognosis

Conclusion

Here, we identified new genes which are vastly overexpressed in patients with AML. These markers allow the monitoring of MRD in AML, some could also serve as therapeutic targets and some might be involved in the pathology and development of this disease.

Acknowledgments

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References


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