Real-time quantitation of minimal residual disease in inv(16)-positive acute myeloid leukemia may indicate risk for clinical relapse and may identify patients in a curable state

Silvia Buonamici, Emanuela Ottaviani, Nicoletta Testoni, Vittorio Montefusco, Giuseppe Visani, Francesca Bonifazi, Marilina Amabile, Carolina Terragna, Deborah Ruggeri, Pier Paolo Piccaluga, Alessandro Isidori, Michele Malagola, Michele Baccarani, Sante Tura, and Giovanni Martinelli

The inv(16) cytogenetic subtype of acute myeloid leukemia (AML) has a relatively good prognosis. Many patients achieve complete remission (CR). The prognostic uncertainty of negative qualitative reverse transcription–polymerase chain reaction (RT-PCR) assays suggests the need to identify prognostically significant critical thresholds by real-time RT-PCR. A reliable and sensitive (10\(^{-5}\)) real-time RT-PCR assay was set up for the evaluation of relevant CBFβ-MYH11/ABL transcript ratios and was applied to the 21 patients with inv(16) AML routinely referred for cytogenetic and molecular monitoring in Sera gnoli Institute (Bologna, Italy) since 1990. Among the 18 patients who underwent ablative chemotherapy, all achieved CR with a 3-year disease-free survival probability of 63% (95% CI, 40%-87%) and no recorded events after 26 months. Five patients had relapses; 2 died of disease and 3 entered second CR. Analysis of the 125 bone marrow (or peripheral blood) samples studied by real-time RT-PCR showed that transcript ratios of samples taken during CR at any time before a relapse were always greater than 0.12%, whereas those of samples taken during first or second CR from patients who did not subsequently have relapses were always less than 0.25%. This suggests that transcript ratios greater than 0.25% may correspond to high risk for relapse, whereas ratios below 0.12% might indicate the patient is in a curable state. If confirmed, such thresholds could open the way to a new phase in post-CR therapeutic decision making for patients with inv(16) AML. (Blood. 2002;99:443-449)

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From the Institute of Hematology and Medical Oncology L. e A. Seràgnoli, University of Bologna, Italy.

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Institute of Hematology (Bologna, Italy) since June 1990. Of these, 19 were consecutive patients treated at the Seràgnoli Institute, whereas the remaining 2 were treated at the Institute of Hematology, University of Udine (patient 15) and the Institute of Hematology, University of Verona (patient 17). All patients provided written, informed consent to participate in the study. Diagnosis was made according to the French-American-British classification. Clinical and cytogenetic data are summarized in Table 1. Eighteen patients (patients 1–18) underwent molecular and cytogenetic analysis at diagnosis and during follow-up after ablative induction and consolidation chemotherapy, including transplantation procedures. Three patients (patients 19–21), who were older than 60, were referred for nonablative chemotherapy and underwent molecular and cytogenetic analysis only at diagnosis. Samples of bone marrow (n = 183) and peripheral blood (n = 5) were collected for routine patient care, according to institutional guidelines. Criteria for CR included morphologically normal bone marrow with less than 5% blasts, neutrophil count less than 1 × 10^9/L, platelet count less than 100 × 10^9/L, normalization of karyotype, and normal physical findings for more than 2 months.

**Cytogenetic analysis**

Cytogenetic analysis was routinely performed using a standard technique with Wright stain banding. At least 20 mitoses were analyzed for each sample.

**Samples and RNA isolation**

Mononuclear cells from samples were obtained by Ficoll-Hypaque density gradient centrifugation and were stored at −80°C in guanidinium thiocyanate. Total cellular RNA was extracted as previously described.

### Table 1. Clinical and karyotype characteristics of patients with inv(16)(p13q22) AML

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex/age, y</th>
<th>Therapy</th>
<th>Type of transcript</th>
<th>Clinical outcome after CT/clinical status</th>
<th>Karyotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F/47</td>
<td>ICE/NOVIA/FLAG</td>
<td>A</td>
<td>CR/relapse, died</td>
<td>46,XX(6)/46,XX,inv(16)(1p32q22)(6)/46,XX,inv(16)(p13q22)(1)</td>
</tr>
<tr>
<td>2</td>
<td>F/51</td>
<td>ICE/NOVIA/FLAG/ABMT</td>
<td>A</td>
<td>CR/AW</td>
<td>46,XX(3)/46,XX,inv(16)(p13q22)(7)</td>
</tr>
<tr>
<td>3</td>
<td>M/34</td>
<td>ICE/NOVIA/ABMT</td>
<td>A</td>
<td>CR/AW</td>
<td>46,XY(1)/46,XY,del(7)(q22),inv(16)(p13q22)(13)</td>
</tr>
<tr>
<td>4</td>
<td>M/38</td>
<td>ICE/NOVIA/ABMT</td>
<td>A</td>
<td>CR/AW</td>
<td>46,XY(2)/46,XY,inv(16)(p13q22)(10)</td>
</tr>
<tr>
<td>5</td>
<td>F/51</td>
<td>ICE/NOVIA/FLAG/FLANG/BMT</td>
<td>A</td>
<td>CR/breast cancer, died</td>
<td>46,XX(1)/46,XX,inv(16)(p13q22)(21)</td>
</tr>
<tr>
<td>6</td>
<td>F/58</td>
<td>ICE/NOVIA/ABMT-PBSC/FLANG/FLANG</td>
<td>A</td>
<td>2nd CR/AW</td>
<td>46,XX(2)/46,XX,inv(16)(p13q22)(28)</td>
</tr>
<tr>
<td>7</td>
<td>M/60</td>
<td>ICE/NOVIA/ABMT-PBSC</td>
<td>A</td>
<td>CR/AW</td>
<td>46,XY(2)/46,XY,inv(16)(p13q22)(19)</td>
</tr>
<tr>
<td>8</td>
<td>M/37</td>
<td>ICE/NOVIA/BMT</td>
<td>A</td>
<td>CR/AW</td>
<td>46,XY(3)/46,XY,inv(16)(p13q22)(16)</td>
</tr>
<tr>
<td>9</td>
<td>M/57</td>
<td>ICE/NOVIA/ABMT</td>
<td>A</td>
<td>CR/AW</td>
<td>47,XY,inv(16)(p13q22), +22(12)/47,XY,t(9;19)(q22q13),inv(16)(p13q22), +22(8)</td>
</tr>
<tr>
<td>11</td>
<td>M/35</td>
<td>ICE/FLANG/FLANG/ABMT-PBSC</td>
<td>A</td>
<td>CR/AW</td>
<td>46,XY(1)/46,XY,inv(16)(p13q22), +9(14)</td>
</tr>
<tr>
<td>12</td>
<td>F/60</td>
<td>ICE/FLANG/FLANG</td>
<td>A</td>
<td>CR/AW</td>
<td>46,XX(1)/46,XX,inv(16)(p13q22), +8(30)</td>
</tr>
<tr>
<td>13</td>
<td>M/49</td>
<td>ICE/FLANG/FLANG/ABMT</td>
<td>A</td>
<td>CR/AW</td>
<td>46,XY(1)/46,XY,inv(16)(p13q22), +8(28)</td>
</tr>
<tr>
<td>16</td>
<td>F/26</td>
<td>ICE/FLAN/FLAN/ABMT</td>
<td>E</td>
<td>CR/AW</td>
<td>46,XX(1)/46,XX,inv(16)(p13q22)(28)</td>
</tr>
<tr>
<td>17</td>
<td>M/17</td>
<td>ICE/FLAN/FLAN</td>
<td>A</td>
<td>CR/AW</td>
<td>46,XY(1)/46,XY,inv(16)(p13q22)(28)</td>
</tr>
<tr>
<td>18</td>
<td>M/16</td>
<td>ICE/FLAN/FLAN</td>
<td>C</td>
<td>CR/AW</td>
<td>46,XY,inv(16)(p13q22)(30)</td>
</tr>
<tr>
<td>19</td>
<td>M/63</td>
<td>Nonablative</td>
<td>A</td>
<td>—</td>
<td>46,XY(3)/46,XY,inv(16)(p13q22)(23)</td>
</tr>
<tr>
<td>20</td>
<td>M/61</td>
<td>Nonablative</td>
<td>A</td>
<td>—</td>
<td>46,XY(2)/46,XY,inv(16)(p13q22)(28)</td>
</tr>
<tr>
<td>21</td>
<td>F/65</td>
<td>Nonablative</td>
<td>A</td>
<td>—</td>
<td>46,XX(1)/46,XX,inv(16)(p13q22)(19)</td>
</tr>
</tbody>
</table>

**Qualitative RT-PCR analysis**

**RT-PCR**. Qualitative RT-PCR was routinely performed at diagnosis and during follow-up as previously described. Whenever possible, these results were confirmed by means of the recently defined BIOMED-1 Concerted Action protocol.

**RT-PCR of ABL control gene**. To assess the quality and quantity of the amplifiable RNA isolated from samples, RT-PCR of ABL gene transcripts was performed as previously described.

**Criteria for qualitative RT-PCR negativity**. Stringent criteria for negativity were applied: no amplification of the CBFβ-MYH11 transcript in 3 independent assays at a sensitivity of at least 10^3 (see below), always accompanied by successful amplification of the ABL transcript. To determine the level of sensitivity of breakpoint sequence amplification, experiments using CBFβ-MYH11-positive RNA (types A, D, E, and C) were conducted by mixing serially diluted total RNA isolated from patients 7, 12, 16, and 18 with the HL60 cell line.

**Reproducibility and accuracy of assays**. Positive and negative controls were included in all assays. In particular, RNA from the time of diagnosis was used for positive control. Negative controls included reactions with no RNA or no complementary DNA (cDNA) or HL60 cell line. Precipitates taken to avoid contamination included the use of a specifically designed UV flow cabinet and PCR-designated pipettes with filtered tips. All tests were conducted twice to confirm the results.

**Real-time RT-PCR**

Real-time RT-PCR was retrospectively performed using a method previously described.

Table 1. Clinical and karyotype characteristics of patients with inv(16)(p13q22) AML

- **Medications**: idarubicin (endovenously infused, 10 mg/m² per day) for days 1, 3, and 5, cytarabine (100 mg/m² per day) continuously infused for 10 days, and etoposide (VePesid; 100 mg/m² per day) for days 1–5. NOVIA (consolidation chemotherapy; cytarabine (500 mg/m² per day) for 6 days (endovenously infused twice a day for 4 hours) plus mitoxantrone (Novantron; 12 mg/m² per day) for 3 days (days 4, 5, 6). FLANG: 5 days of treatment with a 30-minute infusion of fludarabine (30 mg/m² per day) followed 4 hours later by a 4-hour infusion of cytarabine (2 g/m² per day). G-CSF (300 µg/day) was administered subcutaneously before starting fludarabine, continued for 5 days and then given again 1 week after the end of therapy until complete neutrophil recovery. FLANG: 3 days of fludarabine (30 mg/m² per day) as above, followed 4 hours later by a 2-hour infusion of cytarabine (1 g/m² per day) and a 30-minute infusion of mitoxantrone (Novantron; 10 mg/m² per day). Twelve hours before fludarabine infusion, G-CSF (300 µg/day) was administered subcutaneously. This was continued for 3 days and then was restarted as in the FLAG regimen. MEC6 chemotherapy: 6 days of etoposide (VePesid; 100 mg/m² per day), 30-minute infusion of mitoxantrone (Novantron; 10 mg/m² per day), and continuous infusion of cytarabine (100 mg/m² per day), followed by consolidation chemotherapy (MEC4 protocol). MEC4: 4 days of etoposide (VePesid; 100 mg/m² per day), 30-minute infusion of mitoxantrone (Novantron; 10 mg/m² per day), and continuous infusion of cytarabine (100 mg/m² per day).

*Karyotype at relapse. CR indicates complete remission; AW, alive and well.*
Fig. 1. Position of primers and probe for CBFβ-MYH11 transcript types A, D, and E, and for the ABL gene. CBFβ-MYH11 transcript types A, D, and E: the probe (inv(16) Probe) (5'-TTC AAA TTC GCG TGT CCT CCG A)-A and the sense primer (inv(16)A FW) (5'-TTA GCA CAA CAG GCC TTT GAA)-A are positioned in exon 5 of the CBFβ-MYH11 gene. The reverse primers (inv(16)A RW) (5'-CAG GCC CCG CCT GGA)-A, (inv(16)D RW) (5'-GCC CGG CCT CGT TAA GCA T)-A, and (inv(16)E RW) (5'-GCG TCT GCT TAT CTG GAA)-A are positioned in exons 12, 8, and 7 of the MYH11 gene for transcript types A, D, and E, respectively. ABL gene: the probe (inv(16) ABL Probe) (5'-CCA GTA GCA TCT GAG TGT GAA)-A and the reverse primer (inv(16) ABL RW) (5'-TGG GTC GTC CAG CGA GAA)-A are positioned in exon 1b of the ABL gene.

Statistical analysis

Overall survival (OS) and DFS probability were calculated from the date of diagnosis to the last contact or death, and to death or relapse, respectively, according to the Kaplan-Meier method. Comparisons of the CBFβ-MYH11/ABL ratios at diagnosis and relapse and during treatment and CR were performed by the Kruskal-Wallis test. χ² analysis was used for binary variables. All analyses were performed using the SPSS software package (SPSS, Chicago, IL).

Results

Clinical follow-up, survival, and status

Figure 3 reports the clinical outcomes and OS and DFS of the 18 patients who underwent ablative chemotherapy. At a median follow-up of 51 months (range, 4-115 months), 13 (72%) patients are in first CR and 3 (17%) are in second CR. Thirteen (72%) patients achieved qualitative RT-PCR negativity after induction chemotherapy, and 3 (17%) achieved it after consolidation chemotherapy. Figure 3 illustrates that the OS and DFS probabilities at 3 years were 82% (95% confidence interval [CI], 63%-100%) and 63% (95% CI, 40%-87%), respectively. In either case, no events were recorded after the 26th month.

Cytogenetic analysis

Cytogenetic results are summarized in Figure 4A. All 21 patients showed inv(16)(p13q22) at diagnosis, and 5 also had additional karyotypic abnormalities (Table 1). In one patient (patient 1), an additional translocation was detected at relapse [46,XX, inv(16)(p13q22), t(2;17) (q31;p13)].
Qualitative RT-PCR analysis

Qualitative RT-PCR results are summarized in Figure 4A. Assays of all 188 samples from the 21 patients were routinely studied. Of these, 172 samples were also fully studied according to the BIOMED-1 Concerted Action protocol. The 16 samples that turned out to be ABL-negative were excluded from subsequent analysis. Log sensitivities of the transcript primer sets were $10^{-4}$ for types A, C, D, and E.

At diagnosis, 18 patients displayed type A transcript, one had type D, one had type E, and one had a type C transcript (GenBank accession numbers AF249898, AF249897, AF251768, respectively). At follow-up, 9 of 149 samples taken from the 18 patients who received ablative chemotherapy were positive for CBFβ-MYH11 according to the BIOMED-1 Concerted Action protocol. Fifteen of 18 patients became negative after induction chemotherapy. The remaining 3 patients (patients 13, 15, 16) became negative after the first consolidation course. One patient (patient 10), who was found to be positive 3 months after completing consolidation chemotherapy, had insufficient available samples for molecular follow-up before relapse.

Application of real-time RT-PCR for CBFβ-MYH11 analysis

For 3 transcript types (A, D, E) and the ABL gene, it was possible to develop primer–probe combinations that permitted amplification and real-time RT-PCR analysis. In primer–matrix experiments, different ΔRn values were found for each primer combination. Ct values were comparable. The 300/300 mM primer combination was used in all experiments. As can be seen from Figure 1, for the 3 types of transcript, the TaqMan probe and the forward primer were

Figure 3. Overall survival and disease-free survival of 18 patients with inv(16) AML.

Figure 4. Schematic representations of karyotypic and qualitative RT-PCR and real-time RT-PCR follow-up of patients with inv(16) AML. (A) Karyotypic and qualitative RT-PCR follow-up of 21 patients: karyotypic analysis negative (●) or positive (○) for inv(16); molecular analysis negative (△) or positive (◊) for CBFβ-MYH11. (B) Real-time RT-PCR of 18 patients (patients 1, 10, 18 had insufficient material for analysis): samples taken at diagnosis or at the moment of relapse (●); samples taken at any time during or after treatment before relapse (○); samples taken during follow-up when no subsequent relapse was recorded (△). *Samples that fell in the gray zone with CBFβ-MYH11/ABL ratios between 0.12% and 0.25%. Auto-BMT indicates autologous bone marrow transplantation; allo-BMT, allogeneic bone marrow transplantation; and PBSCT, autologous transplantation with peripheral blood stem cells.
located in exon 5 of the CBFβ gene. Reverse primers were located in exons 12, 8, and 7 of the MYH11 gene for transcript types A, D, and E, respectively. The number of target molecules of transcript in each sample was expressed as a percentage ratio between CBFβ-MYH11 and ABL in 6 μL cDNA. Three patients (patients 19-21) were tested at diagnosis only. For 16 patients, transcript levels were quantified in sequential bone marrow or peripheral blood samples, but for 2 patients (patients 1 and 10), insufficient amounts of material precluded transcript quantification at diagnosis and during follow-up.

**Discussion**

Among the distinct subset of AML patients carrying inv(16)(p13q22), a high percentage achieves CR.12,17,18 It is thought that some of these patients may be considered cured. This report on a large series of patients with inv(16) AML examines what, to our knowledge, is the longest clinical, cytogenetic, and molecular follow-up thus far published. Our results suggest that the high proportion of patients who remain in CR for as long as 2 years (89% in our series) seems to have a very good prognosis because none, at the time of writing, have had relapses. To search for prognostic indications at a molecular level, we developed primer-probe combinations that permitted effective real-time RT-PCR quantification of CBFβ-MYH11 transcripts. Our results provide the first evidence that cutoff levels of transcripts probably do exist, below which or above which cure or relapse, respectively, is likely.

The clinical results from our series are fully in line with the concept that long-term CR can be achieved in a high proportion of patients with inv(16) AML. At a median follow-up of 51 months (range, 4-115 months), 16 of 18 (89%) of the patients are stable in either first (13 patients) or second (3 patients) CR, with a DFS probability of 63% at 3 years. No relapses or deaths have been observed after the 26th month. These encouraging data highlight the need to assess whether any or all patients who achieve durable CR can be considered cured.

At qualitative RT-PCR, most of our patients achieved molecular remission, defined as undetectability of the neoplastic transcript at the sensitivity threshold level (1:10^5) permitted by the BIOMED-1 Concerted Action protocol.23 Probably because of different sensitivity methods applied,12,19,21 some authors reported that most patients with AML inv(16) show persistence of minimum residual disease after CR. Using BIOMED-1 criteria, 72% of our patients achieved qualitative molecular remission after induction chemotherapy, and 17% achieved it after consolidation chemotherapy. The ultimate prognostic impact of qualitative RT-PCR negativity is uncertain.12 Our series confirmed that patients who achieve this status are still susceptible to eventual relapse. Indeed, all 3 of our patients who had relapses after receiving ablative chemotherapy had had at least one negative qualitative RT-PCR assay. Like other authors,27 we found that qualitative RT-PCR did not allow cutoff levels to be identified for prognostic purposes.

Using TaqMan technology, we therefore set up a quantitative real-time RT-PCR assay. To our knowledge, this is the first time that real-time RT-PCR has been applied to the analysis of inv(16). Our data demonstrate that the method used is reliable and consistently more sensitive than qualitative RT-PCR (1:10^5 vs 1:10^7). Indeed, a high proportion of the samples in which the CBFβ-MYH11
the prognostic significance of an undetectable CBF\-MYH11 transcript was detectable at real-time RT-PCR were negative at 448 BUONAMICI et al BLOOD, 15 JANUARY 2002 analysis,28 regular study at set time points would not place a major of the cutoff points. If, as seems probable, peripheral blood follow-up should help further define the long-term predictive value and of another at 0.25%, above which relapse is probable. Only 6 assays fell within the intermediate gray zone (Figure 4B). Longer follow-up should help further define the long-term predictive value of the cutoff points. If, as seems probable, peripheral blood samples are thought to be adequate for routine real-time RT-PCR analysis,28 regular study at set time points would not place a major burden on patients.

We also found that some samples were negative at qualitative and quantitative RT-PCR. This finding may shed some light12 on the prognostic significance of an undetectable CBF\-MYH11 transcript in inv(16) AML patients in CR. Considering that none of the patients who reached this status had subsequent relapses, the achievement of real-time RT-PCR negativity may be predictive of eventual cure.

In conclusion, our series suggests that most patients with inv(16) AML can achieve durable CR after ablative chemotherapy and that many achieve negative assays at qualitative RT-PCR. The real-time RT-PCR assay set up by us can be recommended as a reliable and more sensitive method for routine monitoring of minimum residual disease in such patients. Our data suggest that samples with CBF\-MYH11/ABL ratios above 0.25% correspond to a high risk for relapse. In contrast, in the presence of ratios lower than 0.12%, relapse seems more unlikely and patients might be considered to be in a curable state. It is possible that complete negativity at real-time RT-PCR could be associated with a cure from this AML subtype. More generally, our findings suggest that in inv(16) AML, real-time RT-PCR could provide indications regarding the patient's clinical state similar to those that can apparently be obtained (through CBFx) in t(8;21) AML, where high- and low-risk thresholds have already been reported.28

Acknowledgments

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16p13.3q22) in acute myelogenous leukemia. 
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