Multidrug Resistance (mdr1) Gene Expression in Adult Acute Leukemias: Correlations With Treatment Outcome and In Vitro Drug Sensitivity

By Jean-Pierre Marie, Robert Zittoun, and Branimir I. Sikic

Resistance to multiple chemotherapeutic agents has been related to the production of P-glycoprotein, a trans-membrane drug efflux pump that is encoded by the multidrug resistance (MDR) gene mdr1. To investigate whether mdr1 could be involved in clinical resistance to chemotherapy in acute leukemias, we have analyzed retrospectively the RNA from adult acute leukemia cells by slot-blot hybridization with a human mdr1 probe. Units of mdr1 expression were defined by reference to drug-sensitive human sarcoma and K562 leukemia cell lines (1 U) and the highly resistant doxorubicin selected leukemia cells K562/R7 (50 U). We studied 41 adult patients with acute leukemias: 5 acute lymphoblastic leukemias, 23 acute myeloid leukemias, and 13 secondary leukemias or blast crisis of chronic myelogenous leukemia. Expression of 10 U or more of mdr1 was found in 6 of 31 (19%) leukemias at diagnosis, versus 5 of 10 multiple myeloma and leukemias have found low mdr1 expression in most patients at diagnosis.8,11-14

If high mdr1 expression correlates with a poor response to therapy, the prospective identification of such patients could lead to therapeutic strategies to circumvent or overcome MDR. These strategies might include the more intensive use of chemotherapeutic agents not involved in the MDR phenotype, or modulation of MDR by inhibitors of P-gp such as verapamil,9,30 cyclosporine,71 or others.21 In the current study, we correlate mdr1 expression in adult acute leukemias with both treatment outcome and in vitro sensitivity of leukemic clonogenic cells.

Patients. Forty-five specimens were studied from 41 patients with adult acute leukemias (Table 1). The average age was 45 years (range 15 to 82 years). The average peripheral white blood cell (WBC) count was \(115 \times 10^9/L\) (SD, \(\pm 156 \times 10^9/L\)), with \(82 \times 10^9/L\) (SD, \(\pm 119 \times 10^9/L\)) circulating leukemic cells. The mean percent of leukemic cells in the bone marrow was 72% (SD, \(\pm 18\%\)).

There were 13 cases of secondary leukemia (10 after myeloproliferative syndrome, 3 after refractory anemia with an excess of blasts). Among these, 2 were lymphoblastic (CALLA+), and 11 were myeloid. The 28 patients with primary leukemias included 5 lymphoblastic (2 cALL, 1 T-ALL, 1 Burkitt’s, and 1 lymphosarcoma cell leukemia), and 23 myeloid leukemias (3 M1, 6 M2, 1 M3, 8 M4, and 5 M5 according to the French-American-British [FAB] classification).

Specimens were obtained from 31 patients at diagnosis and 10 patients after therapy, which included MDR-related drugs (four in first phase with resistant disease, four in first relapse, two in second relapse). A total of 36 patients received chemotherapy, which included MDR-related drugs and is detailed in Table 1. Treatment for the seven patients with ALL included either Vcr or vindesine (Vind) with prednisone (Pdn) and the addition of doxorubicin (Dnr) and cytosine arabinosine (araC) according to the AML-8 protocol of the E.O.R.T.C., four relapsed AML patients treated with AMSA and high-dose araC, and one with Dnr, Vcr, and Mit.

Fifteen patients obtained a complete remission (CR), according

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Table 1. Clinical Data and mdr1 Expression in Patients With Leukemia

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<th>Patient No.</th>
<th>Age (yr)</th>
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<td></td>
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<td>% (10^6)</td>
<td>BM (%)</td>
<td>Phase</td>
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<td>M5</td>
<td>BM</td>
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</tbody>
</table>

(Continued on following page)

RNA slot-blot analysis of mdr1 expression. Mononuclear cells from bone marrow aspirates or peripheral blood were separated by Ficol-Hypaque density gradient centrifugation and stored at -80°C. Total cellular RNA was prepared according to the acid guanidinium phenol chloroform technique.26 Aliquots of 2.5, 5, and 10 µg of total cellular RNA were dissolved in 100 µL water, 300 µL of a solution of 6.15 mol/L formaldehyde and 10% SSC were added and incubated for 15 minutes at 65°C, and then loaded onto a Zeta-probe nylon blotting membrane (Bio-Rad Laboratories, Richmond, CA) using a slot-blot apparatus (Schleicher and Schuell, Keene, NH). Membranes were heated for 2 hours at 80°C in a moist atmosphere with 6% carbon dioxide. Before plating, the cells were incubated in a humidified chamber for 30 minutes at 37°C in the presence of 5% CO2 and 7.5% humidity. The technique of blast colony formation has been described previously.27 Blast colony formation was performed with methylcellulose (0.8%) and 20% fetal calf serum, using a CFU-L assay of drug sensitivity.

Table 1. Clinical Data and mdr1 Expression in Patients With Leukemia (Cont’d)

<table>
<thead>
<tr>
<th>Patient</th>
<th>Cytologic Classification¹</th>
<th>Source of Cells²</th>
<th>Blood (10%)</th>
<th>BM (%)</th>
<th>Phase²</th>
<th>Chemotherapy³</th>
<th>Outcome⁴</th>
<th>mdr1 Expression⁵</th>
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<tr>
<td>No.</td>
<td>Age</td>
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<tr>
<td>38</td>
<td>81</td>
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<td>NT</td>
<td>NE</td>
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<tr>
<td>39</td>
<td>19</td>
<td>M2</td>
<td>BM</td>
<td>26</td>
<td>63</td>
<td>P1</td>
<td>Dnr/araC</td>
<td>E4</td>
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<tr>
<td>40</td>
<td>67</td>
<td>M2</td>
<td>BM</td>
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<td>P3</td>
<td>Dnr/araC</td>
<td>E2</td>
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<td>BM</td>
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<td>BL</td>
<td>ND</td>
<td>ND</td>
<td>P1 R</td>
<td>NT</td>
<td>NE</td>
</tr>
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<td>31</td>
<td>M5</td>
<td>BM</td>
<td>19</td>
<td>57</td>
<td>P2</td>
<td>Dnr/Mit/Vcr</td>
<td>E2</td>
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<td>M5</td>
<td>BM</td>
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<td>58</td>
<td>P2</td>
<td>Dnr/araC</td>
<td>E4</td>
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</table>

Abbreviation: ND, not detected.

*BC/ALL/M, lymphoid (myeloid) blast crisis of chronic myelogenous leukemia; BC/MMML, blast crisis of chronic myelo-monocytic leukemia; MiL, acute myeloid leukemia secondary to dysmyelopoesis; Lymph L, B-cell acute leukemia with lymphosarcoma cells; Burkitt’s LL, Burkitt’s leukemia/lymphoma; L1 and M1 to M5, FAB classification.

1BL, peripheral blood; BM, bone marrow.

41/+, first evolutive phase, before any treatment; P1 R, resistant to first line treatment; P2, second evolutive phase (prior therapy).

3*Drugs received after mdr1 testing: Vcr, vincristine, 1.4 mg/m²/w × 4 to 6 wk; Pdm, prednimustine; AMSA, amsacrine, 100 to 120 mg/m²/d × 3; HDaraC, cytarabine, 1 to 2 g/12 h × 8 to 12; araC, cytarabine, 100 to 200 mg/d × 7; LDaraC, cytarabine, 20 mg/m²/d × 21; HIU, hydroxyurea, 2 g/d; Dnr, daunorubicin, 45 mg/m² × 3; ADR, doxorubicin, 25 to 50 mg/m² × 4; Vind, vinblastine, 3 mg/m² × 3; Mit, mitoxantrone, 8 mg/m²/d × 5; NT, no treatment.

2CR, complete remission (duration in months); E1, E2, complete or relative resistance, E4, death in aplasia; NE, not evaluable.

1(-), 0 to 1 U; (+/−), 2 to 4 U; (+), 5 to 9 U; (++), ≥ 10 U.

The ratio of the signals for mdr1 and ribosomal RNA was calculated using the area under the curve determined by densitometry. The negative control was MES-SA, a drug-sensitive uterine sarcoma line26 with very low expression of the mdr1 gene (defined as 1 U of expression). The positive control was K562/R7, a resistant leukemic cell line27 that was 75-fold resistant to ADR compared to the parental cell line, K562, with a relative increase of mdr1 mRNA of 50 U. Sample values were expressed as units of expression relative to MES-SA and K562/R7 cells. All the samples were tested at least twice on two different blots with two different batches of probe. The correlation coefficient for separate determination of mdr1 expression by slot blotting was 0.927.

Because of concerns regarding the use of degraded RNA from clinical leukemia specimens, we performed an RNase digestion experiment using intact RNA from an MDR cell line derived from MES-SA cells. RNA was incubated with 0.01, 0.1, and 1.0 µg/mL of RNase A for 15 minutes, and 0.3, 1.0, and 3.3 µg/mL of the RNA were applied to the slot-blotting apparatus in triplicate and hybridized with the mdr1 probe. As expected, progressive degradation of the RNA was evident on ethidium bromide staining after agarose gel electrophoresis. However, degradation of the RNA did not lead to a falsely high or low hybridization signal for the mdr1 gene in this experiment.

CFU-L assay of drug sensitivity. Data for in vitro drug sensitivities are available for only 22 of the 41 specimens because some samples were frozen directly for molecular studies only, and 10% of the samples that were plated did not grow sufficiently under our assay conditions.

The technique of blast colony formation has been described previously.27 Briefly, 2 × 10⁵ T-depleted cells in 0.1 mL of α medium were plated in methylcellulose (0.8%), 20% fetal calf serum (FCS; Flow Laboratories), and 10% phytohemagglutinin-leukocyte conditioned medium in 1-mL microwells (Titertek Laboratories). Eight to 10 microwells were plated for each drug exposure and incubated in a moist atmosphere with 6% carbon dioxide. Before plating, the cells were incubated in α medium containing 10% FCS for 30 minutes with 10⁻⁶ mol/L Dnr or Mit, or for 1 hour with 10⁻⁵ mol/L AMSA or etoposide (VP16). The cells...
were washed and plated as before. For combined Dnr/araC sensitivity, cells were exposed to \(10^{-7}\) mol/L araC in the culture medium after exposure to Dnr. Aggregates of greater than 20 cells were counted at day 7 in at least four wells. The percentage inhibition of CFU-L was calculated by comparison with growth of control plates without drug exposure. Resistance was defined as less than 70% CFU-L inhibition after Dnr, AMSA, or VP16 exposure, and less than 80% inhibition after Dnr/araC or Mit exposure.  

Statistical analysis. Linear correlation was used to test for the reproducibility of blotting and sample values relative to positive and negative controls. Fisher’s exact test was used to test for significant differences between groups. In the analysis, the anthracyclines Dnr and Adr, AMSA, Mit, VP16, Vcr, and Vind were considered to be MDR-related drugs, whereas cyclophosphamides, araC, and Pdn were not considered to be involved in the MDR phenotype.

RESULTS

Figure 1 depicts a representative RNA slot blot hybridized with the 5‘-mdrl (upper row) and ribosomal (lower row) cDNA probes. The levels of mdrl mRNA expression of the 41 patients, divided according to no prior versus prior therapy, are shown in Table 2. The results of slot blotting were very reproducible from blot to blot \((r^2 = .93)\) and were consistent when normalized to either of the control cell lines, MES-SA or K562/R7 \((r^2 = .91)\). Absent or low levels of mdrl expression, 0 to 1 U, were graded as \((-\) and observed in 13 (32%) patients, and moderate elevation of 2 to 4 U \((+/-)\) was seen in 10 (24%). Levels between 5 and 9 U \((+)\) were seen in seven patients (17%), and expression of 10 U or more \((++)\) in 11 cases (27%). Although sample 37b in Fig 1 appears to have a slight positive signal, two other blots of this specimen were negative and it is graded as negative.

High expression of \(\geq 10\) U of mdrl \((++)\) was found in 50% of patients previously treated with MDR-related drugs, compared with 19% of untreated patients \((P = .06)\). No significant difference in expression was seen between primary and secondary leukemias. No correlations were found between mdrl expression and the number of circulating leukemic cells, the percentage of bone marrow blasts, the FAB classification, or the age of the patients. Serial determinations of mdrl expression in the four cases tested before and after treatment showed an increased expression in two patients (Table 3). One patient remained negative for mdrl expression after failure of chemotherapy, and one patient with high expression after Dnr/araC therapy exhibited decreased expression after additional therapy with AMSA and high-dose araC.

Treatment outcome in 36 patients treated with chemotherapy including MDR-related drugs is presented in Table 4. CR was obtained in only 29% of patients expressing 2 U or more of mdrl \((+/-, +, and ++)\), whereas 67% of patients negative for mdrl expression achieved CR \((P = .03)\). This difference is significant, even if the patients who died in aplasia are excluded from the analysis \((33\% v 73\%, P = .04)\). No correlation was found between remission duration and mdrl expression.

In 22 AML patients, in vitro drug sensitivity of leukemic

### Table 2. mdrl Expression (mRNA) in Leukemic Cells of 41 Patients Measured by Slot-Blot Analysis (prior vs no prior therapy with MDR-related drugs)

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<thead>
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<th>Tumor Type (n)</th>
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<td></td>
<td>1</td>
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<td>De novo ALL (5)</td>
<td>(-)</td>
<td>(+/-)</td>
<td>(+)</td>
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<td>De novo AML (23)</td>
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<tr>
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<tr>
<td>Total (41)</td>
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</table>

*Units = ratio of mdrl RNA to ribosomal RNA, compared with control cells; 1 U = negative control cells, K562 and MES-SA; 50 U = Dnr-selected MDR cell line, K562/R.
clonogenic cells was determined and compared with the mdr1 RNA expression measured on the same sample. All 11 cases with in vitro drug resistance exhibited detectable mdr1 expression, while 5 of 11 cases with CFU-L sensitivity to MDR-related drugs did not express increased levels of mdr1 expression (Table 5). The correlation between lack of mdr1 expression and in vitro sensitivity to several MDR-related drugs was statistically significant ($P = .03$).

### DISCUSSION

The most important result of our study is the significant inverse association of mdr1 expression and the achievement of a CR in adult patients with acute leukemias. The CR rate among patients with low mdr1 expression was more than twofold higher than in those with increased mdr1 expression, $67\%$ CR versus $29\%$ (Table 4). The low overall CR rate of $46\%$ is not surprising considering the prevalence of adverse prognostic factors in this group of patients, with an average age of 45 years and a mean peripheral WBC count of $115 \times 10^9/\text{L}$.

The proportion of cases with increased mdr1 mRNA expression is higher in our series than in some other. The variations between studies may be partly explained by the use of different positive control cell lines and the number of units arbitrarily assigned to these controls. Patient selection might also contribute to differing results. An incidence of increased mdr1 mRNA expression similar to our findings has been reported recently for both ALL and AML. It is unlikely that mdr2 expression affected our results because it is not selectively expressed in most normal tissues and human tumor cell lines.

We did not find a significant difference in the level or distribution of mdr1 expression among leukemias before or after therapy, perhaps because there were only 10 samples analyzed after chemotherapy. However, there was a suggestion that high expression may be selected by therapy, with 50% of patients (5 of 10) exhibiting 10 U or more of expression after treatment, while only 19% of untreated patients expressed such high levels ($P = .06$). Three of 10 patients who were clinically resistant to MDR drugs did not exhibit mdr1 expression, implying that other mechanisms of drug resistance were present (Table 2). Recently, high expression of mdr1 has been reported in three of four patients with blast crisis of chronic myelogenous leukemia, and may contribute to the poor response of these patients to chemotherapy.

Although only 19% of leukemias from untreated patients expressed very high levels of mdr1, an additional 19% expressed moderate levels of 5 to 9 U (+), and only 32% of patients were completely negative for mdr1 expression. A low but detectable level of expression might be partly explained by the expansion of a subset of immature normal myeloid cells which express mdr1 RNA. These positive cells were not detected by RNA slot blotting of normal bone marrow, but were demonstrated by in situ RNA hybridization, which can detect mdr1 gene expression in a subset of the normal marrow population, and by the polymerase chain reaction. We are continuing to assess the optimal method for detecting mdr1 expression in clinical specimens.

No significant correlations were found between the levels of mdr1 expression and known adverse prognostic factors in acute leukemia, such as secondary leukemia, patient age, or high WBC count. The remission duration in patients with $mdr \ (+)$ and $mdr \ (\sim)$ leukemic cells did not differ statistically. Any such differences in remission duration might be reduced by the use of consolidation therapy with high-dose araC.

The fact that 29% of patients with detectable mdr1 expression attained a CR can be explained in part by the fact that these patients were also treated with non-MDR drugs such as araC, Pdn, and cyclophosphamide. araC

### Table 3. Sequential Determination of mdr1 mRNA Expression in Four Patients With Acute Myeloid Leukemia

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Prior Treatment</th>
<th>First Specimen mdr1</th>
<th>Subsequent Therapy</th>
<th>Elapsed Time (wk)</th>
<th>Second Specimen mdr1</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>Dnr/araC</td>
<td>++</td>
<td>AMSA/HDaraC</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>21</td>
<td>Untreated</td>
<td>+</td>
<td>Dnr/araC</td>
<td>21</td>
<td>+</td>
</tr>
<tr>
<td>22</td>
<td>Untreated</td>
<td>-</td>
<td>Dnr/araC,AMS/AHaraC</td>
<td>28</td>
<td>-</td>
</tr>
<tr>
<td>37a</td>
<td>Untreated</td>
<td>-</td>
<td>Dnr/araC,AMS/AHaraC</td>
<td>20</td>
<td>+/-</td>
</tr>
</tbody>
</table>

See Table 1 for drug nomenclature.

### Table 4. mdr1 Expression and Clinical Outcome in Patients Treated With a Chemotherapy Including at Least One MDR-Related Drug

<table>
<thead>
<tr>
<th>Treatment Type (n)</th>
<th>Levels of mdr1 RNA Expression</th>
<th>CR (n = 15)</th>
<th>Resistant (n = 16)</th>
<th>Death in Aplasia (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 (--)</td>
<td>2-4 (+/-)</td>
<td>5-9 (+)</td>
<td>10 (+)</td>
</tr>
<tr>
<td>AML treatments</td>
<td>Dnr/araC (25)</td>
<td>5</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>AMSA/HD araC (4)</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>ALL treatments</td>
<td>Vcr/Pdn +/-Dnr (7)</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total (36)</td>
<td>8</td>
<td>2</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>

$P = .03$ for CR v all failures for $mdr = 1 \ v \geq 2 \ U$; $P = .04$ for CR v resistances for $mdr = 1 \ v \geq 2 \ U$. 

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**Table 5. mdr1 Expression and In Vitro Drug Sensitivity of Clonogenic Leukemic Cells (CFU-L) to MDR-Related Drugs in AML Patients**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>mdr1 Expression</th>
<th>CFU-L Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Patients with CFU-L resistant to at least one MDR-related drug</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>++</td>
<td>R R</td>
</tr>
<tr>
<td>30</td>
<td>++</td>
<td>R R S</td>
</tr>
<tr>
<td>38</td>
<td>++</td>
<td>R R</td>
</tr>
<tr>
<td>41</td>
<td>++</td>
<td>R R</td>
</tr>
<tr>
<td>29</td>
<td>+</td>
<td>R R S</td>
</tr>
<tr>
<td>40</td>
<td>+</td>
<td>R S S</td>
</tr>
<tr>
<td>6</td>
<td>+/-</td>
<td>R R R</td>
</tr>
<tr>
<td>9</td>
<td>+/-</td>
<td>R R</td>
</tr>
<tr>
<td>32</td>
<td>+/-</td>
<td>R S S</td>
</tr>
<tr>
<td>34</td>
<td>+/-</td>
<td>R S R S</td>
</tr>
<tr>
<td>39</td>
<td>+/-</td>
<td>R R</td>
</tr>
<tr>
<td>B. Patients with CFU-L sensitive to MDR-related drugs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>++</td>
<td>S S</td>
</tr>
<tr>
<td>12</td>
<td>++</td>
<td>S S</td>
</tr>
<tr>
<td>20</td>
<td>++</td>
<td>S S S S</td>
</tr>
<tr>
<td>21</td>
<td>+</td>
<td>S S S R</td>
</tr>
<tr>
<td>10</td>
<td>+/-</td>
<td>S S S R</td>
</tr>
<tr>
<td>37b</td>
<td>+/-</td>
<td>S S S R</td>
</tr>
<tr>
<td>19</td>
<td>-</td>
<td>S S S S</td>
</tr>
<tr>
<td>22</td>
<td>-</td>
<td>S S S S</td>
</tr>
<tr>
<td>28</td>
<td>-</td>
<td>S S</td>
</tr>
<tr>
<td>33</td>
<td>-</td>
<td>S S</td>
</tr>
<tr>
<td>37a</td>
<td>-</td>
<td>S S</td>
</tr>
</tbody>
</table>

**CFU-L resistance (R) is defined as:** <70% inhibition with 10^{-7} mol/L of Dnr; <80% inhibition with 10^{-6} mol/L of Dnr + 10^{-7} mol/L of AraC; <80% inhibition with 10^{-5} mol/L of Mit; or <70% inhibition with 10^{-6} mol/L of AMSA or VP16. mdr1 expression is defined as: (–), 0 to 1 U; (+/–), 2 to 4 U; (+), 5 to 9 U; (++), >10 U.

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Multidrug resistance (mdr1) gene expression in adult acute leukemias: correlations with treatment outcome and in vitro drug sensitivity

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