Favorable Prognosis of Hyperdiploid Common Acute Lymphoblastic Leukemia May Be Explained by Sensitivity to Antimetabolites and Other Drugs: Results of an In Vitro Study


DNA hyperdiploidy is a favorable prognostic factor in childhood acute lymphoblastic leukemia (ALL). The explanation for this prognostic significance is largely unknown. We have studied whether DNA ploidy was related to cellular resistance to 12 drugs, assessed with the methyl-thiazol-tetrazolium assay, in samples of 74 children with common (CD10+ precursor B-cell) ALL. Sixteen patients had hyperdiploid ALL cells and 58 patients had nonhyperdiploid ALL cells. Hyperdiploid ALL cells were more sensitive to mercaptopurine (median, 9.0-fold; \( P = .000003 \)), to thioguanine (1.4-fold; \( P = .023 \)), to cytarabine (1.8-fold; \( P = .016 \)), and to l-asparaginase (19.5-fold; \( P = .022 \)) than were nonhyperdiploid ALL cells. In contrast, these two ploidy groups did not differ significantly in resistance to prednisolone, dexamethasone, vincristine, vindesine, daunorubicin, doxorubicin, mitoxantrone, and teniposide. The percentage of S-phase cells was higher (\( P = .05 \)) in the hyperdiploid ALL samples (median, 8.5%) than in the nonhyperdiploid ALL samples (median, 5.7%). However, the percentage of cells in S-phase was not significantly related to in vitro drug resistance. We conclude that the favorable prognosis associated with DNA hyperdiploidy in childhood common ALL may be explained by a relative sensitivity of hyperdiploid common ALL cells to antimetabolites, especially to mercaptopurine and to l-asparaginase.

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Each drug was tested in 6 concentrations in duplicate, with concentrations of 6TG with drugs) were used to blank the reader, and 6 wells with cells in medium without drugs were used to determine the control by dead cells. The formazan crystals formed were dissolved with CO2 for 4 days at 37°C. Then, 10 µL of MTT solution (5 mg/mL) was added to the plates. After shaking the plates until the cell pellet was resuspended, they were incubated for 6 hours. The tetrazolium salt MTT is reduced to a colored formazan by living cells but not by dead cells. The formazan crystals formed were dissolved with 100 µL acidified isopropanol. The optical density of the wells, which is linearly related to the cell number, was measured with an EL-312 microplate spectrophotometer (Bio-tek Instruments Inc, Winooski, VT) at 562 nm. Leukemic cell survival (LCS) was calculated from the dose-response curve and used as measure of resistance. Results were considered evaluable in case of an optical density of ≥0.050 and ≥70% leukemic cells in the control wells (leukemic cells without drug), which assures reliable test results.T1-T6

Eighty microliters of cell suspension (2 × 10⁶ cells/mL) was added to 20 µL of the various drug solutions in 96-well microculture plates. Each drug was tested in 6 concentrations in duplicate, with concentration ranges as reported previously.T6 These concentrations do not necessarily stay the same during the 4 days of incubation. Experiments (not shown here) showed no differences between 6MP and 6TG regarding their in vitro behavior (stability and adherence to the walls of the plates). Wells with culture medium only (for 6MP and 6TG with drug) were used to blank the reader, and 6 wells with cells in medium without drugs were used to determine the control cell survival and to calculate the coefficient of variation of control wells. The plates were incubated in humidified air containing 5% CO2 for 4 days at 37°C. Then, 10 µL of MTT solution (5 mg/mL) was added to the plates. After shaking the plates until the cell pellet was resuspended, they were incubated for 6 hours. The tetrazolium salt MTT is reduced to a colored formazan by living cells but not by dead cells. The formazan crystals formed were dissolved with 100 µL acidified isopropanol. The optical density of the wells, which is linearly related to the cell number, was measured with an EL-312 microplate spectrophotometer (Bio-tek Instruments Inc, Winooski, VT) at 562 nm. Leukemic cell survival (LCS) was calculated by the equation: LCS = (mean optical density treated wells/mean optical density control wells) × 100%. The LCS, the drug concentration lethal to 50% of the cells, was calculated from the dose-response curve and used as measure of resistance. Results were considered evaluable in case of an optical density of ≥0.050 and ≥70% leukemic cells in the control wells (leukemic cells without drug), which assures reliable test results.T1-T6

The intra-assay (duplicates) and inter-assay (repeated testing of a sample) variation in LCS values is less than 1 dilution step for each drug. The median coefficient of variation of the control wells was 5.2% (range, 0.9% to 15.3%).

DNA ploidy and S-phase determination. Flow cytometric analysis of cellular DNA content and cell cycle distribution was performed with an pulse cytophotometer (ICP-11, Thye AG, Göttingen, Germany) on ethidium bromide-stained cells as described previously.6 The DNA index was defined as the modal DNA content of leukemic cells compared with that of reference normal lymphocytes. Patients were divided in hyperdiploid (DNA index, ≥1.16 and ≤1.35) and nonhyperdiploid (DNA index, <1.16 or >1.35). We thus attempted to exclude near-triploid and near-tetraploid cases (with a DNA index of ≥1.35) from the hyperdiploid group, because these patients have a less favorable prognosis.7 The percentage of S-phase cells was determined by planimetry of DNA cell readings, and was expressed as a percentage of all cells, irrespective of DNA content.

Statistics. Computer equipment was provided by Olivetti Nederland BV (Leiden, The Netherlands). The Wilcoxon’s ranking test for unpaired data and the χ² test were used for two-tailed testing at a level of significance of .05. The Spearman’s rank correlation test (parameter ρ) was used to study the relation between the percentage of S-phase cells and LC50 values.

RESULTS

Sixteen (22%) of the 74 patients had hyperdiploid ALL cells and 58 patients had nonhyperdiploid ALL cells. Patient characteristics of the two groups are shown in Table 1. These characteristics did not differ significantly, except that hyperdiploidy was more frequent in male patients (P = .04). However, for 14 nonevaluable cases, the white blood cell counts (WBC) of the 9 hyperdiploid cases were lower (median, 6.2 × 10⁹/L; range, 3.2 to 41.9 × 10⁹/L) than those of the 5 nonhyperdiploid cases (median, 23.8 × 10⁹/L; range, 4.8 to 89.3 × 10⁹/L). Patient characteristics other then WBC did not differ between the ploidy groups in these nonevaluable cases. The MTT assay results of these 14 samples were not evaluable because of an optical density of less than 0.050 (n = 7) or because of less than 70% of leukemic cells in the control wells (n = 7). The failure rate was thus significantly (P = .001) higher in hyperdiploid c-ALL samples (9/25) than in nonhyperdiploid c-ALL samples (5/63). In agreement with this observation, we found that the control cell survival in the successfully tested samples was lower (P = .003) in hyperdiploid samples (median, 46%; range, 26% to 69%) than in nonhyperdiploid samples (median, 68%; range, 23% to 135%). Similarly, the percentage of leukemic cells in control wells after 4 days of culture was lower (P = .005) in hyperdiploid samples (median, 80%; range, 71% to 95%) than in nonhyperdiploid samples (median, 92%; range, 71% to 99%). At the start of culture, these median percentages were both 94%.

The hyperdiploid and nonhyperdiploid c-ALL samples showed a large overlap of individual LC50 values for all drugs, except for 6MP. Hyperdiploid c-ALL samples were significantly more sensitive to 6MP (median, 9.0-fold; P = .000003), to 6TG (1.4-fold; P = .023), to ARA-C (1.8-fold; P = .016), and to ASP (19.5-fold; P = .022) than were nonhyperdiploid c-ALL samples. Figure 1 shows these differences for individual patients. In addition to these significant differences, hyperdiploid c-ALL samples were not significantly more sensitive to the structurally related drugs PRD (6.3-fold) and DXM (8.6-fold). For the remaining drugs, small and nonsignificant differences were observed (Table 2). In some samples, not all drugs could be tested because of the lack of material. However, 6MP and 6TG were successfully tested in the same specimens. Moreover, an analysis including only those samples in which all three antimetabolites (6MP, 6TG, and ARA) were successfully tested gave results very similar to those shown in Table 2 (data not shown).
The median percentage of S-phase cells was 8.5% in 13 hyperdiploid c-ALL samples (range, 4.2% to 20%) and 5.7% (range, 0.2% to 30%) in the nonhyperdiploid c-ALL samples ($P = .05$; Fig 2). There was no significant correlation (all $P$ values $> .20$) between the percentage of S-phase cells and LC50 values for PRD ($P = .04$), DXM ($P = .04$), VCR ($P = .15$), VDS ($P = .03$), DNR ($P = .01$), DOX ($P = .12$), MIT ($P = .20$), TEN ($P = .08$), ASP ($P = .10$), 6TG ($P = .05$), and ARA-C ($P = .06$). Only for 6MP was a statistically borderline significant correlation observed ($P = .30$; $P = .05$).

**DISCUSSION**

DNA hyperdiploidy is strongly and independently associated with a good prognosis in childhood ALL. The reason for this prognostic significance is largely unknown, but it may be related to differences in cellular drug resistance. Cellular drug resistance is one of the main determinants of the clinical outcome after chemotherapy, together with the pharmacokinetics of the administered drugs and with the regrowth or relapse potential of minimal residual cells. Recently, Whitehead et al reported that lymphoblasts of 13 children with hyperdiploid ALL accumulated higher levels of methotrexate polyglutamates (MTXPG) than those in B-lineage lymphoblasts of 34 children with other ploidy. The investigators suggested that these high levels may increase the cytotoxicity of MTX. Previously, the same investigators had reported that children with B-lineage ALL, whose lymphoblasts accumulated high levels of MTXPG in vitro, had a better prognosis than did children with lower levels. Otherwise, Pinkel reported in 1987 already that hyperdiploid c-ALL had a high cure rate with methotrexate and mercaptopurine.

To the best of our knowledge, the present study is the first to report on a direct relation between DNA ploidy and cellular drug resistance in childhood ALL. Resistance to 12 drugs, commonly used in the treatment of this disease, was investi-
gated in samples of 74 children with newly diagnosed c-ALL. Hyperdiploidy is found mainly in this immunophenotypic subgroup.\textsuperscript{24,25} An analysis stratified for immunophenotype is also indicated because immunophenotype itself is related to cellular drug resistance in childhood ALL.\textsuperscript{26} MTX was not included in the panel because this drug shows no dose-dependent cytotoxicity in ALL patient samples in nonclonogenic cell culture drug resistance assays. A possible explanation for this is described elsewhere.\textsuperscript{27} Hyperdiploid c-ALL samples were more sensitive to all antimetabolites tested (6MP, 6TG, and ARA-C) than were nonhyperdiploid c-ALL samples. Hyperdiploid samples were also more sensitive to ASP. Resistance to each of the other drugs did not differ significantly between both ploidy groups. However, it should be noted, that a limited number of samples has been tested, especially in the hyperdiploid group. Some of the nonsignificant differences might be clinically relevant and need confirmation in a larger study.

The reason for the relative sensitivity of hyperdiploid c-ALL cells to antimetabolites is unknown. It has been attributed to a higher percentage of S-phase cells in hyperdiploid than in nonhyperdiploid ALL.\textsuperscript{4} In the present study, the percentages of S-phase cells in the hyperdiploid c-ALL samples were higher than those in the nonhyperdiploid c-ALL samples. However, in a limited number of samples (up to 38), there was no significant and strong relation between the percentage of S-phase cells and in vitro resistance to any of the drugs tested.

It has been suggested that hyperdiploid ALL cells are more sensitive to glucocorticoids, because of their tendency towards terminal differentiation.\textsuperscript{4} Both in vitro and in vivo sensitivity to glucocorticoids is associated with a favorable prognosis.\textsuperscript{13,28,29} We found indeed that hyperdiploid c-ALL samples were more sensitive to PRD (median, 6-fold) and DXM (median, 9-fold) than were nonhyperdiploid samples. However, the differences were not statistically significant. The difference in resistance to ASP was significant, with hyperdiploid c-ALL cells being a median of 19.5-fold more sensitive. It seems of interest to study whether there are differences in asparagine synthetase between hyperdiploid and nonhyperdiploid c-ALL cells.

Hyperdiploid c-ALL samples were a median of 9.0-fold more sensitive to 6MP, but were only 1.4-fold more sensitive to 6TG than were nonhyperdiploid c-ALL samples. This discrepancy between 6MP and 6TG, two closely related thiopurines, is interesting. Both drugs are intracellularly metabolized into thioguanine nucleotides, which are subsequently incorporated in DNA and RNA. However, the conversion of 6MP requires two enzymes more than the conversion of 6TG, namely inosinate (IMP) dehydrogenase and guanosine synthetase. The rate-limiting enzyme is IMP dehydrogenase.\textsuperscript{30} In addition, both 6TG and 6MP are catabolized by thiopurine methyltransferase, but this enzyme has a higher affinity for 6MP.\textsuperscript{31} It is tempting to speculate that hyperdiploid and nonhyperdiploid c-ALL cells differ in the activity of one or more of these enzymes. The favorable prognostic value of hyperdiploidy is associated with nonrandom numerical chromosome gains, including chromosomes 4, 6, 10, and 21.\textsuperscript{32,33} The genes encoding for the enzymes involved in the intracellular metabolism of antimetabolites may localize to these chromosomes. However, an extensive literature

### Table 2. Relationship Between DNA Ploidy Determined by Flow Cytometry and Cellular Drug Resistance Determined With the Colorimetric MTT Assay in Untreated Childhood c-ALL

<table>
<thead>
<tr>
<th>Drug</th>
<th>DNA Ploidy</th>
<th>LC50 Values (µg/mL, ASP IU/mL)</th>
<th>Median</th>
<th>Range</th>
<th>N</th>
<th>P-Values</th>
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</thead>
<tbody>
<tr>
<td>PRD</td>
<td>Nonhyperdiploid</td>
<td>2.20 (0.05-1500)</td>
<td>51</td>
<td>.17</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Hyperdiploid</td>
<td>0.35 (0.05-134)</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DXM</td>
<td>Nonhyperdiploid</td>
<td>0.25 (0.003-6)</td>
<td>47</td>
<td>.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hyperdiploid</td>
<td>0.03 (0.002-12)</td>
<td>12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VCR</td>
<td>Nonhyperdiploid</td>
<td>1.48 (0.05-50)</td>
<td>53</td>
<td>.63</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hyperdiploid</td>
<td>0.72 (0.09-38.3)</td>
<td>13</td>
<td></td>
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<tr>
<td>VDS</td>
<td>Nonhyperdiploid</td>
<td>2.59 (0.05-50)</td>
<td>40</td>
<td>.35</td>
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<tr>
<td></td>
<td>Hyperdiploid</td>
<td>1.56 (0.05-37.8)</td>
<td>8</td>
<td></td>
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<tr>
<td>DNR</td>
<td>Nonhyperdiploid</td>
<td>0.12 (0.003-1.3)</td>
<td>52</td>
<td>.32</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Hyperdiploid</td>
<td>0.13 (0.002-1.1)</td>
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<td></td>
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<tr>
<td>DOX</td>
<td>Nonhyperdiploid</td>
<td>0.39 (0.10-1.3)</td>
<td>41</td>
<td>.34</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hyperdiploid</td>
<td>0.33 (0.06-1.2)</td>
<td>11</td>
<td></td>
<td></td>
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<tr>
<td>MIT</td>
<td>Nonhyperdiploid</td>
<td>0.06 (0.001-1.0)</td>
<td>42</td>
<td>.65</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hyperdiploid</td>
<td>0.05 (0.001-1.0)</td>
<td>12</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>TEN</td>
<td>Nonhyperdiploid</td>
<td>0.32 (0.12-3.3)</td>
<td>41</td>
<td>.21</td>
<td></td>
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<tr>
<td></td>
<td>Hyperdiploid</td>
<td>0.25 (0.14-3.0)</td>
<td>12</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>ASP</td>
<td>Nonhyperdiploid</td>
<td>0.39 (0.002-10)</td>
<td>48</td>
<td>.022</td>
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<tr>
<td></td>
<td>Hyperdiploid</td>
<td>0.02 (0.002-8.2)</td>
<td>12</td>
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<tr>
<td>6MP</td>
<td>Nonhyperdiploid</td>
<td>216.7 (15.6-500)</td>
<td>47</td>
<td>.000003</td>
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</tr>
<tr>
<td></td>
<td>Hyperdiploid</td>
<td>24.2 (15.6-42.8)</td>
<td>12</td>
<td></td>
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</tr>
<tr>
<td>6TG</td>
<td>Nonhyperdiploid</td>
<td>7.7 (1.6-50)</td>
<td>47</td>
<td>.023</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hyperdiploid</td>
<td>5.7 (2.2-11.0)</td>
<td>12</td>
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<tr>
<td>ARA-C</td>
<td>Nonhyperdiploid</td>
<td>0.51 (0.07-2.5)</td>
<td>45</td>
<td>.016</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hyperdiploid</td>
<td>0.29 (0.03-2.5)</td>
<td>14</td>
<td></td>
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</tr>
</tbody>
</table>

Cases were classified as hyperdiploid if the DNA index was 1.16 to 1.36 and as nonhyperdiploid if the DNA index was <1.16 or >1.35.
search did not show the chromosomal locations of the enzymes mentioned above.

One concern in our study is that the success rate of the MTT assay was lower in hyperdiploid samples. Moreover, the control cell survival and the percentage of leukemic cells after 4 days of culture were lower in successfully tested hyperdiploid samples than in nonhyperdiploid samples. This finding is in agreement with preliminary results presented by Campana et al., who reported that hyperdiploid ALL cells were more likely to die in vitro, via induction of apoptosis. Thus, it may be that hyperdiploid c-ALL samples are actually even more drug sensitive than are nonhyperdiploid c-ALL samples than our study shows, because leukemic cells that die spontaneously (not drug-induced) by apoptosis may also be relatively sensitive to the cytotoxicity of anticancer agents. Most anticancer agents appear to kill malignant cells by apoptosis.

We conclude that hyperdiploid c-ALL samples are more sensitive to antimetabolites (especially to 6MP) and to ASP than are nonhyperdiploid c-ALL samples. This difference may contribute to the more favorable prognosis associated with DNA hyperdiploidy. The observation of other investigators that hyperdiploid ALL cells, compared to ALL cells of other ploidy, accumulate higher levels of MTXPG and therefore are likely to be more sensitive to MTX supports the general conclusion that the prognostic significance of DNA ploidy in childhood ALL is most likely to be mainly explained by its relation with antimetabolite resistance.

ACKNOWLEDGMENT


REFERENCES

6. Secker-Walker LM, Lawler SD, Hardisty RM: Prognostic im-


Favorable prognosis of hyperdiploid common acute lymphoblastic leukemia may be explained by sensitivity to antimetabolites and other drugs: results of an in vitro study

GJ Kaspers, LA Smets, R Pieters, CH Van Zantwijk, ER Van Wering and AJ Veerman