Detection of Trisomy 12 in Chronic Lymphocytic Leukemia by Fluorescence In Situ Hybridization To Interphase Cells: A Simple and Sensitive Method

By John Anastasi, Michelle M. Le Beau, James W. Vardiman, Anthony A. Fernald, Anthony A. Larson, and Janet D. Rowley

Trisomy 12 is the most common cytogenetic abnormality in chronic lymphocytic leukemia (CLL), and a number of studies have suggested that it may be an adverse prognostic indicator. We have evaluated the usefulness of fluorescence in situ hybridization with a chromosome 12-specific probe as a simple means for detecting trisomy 12 in interphase cells. Forty cases of B-cell CLL previously studied with conventional cytogenetic techniques were analyzed with a biotinylated probe to the centromeric region of chromosome 12. Thirty of these retrospective cases could be reevaluated with in situ hybridization. Our analysis showed three hybridization signals (ie, trisomy 12) in interphase cells from seven of seven cases found previously to have trisomy 12. Trisomy 12 was also detected in five additional cases: in one case thought to have a normal karyotype, in two cases that had been inadequate for routine cytogenetic analysis, and in two cases that had been found to have an abnormal karyotype without trisomy 12. In a prospective series of 20 newly accrued CLL cases, all cases were analyzed successfully by in situ hybridization and six (30%) showed trisomy 12. We were able to perform the analysis on routinely prepared and previously Wright-stained peripheral blood smears. We conclude that fluorescence in situ hybridization is a simple means for the detection of trisomy 12 in CLL. The technique is more sensitive than conventional cytogenetic analysis and would be a useful tool in clinical studies.

© 1992 by The American Society of Hematology.

MATERIALS AND METHODS

Patient specimens. The retrospective study was performed on cells from a series of B-cell CLL cases that we had previously studied with conventional cytogenetic techniques. The series included seven cases shown to have trisomy 12, nine with clonal abnormalities other than trisomy 12, eight with nonclonal abnormalities, seven with normal chromosomes, and nine that had been inadequate because no metaphase cells were seen. Cells from this series of cases had been collected from peripheral blood, bone marrow aspirates, bone core biopsies, and/or lymph node samples. Details regarding the selection of patients, morphology, clinical data, and preparation of specimens are described in the previous report. Specimens that had been processed for conventional cytogenetic studies had been fixed in methanol:glacial acetic acid (3:1 vol:vol) and had been stored at -20°C for up to 12 years.

The prospective study was performed on peripheral blood specimens from 20 patients with CLL. Red blood cells (RBCs) in EDTA-anticoagulated blood were lysed with ammonium chloride (0.16 mol/L) in potassium carbonate (7.2 mmol/L), and EDTA (1 mmol/L) (pH 7.3). The resulting preparation was incubated with hypotonic potassium chloride (0.075 mol/L) for 8 minutes and then centrifuged. The pellets were resuspended and then fixed (three times) with methanol:glacial acetic acid (3:1 vol:vol). The fixed cells were stored at -20°C up to the time of hybridization. In selected cases, the analysis was performed on previously Wright-stained cells, as reported earlier.

Control specimens were obtained from five patients undergoing bone marrow harvest for autologous bone marrow transplantation. The primary malignancies of these patients were nonhematopoietic, and the bone marrows had been shown to be free of tumor. The specimens were processed in a manner similar to that described for the prospective study. Three hundred cells were scored for each specimen. To determine if long-term storage affected the hybridization, we also studied 10 specimens that had been processed for conventional cytogenetics and stored in fixative at -20°C for 5 to 12 years. These specimens were from patients with nonmalignant conditions; those cases that were analyzed showed only normal metaphase cells.

Hybridization. Hybridization was performed on cells dropped onto glass slides that were then dried in an oven for 1 to 2 hours at...
TRISOMY 12 IN CLL

60°C. The hybridization methods were similar to those described previously. The probe used was a biotinylated DNA probe specific for the α-satellite repeat sequences in the centromeric region of chromosome 12 (Oncor, Gaithersburg, MD). The hybridized probe was detected with fluorescein-conjugated avidin (Vector Laboratories, Burlingame, CA).

Analysis was performed by enumeration of hybridization signals in 100 to 300 cells. We considered those cells that had either no signal or an ambiguous signal as unanalyzable and combined their number when scoring. The percentage of cells with three signals was calculated from the total number of analyzable cells. When fewer than 50 cells could be scored successfully, we considered the case inadequate. In cases 8 through 40 of the retrospective series we searched for metaphase cells in the in situ preparations. When mitoses were found to contain a number of signals other than two, signal or an ambiguous signal as unanalyzable and combined their number when scoring in 100 to 552.

Survival curves. Survival curves comparing patients with and without trisomy 12 were first plotted for data from the conventional metaphase analysis and then for data from the interphase study. Survival was measured from the date of diagnosis to the date of death or to the date of last follow-up. We studied cases 15 and 17 to compare interphase with metaphase cytogenetics, but these were excluded from the survival curves because the patients had prolymphocytic leukemia without antecedent CLL.

RESULTS

Controls. Analysis of the five freshly obtained control cases demonstrated 0, 1, 2, and 4 hybridization signals in an average of 0.7%, 5.6%, 93.1%, and 0.1% of cells, respectively (Table 1A). In these control specimens, three signals, which were considered as an indication of "false trisomy," were seen in 0.5% ± 0.3% (mean ± 1 SD) of the cells. These data are consistent with the results of others. Thus, the finding of three signals in 2% or more of the cells of test specimens was considered sufficient for indicating trisomy 12. Two of the 10 archived specimens used as controls were not analyzable due to degeneration of the nuclei. However, in the remaining eight the results were similar to the fresh specimens in that the percentage of cells with "false trisomy" was not increased. The number of nuclei that could not be scored in these specimens was greater (see Table 1B) and appeared to correlate with increasing storage time.

Retrospective study. Hybridization in the retrospective series was inadequate in 10 cases (see Table 2). In these cases there were either insufficient cells for analysis or uninterpretable results because of marked degeneration of nuclei in the stored samples. Populations of cells with three hybridization signals were detected in each of the seven cases in which trisomy had been detected cytogenetically (Table 2 and Fig 1).

Among the nine cases that had clonal abnormalities other than trisomy 12, numerical abnormalities of chromosome 12 were found in two. In one of these (case 12), trisomy 12 was detected in 24% of interphase cells (Fig 2A), but was also present in six of seven metaphase cells seen on the same in situ hybridization preparation (Fig 2B). Review of the karyotype and correlation with the in situ findings demonstrated that the extra copy of chromosome 12 was a ring chromosome of previously undetermined origin (Fig 2C). The ring chromosome was present in all 21 abnormal metaphase cells of this patient analyzed in the cytogenetic study.

In the second of these cases (case 10), three hybridization signals were noted in 10% of cells, and 4, 5, or 6 signals were seen in an additional 26% (Fig 3A and B). This suggested that trisomy 12 occurred in both hyper-diploid and hyper-tetraploid clones. Additional hybridization with a probe directed to the centromeres of the X chromosome and of chromosome 17 confirmed the presence of a tetraploid population accounting for 30% of the total number of cells. The findings from interphase analysis could not be correlated with the karyotype in this case because we could not identify any metaphase cells in the material remaining from this patient. In the initial cytogenetic analysis of this case, 103 slides from 18 different cultures of both bone marrow and peripheral blood were scored. Of 23 cells karyotyped, 11 cells had an abnormal karyotype (Table 2, footnote †). Only one of the metaphase cells was tetraploid, and none had any unidentified marker chromosomes.

Among the nine cases that initially had been found to be inadequate for karyotyping, five were analyzable by the in situ method. Two cases with trisomy 12 (cases 32 and 35) were identified, and the abnormal cells constituted 44% and 59% of the total number of cells, respectively. Of the six cases that had shown only normal metaphase cells initially, one (case 28) showed trisomy 12 in 59% of cells. Metaphase cells from the same sample of peripheral blood from this case had shown only 46,XY cells when studied by conventional techniques.

Survival. With the exception of one patient who was diagnosed in 1947, all patients had the diagnosis of CLL made between 1966 and 1982. The median follow-up time from the date of diagnosis was 51 months (range 6 to 552.
trisomy 12 (Fig 4B). However, statistical analysis did not show a statistically significant difference between the two curves ($P = .14$).

**Prospective study.** In the prospective series (Table 3), all cases were analyzable. Trisomy 12 was detected in 6 of the 20 cases (30%). Trisomic cells ranged from 48% to 72% of the total number of cells in each case. The percentage of cells with zero or uninterpretable signals (mean, 0.7%) was lower than that noted in the stored specimens (mean, 11.0%). This was similar in the controls and was not...
TRISOMY 12 IN CLL

Figs 1 through 3. Fig 1. An interphase cell from case 5, showing three hybridization signals that indicate trisomy 12. An extra chromosome 12 had been detected by conventional analysis of metaphase cells in this case. Fig 2A. Two interphase cells from case 12, each with three hybridization signals or trisomy 12. An extra copy of chromosome 12 had not been detected by metaphase analysis in this case. Fig 2B. A metaphase cell from the same preparation as (A). Three hybridization signals are present; two are in medium-sized chromosomes (small arrows) that are consistent with chromosome 12, and the third is in a small chromosome (large arrow) consistent with the ring chromosome seen in metaphase cells. Fig 2C. Partial karyotype (quinacrine-stained) from case 12 demonstrating the two copies of chromosome 12 and the initially unidentified ring chromosome. Fig 3A and B. Cells with three and six hybridization signals, respectively, from case 10. The extra copies of chromosome 12 detected by interphase cytogenetic analysis in this case could not be correlated with the karyotype (see Table 2, footnote, and Discussion).

unexpected in that degradation of cell nuclei occurs during storage.

In two cases showing trisomy 12 in the prospective series, we demonstrated that the hybridization could be performed on the previously Wright-stained peripheral blood smear from the clinical laboratory (Fig 5, A and B) and that trisomy 12 could be detected as easily in each case. However, we found that the hybridization was successful only if the stained slides were less than 3 days old.

DISCUSSION

The results of this study demonstrate that fluorescence in situ hybridization with a probe to chromosome 12 is a more sensitive method for detecting trisomy 12 in CLL than is conventional cytogenetic analysis of metaphase cells. The five additional cases detected represent an increase of 12% or 17% (5 of 40 total cases or 5 of 30 analyzable cases), for an overall incidence of trisomy 12 of 30% or 40% (12 of 40 total cases or 12 of 30 analyzable cases). The inability to study all cases or all cells in each case in the retrospective series was probably attributable to cell deterioration as a result of up to 12 years of storage in fixative. This may have led to underestimation of trisomy 12. All of the freshly prepared specimens from the prospective cases were analyzable.

The sensitivity of detecting trisomy 12 was increased over that of conventional cytogenetic analysis because we were able to detect the trisomy in cases in which conventional cytogenetics was unable to detect the abnormality. Of the six cases analyzable by in situ hybridization that had shown a normal karyotype, one demonstrated trisomy 12 in 59% of interphase nuclei; 32 metaphase cells had been identified in this case, and 25 were analyzed in detail. The discrepancy between the results of metaphase and interphase analyses in this case corroborates the work of Knuutila et al,9 which demonstrated that the normal metaphase cells in some cases of CLL are dividing, reactive T cells that are not a part of the neoplastic clone. It also corroborates the recent report by Perez Losada et al10 in which 2 of 13 cases of B-cell CLL with a normal karyotype were found to have trisomy 12 by interphase cytogenetic analysis.

In the present study, the utility of interphase cytogenetics was further illustrated as we were able to demonstrate trisomy 12 among cases that had been inadequate for cytogenetic study and among those that had an abnormal karyotype without trisomy 12. Of five cases inadequate for karyotype analysis, two showed trisomy 12 by interphase study. Of eight cases with abnormal karyotypes without trisomy 12, two cases (cases 10 and 12) showed populations of cells with three hybridization signals. In case 12, in situ hybridization detected trisomy 12 in interphase cells and demonstrated, in addition, that a ring chromosome, the chromosomal origin of which was initially not identified, actually represented the extra copy of chromosome 12. The extra copies of chromosome 12 noted in case 10 could not be correlated with the karyotype because no metaphase cells were found in the material remaining from this patient.

An interesting observation regarding these last two cases is that both exhibited a complex karyotype, including a t(14;19) (q32;q13). Thus, both cases had the t(14;19) and trisomy 12. These two chromosomal abnormalities were
Fig 4. Survival curves for patients with and without trisomy 12 in the retrospective series. Survival was determined from the date of diagnosis. Panel (A) was plotted using data from the initial metaphase analysis. There is no difference between the two patient groups (+12 n = 7, no +12 n = 22, P = .32). Panel (B) was constructed with data from the interphase analysis. In this plot, the two curves have moved apart, illustrating a trend for poorer survival in patients with trisomy 12 (+12 n = 12, no +12 n = 17, P = .14).

also detected cytogenetically in a third case (case 5). We know of five other cases of CLL with a t(14;19), three of which have also trisomy 12 (J.D.R., unpublished observations, April 1990). Thus, the t(14;19) appears to be commonly associated with trisomy 12. The nature of the relationship of the two abnormalities is a subject for additional study.

Whether the increased incidence of trisomy 12 detected by interphase cytogenetic analysis will have a bearing on the clinical significance of trisomy 12 is open to question. When we reviewed our survival data in light of the interphase analysis, we found a trend indicating poorer survival associated with trisomy 12, whereas no trend had been noted with data from the initial conventional cytogenetic study. Thus, similar retrospective interphase cytogenetic analyses of previously published large series might further clarify the clinical significance of trisomy 12 in CLL. Interphase cytogenetics would also facilitate prospective analyses of large series of CLL patients. Our illustration that the analysis can be performed on previously stained slides demonstrates how easily the technique can be integrated into the clinical laboratory assessment of such patients.

Although in situ hybridization with a probe to a particular chromosome may be a more sensitive means than

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Total Analyzed</th>
<th>0*</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>% +12</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>200</td>
<td>3</td>
<td>11</td>
<td>186</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2.</td>
<td>200</td>
<td>4</td>
<td>14</td>
<td>182</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3.</td>
<td>200</td>
<td>0</td>
<td>9</td>
<td>191</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4.</td>
<td>200</td>
<td>0</td>
<td>7</td>
<td>97</td>
<td>96</td>
<td>0</td>
<td>48</td>
</tr>
<tr>
<td>5.</td>
<td>300</td>
<td>0</td>
<td>10</td>
<td>139</td>
<td>151</td>
<td>0</td>
<td>53</td>
</tr>
<tr>
<td>6.</td>
<td>200</td>
<td>0</td>
<td>0</td>
<td>56</td>
<td>144</td>
<td>0</td>
<td>72</td>
</tr>
<tr>
<td>7.</td>
<td>200</td>
<td>2</td>
<td>10</td>
<td>188</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8.</td>
<td>200</td>
<td>2</td>
<td>10</td>
<td>188</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9.</td>
<td>200</td>
<td>0</td>
<td>10</td>
<td>189</td>
<td>1</td>
<td>0</td>
<td>0.5†</td>
</tr>
<tr>
<td>10.</td>
<td>200</td>
<td>0</td>
<td>10</td>
<td>52</td>
<td>136</td>
<td>0</td>
<td>69</td>
</tr>
<tr>
<td>11.</td>
<td>300</td>
<td>2</td>
<td>12</td>
<td>281</td>
<td>5</td>
<td>0</td>
<td>1.6†</td>
</tr>
<tr>
<td>12.</td>
<td>200</td>
<td>2</td>
<td>5</td>
<td>74</td>
<td>119</td>
<td>0</td>
<td>59</td>
</tr>
<tr>
<td>13.</td>
<td>200</td>
<td>0</td>
<td>13</td>
<td>185</td>
<td>2</td>
<td>0</td>
<td>1.0†</td>
</tr>
<tr>
<td>14.</td>
<td>200</td>
<td>0</td>
<td>11</td>
<td>189</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>15.</td>
<td>200</td>
<td>0</td>
<td>7</td>
<td>80</td>
<td>113</td>
<td>0</td>
<td>56</td>
</tr>
<tr>
<td>16.</td>
<td>200</td>
<td>1</td>
<td>14</td>
<td>185</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>17.</td>
<td>200</td>
<td>0</td>
<td>10</td>
<td>189</td>
<td>1</td>
<td>0</td>
<td>0.5†</td>
</tr>
<tr>
<td>18.</td>
<td>200</td>
<td>0</td>
<td>10</td>
<td>190</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>19.</td>
<td>200</td>
<td>0</td>
<td>5</td>
<td>195</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>20.</td>
<td>200</td>
<td>0</td>
<td>9</td>
<td>190</td>
<td>1</td>
<td>0</td>
<td>0.5†</td>
</tr>
</tbody>
</table>

*Includes cells with no signal and cells with ambiguous signals.
†Not different from 2 SD of mean for controls.
conventional cytogenetic study for detecting a numerical abnormality of that chromosome, the interphase technique can provide only limited information about the karyotype. That is, other important chromosome anomalies would go undetected with interphase analysis directed only at a targeted abnormality. Thus, it may be best to use this method in conjunction with conventional analysis. However, newer techniques are making it possible to use a number of probes together and thus to score simultaneously for multiple aberrations, including translocations as well as numerical abnormalities.20

From this work we conclude that in situ hybridization with a probe to chromosome 12 is a simple means for the detection of trisomy 12 in CLL. Furthermore, we conclude that it is a more sensitive method for detection of this numerical abnormality than is conventional cytogenetic analysis of metaphase cells. The technique has great potential in identifying patients with trisomy 12 for larger clinical correlations, for new clinical trials, and for additional cytogenetic and molecular studies.

ACKNOWLEDGMENT

The authors thank Dr Mary Lourdes Bird for her help with the survival data, Margie Isaacson for help with data management, Rosemarie Mick, MS, for statistical computations, and Elisabeth Lanzl for editing.

REFERENCES

Detection of trisomy 12 in chronic lymphocytic leukemia by fluorescence in situ hybridization to interphase cells: a simple and sensitive method

J Anastasi, MM Le Beau, JW Vardiman, AA Fernald, RA Larson and JD Rowley

Updated information and services can be found at:
http://www.bloodjournal.org/content/79/7/1796.full.html
Articles on similar topics can be found in the following Blood collections

Information about reproducing this article in parts or in its entirety may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:
http://www.bloodjournal.org/site/subscriptions/index.xhtml