Clonal analysis of progenitor cells by interphase cytogenetics in patients with acute myeloid leukemia and myelodysplasia

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Interphase cytogenetics was used to investigate the clonal origin of bone marrow (BM) cells, peripheral blood (PB) cells, and in vitro cultured progenitor cells of five patients with acute myeloid leukemia (AML) and myelodysplasia (MDS). A new in situ hybridization (ISH) technique was used to examine the origin of the progenitor cells. Two patients with, respectively, trisomy 8 and polyploidy as ISH marker were studied both at presentation and during remission. At presentation, the in vitro cultured clusters of both cases appeared diploid. Therefore, despite the abnormal growth patterns, the cultured progenitors could have been residual normal cells. Alternatively, they could have originated from a preleukemic clone with a normal karyotype. In both cases abnormal BM and/or PB cells (less than 6%) were detected with ISH during remission, indicating partially or completely clonal remissions in these patients. Both patients have relapsed. One patient with trisomy 10 as ISH marker was analyzed during myelodysplastic phase and after progression to AML. On both occasions, abnormally appearing clusters were cultured. However, only part of the clusters carried trisomy 10. The presence of a subclone characterized by trisomy 10 and an abnormally growing (pre)leukemic clone without trisomy 10 may explain this observation. Monosomy 1 and 17 were respectively used as ISH markers in two other AML patients. All in vitro cultured clusters carried the numerical abnormalities. Long-term liquid cultures of these leukemias were performed for 10–20 days. In both cases, no residual normal clonogenic cells could be detected. Therefore, the selective growth advantage of normal progenitor cells in long-term marrow cultures could not be demonstrated in these two patients with leukemia. This paper illustrates the usefulness of ISH to study the biology of AML at the clonogenic level during preleukemic phase, active disease, remission, and under in vitro culture conditions which allows individual analysis of large numbers of small aggregates and single cells in culture.

Keywords: acute myeloid leukemia; interphase cytogenetics; myelodysplasia; progenitor cells

Introduction

Acute myeloid leukemia (AML) may be considered as a clonal expansion of a single transformed cell.1,2 The origin of the leukemic clone may be either a multipotent hematopoietic stem cell (HSC) or a cell restricted to the granulocyte–monocyte pathway.1,2 In myelodysplastic syndrome (MDS), the abnormal clone is assumed to arise at stem cell level.3 In the majority of AML/MDS cases, progenitor cells can be cultured in vitro.4–6 The origin of these clonogenic cells may be difficult to assess, since colonies derived from (pre)leukemic progenitors are not always morphologically distinguishable from those derived from normal progenitors (7).

Several techniques have been developed to determine the clonal origin of hematopoietic cells. In heterozygous females, the X-chromosome-linked enzyme glucose-6-phosphate dehydrogenase (G6PD) or restriction fragment length polymorphisms (RFLP) or other X-chromosomal enzymes can be used as a genetic marker.1,2,8 Karyotyping and surface marker expression analysis offer alternative approaches.2,6,10 Progenitors of AML patients at presentation are usually derived from the malignant clone. Occasionally, the myeloid colonies appeared to originate from normal stem cells.7 During remission, in most patients restoration of nonclonal hematopoiesis and repopulation of the marrow by normal stem cells has been observed. Clonal remissions have also been observed3,10,11

The results obtained with long-term bone marrow culture (LTBMC) of BM cells from AML patients additionally support the presence of residual normal stem cells in leukemic patients during active disease. In most cases, leukemic colonies and clusters were no longer detectable after 1 to 4 weeks of culture.12–14 The few reports about progenitors in MDS suggest the existence of a transformed stem cell either with or without an abnormal karyotype.5,15

Recently, we described a method to determine chromosomal aberrations by ISH in myeloid progenitor cells cultured in agar.16,17 The present study investigates the genetic clonality of myeloid progenitor cells and nucleated cells in five patients with AML and MDS at different stages of their disease.

Patients and methods

Patient selection

Patients with de novo AML or AML after a myelodysplastic phase characterized by a numerical chromosomal aberration were selected for this study. AML and MDS were diagnosed according to the French–American–British classification.18,19 Patient 1 was assessed at diagnosis and in complete remission. He relapsed 6 months after the last analysis (May 1992). Patient 2 presented with a leukemoid reaction during an exacerbation of her ulcerative colitis. This was followed by a spontaneous remission of 5 months without any antileukemic therapy. Patient 3 was investigated during the myelodysplastic phase and after leukemic transformation. Patients 4 and 5 were studied at presentation of AML. The relevant clinical and hematological data are shown in Table 1.

Cell collection, cryopreservation and thawing

Bone marrow cells were collected, cryopreserved in liquid nitrogen and thawed as described in detail elsewhere.20 For ISH, cells were fixed in 70% ethanol (−20°C) and stored at −30°C until further use.
Table 1  Clinical data

<table>
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<tr>
<th>Case no.</th>
<th>Date</th>
<th>Disease status</th>
<th>Age (years)</th>
<th>Sex</th>
<th>WBC (10^3/l)</th>
<th>Hb (mmol/l)</th>
<th>Platelets (10^3/l)</th>
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<th>BM % blasts</th>
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<td>43</td>
<td>M</td>
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<td>6.8</td>
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<td>1</td>
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<td>34</td>
<td>F</td>
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<td>36</td>
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<td>9/87</td>
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<td></td>
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<tr>
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<td>M</td>
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<td>7.1</td>
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<td>12</td>
<td>12</td>
<td>33</td>
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<td>29</td>
<td>73</td>
<td>75</td>
<td>M2</td>
</tr>
</tbody>
</table>

M, male; F, female; WBC, white blood cells; Hb, hemoglobin; BM, bone marrow; FAB, French-American-British classification; NA, not available.

**Human recombinant growth factors**

Human recombinant interleukin 3 (IL-3) and human recombinant granulocyte–monocyte colony-stimulating factor (GM-CSF) were kindly donated by Sandoz (Uden, The Netherlands). Human recombinant granulocyte–colony-stimulating factor (G-CSF) was a kind gift from Behring (Marburg, Germany). Final concentrations of 40 ng/ml, 20 ng/ml, and 5 ng/ml, respectively, were used and resulted in plateau stimulation.

**Clonogenic assay (CFU-C)**

Cells were cultured in Iscove’s medium, supplemented with 20% FCS, 50 IU/ml penicillin, 50 µg/ml streptomycin, and 0.3% bacto-agar (Difco, Detroit, MI, USA). The cells were stimulated with the combination of IL-3, GM-CSF and G-CSF. Duplicates were cultured in 35 X 10 mm culture dishes (Costar, Cambridge, MA, USA) at 37° C in a fully humidified atmosphere containing 5% CO2. In order to render the cells better accessible for the DNA probes, a reduced volume of the culture (0.075 M KCl), the cells were exposed to a hypotonic solution (0.075 M KCl) for 15 min. Slides were prepared according to routine cytogenetic procedures. Karyotyping was performed using the GTG technique.

**Karyotyping**

Metaphase spreads were obtained from BM or PB cells after culturing in RPMI 1640 (Flow Laboratories, McLean, VA, USA) for 1 or 24 h. Colcemid was present during the last hour of the culture. Before fixation in methanol : glacial acetic acid (3 : 1), the cells were exposed to a hypotonic solution (0.075 M KCl) for 15 min. Slides were prepared according to routine cytogenetic procedures. Karyotyping was performed using the GTG technique.

**In situ hybridization**

ISH was performed on cell suspensions and dried agar cultures as described before. For good penetration of the DNA probes and antibodies, a proteolytic digestion step with pepsin (P7000; Sigma) was performed at a concentration of 100 µg/ml in 0.01 M HCl for 20 min at 37° C. The nuclei were post-fixed in 4% formaldehyde in PBS for 20 min at 4° C. Ten microliters of the hybridization mixture (60% formamide, 0.6 M NaCl 60 mM sodium citrate (2 x SSC), 10% dextran sulphate and a probe concentration of 1 ng/µl) was applied under a coverslip. Denaturation of target DNA and probe was carried out simultaneously at 70° C for 2.5 min in a moist chamber and hybridization was performed for 2–16 h at 37° C. Subsequently, the hybridized probes were detected by horseradish peroxidase (HRP)-conjugated avidin (Dakopatts, Glostrup, Denmark). Finally, the DNA probe was visualized with 0.5 mg/ml 3,3-diaminobenzidin tetrahydrochloride (DAB; Sigma), 0.65% imidazole (Merck), 0.015% H2O2 (Merck) at pH 7.8 in PBS. As controls, the samples were hybridized with a chromosome-specific DNA probe for which no chromosomal aberration was detected. In these controls the cells from suspension preparations demonstrated one ISH signal for a target chromosome in 5–10% of the cells, and three ISH signals in <2% of the cells. Of these suspension preparations, 200 nuclei per slide were counted. The agar preparations were evaluated by counting 100 single cells and approximately 50 aggregates. In these controls, at least 90% of the cells contained ISH signals. The percentages of cells containing the euploid number of ISH signals for the control DNA probe-targets varied between 85 and 90%.

**Liquid culture**

Cells were suspended in Iscove’s medium supplemented with 20% FCS, 50 IU/ml penicillin, 50 µg/ml streptomycin and a combination of IL-3, GM-CSF and G-CSF. The liquid cultures were maintained in an incubator (37° C, 5% CO2 and fully humidified atmosphere) for a period of up to 20 days. At regular intervals, part of the cells were collected, washed and used for clonogenic assay and ISH.
Results

Case 1

Presentation of AML: At presentation, 12 of 13 analyzed BM cell metaphases showed trisomy 8 by karyotyping (Table 2). With ISH, trisomy 8 was found in 71% of the BM cells. A leukemic growth pattern was observed with small clusters consisting of 5–25 immature myeloid cells. After hybridization with the probe for chromosome 8, disomy was found in all analyzed clusters, whereas 14% of the single cells in the semi-solid culture had trisomy 8.

Complete remission: In May 1991, karyotyping revealed only normal BM cells, whereas no metaphases could be detected in the PB. However, with ISH, 6% of the BM cells showed trisomy 8. Both BM and PB exhibited a normal in vitro growth pattern. The colonies appeared morphologically normal and were diploid when analyzed with the probe for chromosome 8. By contrast, in the BM culture, respectively 19 and 4% of the single cells in agar had trisomy and tetrasomy for chromosome 8, whereas the single cells of the PB cultures showed trisomy and tetrasomy 8 in 12 and 2% respectively.

In November 1991 only PB was analyzed. No metaphases were detected. ISH on cell suspension was not performed. A normal growth pattern was observed demonstrating disomy 8 in all aggregates, whereas part of the single cells in culture had trisomy 8 (7%).

Table 2 Bone marrow and blood (case nos 1, 2 and 3)

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Date</th>
<th>Disease status</th>
<th>Cell sample</th>
<th>Suspension Karyotype</th>
<th>ISH (%)</th>
<th>Semi-solid ISH colonies/clusters</th>
<th>ISH, single cells (%)</th>
<th>CFU-C&lt;sup&gt;c&lt;/sup&gt; colonies/clusters</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>2/88</td>
<td>AML</td>
<td>BM</td>
<td>46,XY/47,XY,+8 (n = 1/12)&lt;sup&gt;a&lt;/sup&gt; (92%)</td>
<td>trisomy 8 (71)</td>
<td>disomy 8 (n = 17)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>trisomy 8 (14)</td>
<td>0/89</td>
</tr>
<tr>
<td></td>
<td>5/91</td>
<td>CR</td>
<td>BM</td>
<td>46,XY (n = 32)</td>
<td>trisomy 8 (6)</td>
<td>disomy 8 (n = 21)</td>
<td>trisomy 8 (19)</td>
<td>99/34</td>
</tr>
<tr>
<td></td>
<td>5/91</td>
<td>CR</td>
<td>PB</td>
<td>no metaphases found</td>
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<td>disomy 8 (n = 18)</td>
<td>trisomy 8 (12)</td>
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<tr>
<td></td>
<td>11/91</td>
<td>CR</td>
<td>PB</td>
<td>no metaphases found</td>
<td>NA</td>
<td>disomy 9 (n = 18)</td>
<td>trisomy 8 (7)</td>
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<tr>
<td></td>
<td>9/87</td>
<td>AML</td>
<td>BM</td>
<td>polyploid (about 96 chromosomes, n = 6) (100%)</td>
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<td>disomy 1 (n = 38)</td>
<td>trisomy 1 (6)</td>
<td>4/5</td>
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<td>CR</td>
<td>BM</td>
<td>46,XX/+8 (n = 2/14) (13%)</td>
<td>trisomy 1 (2) tetrasomy 1 (3)</td>
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<td>BM</td>
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<td>disomy 10 (n = 6)</td>
<td>trisomy 10 (35)</td>
<td>7/116</td>
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<tr>
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<td>5/92</td>
<td>AML</td>
<td>BM</td>
<td>no metaphases found</td>
<td>trisomy 10 (16)</td>
<td>disomy 10 (n = 14)</td>
<td>trisomy 10 (17)</td>
<td>1/43</td>
</tr>
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<td></td>
<td></td>
<td>disomy 10 (n = 18)</td>
<td>trisomy 10 (37)</td>
<td>1/43</td>
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<td></td>
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<td></td>
<td></td>
<td>disomy 10 (n = 23)</td>
<td>trisomy 10 (7)</td>
<td>18/2160</td>
</tr>
</tbody>
</table>

<sup>a</sup>ISH, in situ hybridization; BM, bone marrow; PB, peripheral blood; CR, complete remission; NA, not analyzed.

Case 2

At leukemic presentation, all investigated BM metaphases appeared to be polyploid, containing about 96 chromosomes (Table 2). For ISH analysis, we applied the probe for chromosome 1 in this particular case.

'Leukemoid reaction': During 'leukemoid reaction', a normal karyotype was found in nine bone marrow metaphases (Table 2). In contrast, ISH revealed that 4% of the BM cells contained three or four ISH signals with the chromosome 1 probe. The number of colonies in the semi-solid culture was depressed. Morphology and size of the colonies appeared normal. All analyzed colonies were disomic, whereas 8% of the single cells in agar contained three or four ISH signals.

Presentation: During overt leukemia, when all analyzed BM metaphases were polyploid, tri- or tetrasomy 1 was found by ISH in 62% of the BM cells. Only very few colonies and clusters could be cultured in agar. They appeared to have normal morphology and size and were disomic when hybridized with the probe for chromosome 1. Sixty-six percent of the single cells in those cultures were tri- or tetrasomic.

Complete remission: In February 1988, both normal and polyploid cells were detected by cytogenetic analysis. ISH revealed tri- or tetrasomy 1 in 5% of the BM cells. Normal colony numbers were observed. Their morphology and size also appeared normal. All analyzed colonies were disomic,
whereas 2% of the single cells in the semi-solid culture were tri- or tetrasonic.

**Case 3**

**MDS:** Karyotyping of the BM revealed that eight of 32 analyzed metaphases had trisomy 10 (Table 2). With ISH, 26% of the BM cells had trisomy 10. In the PB cells, on which no karyotyping was performed, 14% of the cells showed trisomy 10 by ISH. Both BM and PB cells were growing as clusters consisting of 5–35 immature cells. Although morphologically not distinguishable, both clusters with and without trisomy 10 were observed by ISH in these cultures. The single cells in the BM and PB cultures demonstrated a trisomy for chromosome 10 in 35 and 37%, respectively.

**Presentation:** At leukemic presentation, no BM metaphases were detected. By ISH, 16% of the BM cells had trisomy 10. The clusters appeared morphologically identical to the clusters cultured during MDS phase. Trisomy 10 was found in seven of 30 clusters (Figure 1), whereas 8% of the single cells in culture showed trisomy 10.

In the following two cases bone marrow was cultured in liquid in the presence of IL-3, GM-CSF and G-CSF for up to 20 days to investigate whether residual normal progenitors had a growth advantage compared to the leukemic clone.

**Case 4**

**Presentation:** At leukemic presentation, karyotyping of BM cells revealed multiple abnormalities in almost all metaphases (Table 3). The probe for chromosome 1 was selected as a marker for ISH. Monosomy 1 was found in 71% of the BM cells. The in vitro growth pattern consisted of small clusters (5–20 cells) consisting of immature cells. All analyzed clusters and 88% of the single cells showed monosomy 1.

**Liquid culture:** Liquid culture for 10 days did not significantly influence the number of abnormal cells and clusters. Ninety-one percent of the cells in suspension showed monosomy 1. Again, only immature appearing small clusters could be cultured, and all showed monosomy for chromosome 1. The single cells in the semi-solid assay demonstrated a monosomy 1 in 91%.

**Case 5**

**Presentation:** Nine out of 10 metaphases showed multiple chromosomal abnormalities (Table 3). In this case, monosomy 17 was chosen as ISH marker. Monosomy 17 was detected by ISH in 96% of the BM cells. The clusters consisted of 5–25 immature cells and all were monosomic for chromosome 17. Moreover, monosomy 17 was found in 82% of the single cells in the semi-solid assay.

**Liquid culture:** The high plating efficiency allowed in liquid culturing of the leukemic cells for up to 20 days. Liquid culture cells and agar cultures (both single cells and clusters) were analyzed at regular intervals. During the whole period the proportion of abnormal cells in liquid remained essentially the same (95–99%). At any time, only aggregates with monosomy 17 were detected by ISH (see Figure 1a).

**Discussion**

Five patients with de novo AML or AML after a myelodysplastic phase and abnormal clones characterized by numerical chromosomal abnormalities were the subject of this study. In all cases, the chromosomal aberrations observed by cytogenetic analyses could also be detected by ISH. The percentage of abnormal cells was within the same range for both methods taking into account the following considerations. Conventional cytogenetic analysis examines fewer cells than ISH and this may result in an less accurate estimation of the real number of abnormal cells. Furthermore, conventional cytogenetic analysis is performed on dividing cells, which may lead to a selection for the abnormal subpopulation. Non-dividing interphase lymphocytes are included in the ISH analysis and cannot be analyzed by karyotyping. The lymphoid lineage is usually not a part of the abnormal clone and thus may account for the lower incidence of abnormal cells found with ISH.

The detection level of ISH analysis is more sensitive than conventional cytogenetic analysis, especially when trisomic or polyploid markers are used. The number of ISH signals is constant during the whole cell cycle, and in normal diploid cells the number of cells with four ISH signals is maximally 1%. The sensitivity level of three ISH signals is at the level of 2–4%. ISH analysis detected 4% abnormal cells during the ‘leukemoid’ reaction of patient 2, while the nine metaphases revealed 46 normal chromosomes. This result may be regarded as of borderline significance, but is further supported by the presence of 8% trisomic or tetrasonic cells in the agar culture of this bone marrow. Remission marrow of patient 1 contained only normal metaphases and 6% abnormal interphase cells as measured with ISH. This patient had persisting abnormal cells in culture 6 months later and consequently relapsed. Similar observations have been made in G6PD and RFLP studies.
The ISH marker for abnormal cells was not detected in the cells of the clusters of patients 1 and 2. In both cases the bone marrow and blood cultures contained single cells with numerical abnormalities. The clusters could be the progeny of normal progenitor cells which exhibit an abnormal growth pattern due to influences of adjacent leukemic cells. In the majority of reported cases, myeloid progenitors are part of the leukemic clone at presentation. However, in a few patients, normal CFU-GM have been detected.\textsuperscript{7} It is important to realise that the ISH method enables the evaluation of small clusters and single cells in the cultures in situ while the methods based on G6PD isoenzymes or genetic analysis require micromanipulation of the colonies. The latter analyses can only be performed on larger colonies and this may have influenced the results. If the clusters in cases 1 and 2 did originate from normal cells, then the in vitro growth pattern and morphology by itself are not sufficient to determine the origin of progenitor cells. An alternative explanation could be that these progenitor cells represent a part of the leukemic clone without the numerical aberration. This concept can be explained by a multistep leukemogenesis with a preleukemic stage; an early step causes clonal proliferation and a later step results in a chromosomal abnormality in descendants of these progenitors.\textsuperscript{8,11}

In case 3 the progression from MDS to AML could be observed. The abnormal but identically appearing clusters could be distinguished in two subpopulations: one consisting of progenitors with and the other without trisomy 10 (Table 2). No increase in the percentages of either BM cells or progenitor cells increased. This observation matches the theory that MDS arises from a transformed progenitor cell and progresses from a preleukemic state to overt leukemia by successive genetic changes.\textsuperscript{3,15} In that case, all cultured clusters belonged to a (pre)leukemic clone with abnormal growth characteristics, but only part of this clone obtained the chromosomal aberration trisomy 10. Patients 4 and 5 were selected because conventional cytogenetic analysis revealed a subpopulation of normal metaphases (Table 2). All cultured clusters from these patients contained the numerical ISH abnormality. This was in agreement with the observed leukemic growth pattern and undifferentiated appearance of the clusters, and confirmed the leukemic origin of these myeloid progenitors. Although in both cases part of the BM cell metaphase spreads and interphase nuclei appeared normal, no residual normal clonogenic cells could be detected after long-term culture in liquid. In the majority of reported cases, long-term marrow cultures provide a selective growth advantage for normal progenitor cells, but in some AML cases, as in patients 4 and 5, persistence of the leukemia has been observed.\textsuperscript{12-14}

Forty to 70\% of the AMLs and 25–50\% of the MDSs have numerical chromosomal abnormalities, indicating that a substantial number of patients with AML and MDS can be analyzed by ISH using centromere associated DNA probes.\textsuperscript{23,24} Moreover, probes against fusion genes, such as \textit{bcr-abl} gene, have become available.\textsuperscript{25} This may further increase the number of patients which can be studied by ISH techniques. By performing ISH on agar cultures, the cytological architecture remains intact. This enables individual analysis of large numbers of aggregates and single cells in the culture. Sample errors are avoided since removal of colonies is not necessary. In contrast to the conventional techniques, not only large colonies but also small clusters can be analyzed. Furthermore, it is less time consuming to investigate large numbers of aggregates. Therefore, ISH may help to clarify the complex biology of MDS and AML both during ‘full-blown’ situation and clinical remission. In addition, it can be applied to monitor in vitro culture systems. Additional studies are needed to explain the nature of small aggregates of some patients which have a normal diploid ISH signal.

### References


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<th>Case no.</th>
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<th>Suspension</th>
<th>Semi-solid</th>
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<td>monosomy 1 (n = 35)\textsuperscript{a}</td>
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<td>presentation</td>
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</table>

ISH, \textit{in situ} hybridization; NA, not analyzed.

\textsuperscript{a}Number of mitosis.

\textsuperscript{b}Number of analyzed aggregates.

\textsuperscript{c}Number of CFU-C per 2 × 10\textsuperscript{6} NC.
23 Yunis JJ. Recurrent chromosomal defects are found in most patients with acute non-lymphocytic leukemia. Cancer Genet Cytogenet 1984; 11: 125-137.