Long-Term and Dependent Growth of Human Leukemic Blast Cells with Granulocyte-Macrophage Differentiation in Vitro

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ABSTRACT

A clonal growth of leukemic cells from the bone marrow of a patient with acute myeloid leukemia was observed in vitro for more than 20 months. Cytochemical and electron microscopic studies of the cells growing in vitro demonstrated that they were blast cells, differentiated granulocytes, and macrophages. They showed complete dependence on granulocyte-macrophage colony-stimulating factor for colony formation in agar. In addition to the presence of granulocytic colonies, some showed granulocyte-macrophage characteristics, suggesting that bipotential cells were also involved in long-term growth. Initially, they showed localized proliferation on or around giant fibroblast-like cells. Even after constant growth was established, attempts to transfer these cells were unsuccessful, and their growth was confined to the original flasks. These observations seem to indicate that their growth was not autonomous but dependent on the adherent cells in the flasks. This was also supported by a coculture experiment in which the cells were demonstrated to proliferate for 4 months only in the presence of normal bone marrow particles and bone marrow particle-derived feeder layers. These results suggest that, in some cases, long-term growth of leukemic cells can be induced in vitro by the cocultivation of bone marrow stromal cells.

INTRODUCTION

Numerous attempts have been made to establish human leukemic cell lines. Although there have been many reports describing the establishment of lymphoid cell lines, attempts to obtain myeloid cell lines have met a lot of difficulties until quite recently. Lozzio and Lozzio (18) first established a myeloid cell line (K 562) from a patient with chronic myeloid leukemia in blast crisis; the cells were proved recently to differentiate along the erythroid pathway (2, 14). Additionally, 2 myeloid cell lines, HL-60 and KG-1, with the capacity to differentiate along macrophage and granulocytic pathways have been reported (7, 15). Cells from each of these cell lines have been shown to proliferate autonomously in vitro. On the contrary, proliferation of myeloid leukemic cells in the primary culture requires, in most cases, certain factors including GM-CSF2 (13), phytohemagglutinin (9) or conditioned medium of lymphocytes stimulated with phytohemagglutinin (20), and conditioned medium of fibroblasts (10). These results suggest that additional changes in the original leukemic cells may be indispensable for their autonomous proliferation in vitro.

Recently, Dexter et al. (8) have introduced a new method with which prolonged proliferation and differentiation of normal murine bone marrow cells can be induced in vitro. Their growth was found to be dependent on adherent cells in the flask prepared by the previous seeding of bone marrow cells. Similar systems may have the potential to culture human stem cells for a long time. With this method, some dependent leukemic cells could also be cultivated, and factors mandatory for the growth of these leukemic cells could be studied as suggested by Gartner and Kaplan (11). This would lead to a new insight in the regulatory mechanisms of the leukemic cell growth and provide an effective method of establishing leukemic cell lines.

We observed prolonged proliferation and differentiation of human hematopoietic cells of leukemic origin in vitro for more than 20 months from bone marrow of a patient with acute myeloid leukemia; their growth was not autonomous and seemed to be dependent on the cells adherent to the culture flasks. Bone marrow fibroblast-like cells were the most likely candidate for the helper cells in vitro.

MATERIALS AND METHODS

Case Report. A 38-year-old Japanese woman was found to have a marked leukocytosis when she consulted a hospital in January 1979. Then, she was referred to our hospital. Physical examination on admission revealed slight hepatomegaly without lymphadenopathy and splenomegaly. Hematological examination revealed the following: hemoglobin, 12.4 g/dl; RBC, 5.1 x 1012/liter; WBC, 58 x 109/liter; platelet, 150 x 109/liter. Differentials were as follows: immature blasts with prominent nucleoli, 50%; myelocytes, 4%; metamyelocytes, 6%; bands, 12%; segmented forms, 7%; monocytes, 5%; and lymphocytes, 16%. Bone marrow smears and histological specimens showed hypercellular marrow with infiltration of blast cells (93%). A few blasts had azurophilic granules, and peroxidase reaction in these blasts was strongly positive. A diagnosis of acute myeloid leukemia was made. Despite the intensive chemotherapy including 1-/S-D-arabinofuranosylcytosine, daunorubicin, 6-mercaptopurine, and prednisolone, and supportive care, she did not enter complete remission and died in August 1979.

Culture of Leukemic Cells. Bone marrow cells were aspirated into a heparinized syringe on June 1, 1979, when leukemic regrowth was noted in the bone marrow after the sixth course of chemotherapy including 1-β-D-arabinofuranosylcytosine (100 mg for 7 days), daunorubicin (40 mg for 3 days), 6-mercaptopurine (50 mg for 6 days), and prednisolone (30 mg for 7 days). The cells were washed, suspended in culture medium, and seeded approximately at a concentration of 2 x 107/ml into plastic culture flasks (Corning Glass Works, Corning, N. Y.), and cultured at 37° in 5% CO2 in air with 100% humidity. The culture medium consisted of Roswell Park Memorial Institute Tissue Culture Medium 1640 (Grand Island Biological Co., Grand Island, N. Y.) containing 20% FCS (Flow Laboratories, Stanmore, Australia), penicillin (50 units/ml), and streptomycin (50 µg/ml). Half of the medium was changed when the color changed to yellow. After constant proliferation was established, half of the medium was changed every 7 to 10 days. Growth was observed constantly by an inverted microscope.

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2 The abbreviations used are: GM-CSF, granulocyte-macrophage colony-stimulating factor; FCS, fetal calf serum; BMP, bone marrow particle; CFU-GM, precursor cell of granulocyte and macrophage.

Received June 4, 1982; accepted February 25, 1983.

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Colonies of the Cells Obtained from Culture Flasks. The cells in the harvested medium were washed and suspended in fresh medium. Then, they were cultured in agar identical to those from normal human bone marrow using cystic fluid as a source of GM-CSF (23). Cell aggregates of more than 50 cells were counted as colonies by the aid of an inverted microscope after 7 to 14 days of culture.

Morphological and Cytochemical Examination of the Cells Pro liferating in the Flasks and Those Forming Colonies in Agar. Floating cells in the flasks were harvested, and smears of the cells were prepared by cytocentrifugation (Shandon Southern Products, Ltd., Runcorn, Cheshire, England). The cells were stained with Wright-Giemsa, myelo peroxidase, Sudan Black B, periodic acid-Schiff, alkaline phosphatase, α-naphthyl acetate esterase, and naphthol AS-D chloroacetate esterase. The morphology or cytochemistry of the cells in colonies was determined as described by Kubota et al. (16) with modification. Briefly, a culture dish containing 1 ml of agar plate was soaked in 0.9% sodium chloride solution. With slight agitation, the agar plate was detached from the dish and floated in the solution. Then, it was carefully transferred onto a slide glass. The agar plate was covered with a sheet of fine nylon mesh followed by several sheets of filter paper. The plate was dried with a hair dryer. Another slide glass was placed over the plate and pressed evenly downward. Then, the nylon mesh, filter paper, and upper slide glass were jointly detached from the lower slide glass carefully. The thin agar film on the lower slide glass was dried and stained with Wright-Giemsa, etc. Surface immunoglobulins were examined as described (12). Epstein-Barr virus nuclear antigen was examined as described by Yamamoto et al. (24).

Electron Microscopic Examination. Cells harvested were centrifuged to form a pellet. They were dehydrated by ethanol and embedded in Epon 812. Thin sections were cut by a diamond knife on an ultramicrotome (Model 8800; LKB Institutes, Inc., Bromma, Sweden) and stained doubly with uranyl acetate and lead citrate. Observations were made by an electron microscope (Model H-300; Hitachi Ltd., Hitachi, Japan) at an accelerating voltage of 75 kV.

Chromosome Examination. Chromosome studies on fresh bone marrow cells were made by short-term culture (12 to 24 hr) in Roswell Park Memorial Institute Tissue Culture Medium 1640 supplemented with 10% FCS on February 24, April 20, and May 10, 1979. Chromosome studies of the cells found to grow again after 3 months of culture were performed on 3 occasions (195th, 339th, and 637th days of culture). The cells were harvested following exposure to Colcemid (0.1 µg/ml) for the first 1.5 hr to harvest. On all occasions, chromosome analyses were performed in the conventional Giemsa-stained and the Q-banded preparations (6); the chromosome analyses were made on at least 10 karyotypes.

Coculture with Normal Bone Marrow Particles. Bone marrow cells were aspirated into a heparinized syringe from normal volunteers who gave informed consent. They were diluted with the same volume of culture medium and centrifuged at 200 x g for 10 min at 4°C. Then, floating BMPs on the surface were collected using a capillary pipet and cultured in 10 ml of culture medium. No attempt was made to disrupt the particles. Usually, 2 culture flasks were established from approximately 1 ml of bone marrow aspirate. In a day or 2, BMPs were observed to adhere to the plastic surface when leukemic cells freshly harvested from the long-term cultures were added to the flasks. For the first month of these cocultures, fresh medium (10 ml) was added when the color of the culture medium changed to yellow. Thereafter, 10 ml of the medium were exchanged for the fresh medium (10 ml) every 7 to 10 days. Fibroblast-like cells were usually observed to spread radially from the central BMPs, and they formed approximately confluent layers in 3 weeks.

RESULTS

Growth of the Cells. Shortly after the start of liquid cultures, bone marrow cells showed an increase in cell number, and the growth of the cells was observed for approximately 3 weeks. Morphologically, these cells were mostly blasts and atypical promyelocytes. Terminally differentiated granulocyes were rarely observed. Thereafter, there was a decline in cell number, and in 2 months of cultures, floating viable cells became hardly countable. Scattered fibroblast-like cells were found by the end of the first week of culture and were observed to grow for another 2 weeks. Fibroblast-like cells may be defined as follows: spindle-shaped adherent cells with typical fibroblast-like morphology and large adherent cells with much wider cytoplasm and a large nucleus containing usually one prominent nucleolus. Few fat cells and endothelial cells were recognized in the flasks.

After 3 months of culture, cell growth was found in 2 of 6 flasks initiated simultaneously. The cells seemed to proliferate initially on or around giant fibroblast-like cells. On phase contrast microscopy, one giant cell had occasionally several numbers of oval or fusiform small cells tightly attaching to its surface (Fig. 1A). As the growth of the cells continued, an increasing number of cells was found to proliferate in suspension. They were round in shape and proliferated mostly as single cells. These cells were designated as SAM cells.5 A few clusters of large cells with prominent cytoplasmic projections were also observed among them. Maximal cell concentration of SAM cells was about 5 x 10^9/ml. Numerous attempts to transfer SAM cells to new flasks were mostly unsuccessful. These included culturing them in 6 kinds of synthetic media containing FCS or horse serum or culturing them with 4 batches of GM-CSF or conditioned medium reported to be stimulatory for leukemic cells. Seeding of the cells over feeder layers of human fetal lung fibroblasts or bovine endothelial cells was also unsuccessful. Repeated seeding of SAM cells (approximately 5 x 10^9 cells/week for 4 weeks) with or without their conditioned media into the same flask sustained the growth of the cells for about 1 or 2 months after the last seeding, but longer growth was not observed. Addition of their conditioned medium could not support their growth either. Failure to transfer SAM cells to new flasks and prominent growth over fibroblast-like cells suggested that the growth of SAM cells might be dependent on some factor(s).

The main cause of the failure seemed to be the differentiation of the cells upon the transfer to new flasks. Two patterns of differentiation were noted in these cases. One was that floating cells proliferated first for a few days after seeding and then stopped growing, and almost all cells became positive for naphthol AS-D chloroacetate stain. Since the increase in the percentage of specific esterase-positive cells was noted before the apparent decrease in cell viability (data not shown), this seemed to indicate differentiation of the cells. As a result, smaller round cells had accumulated in the bottom of the flask. The other is that, after a few days, almost all transferred cells adhered to the surface of the flask and became large cells with cytoplasmic projections or macrophage-like cells (Fig. 1B). They were at least 2 to 3 times larger in diameter than round cells proliferating in the original flasks. These cells did not proliferate actively and survived even 3 months or longer.

Characteristics of the Cells. Morphologically, SAM cells seemed to be blast-like cells with no cytoplasmic granule and granulocytic cells at several stages of maturation on smears stained with Wright-Giemsa (Fig. 1, C and D). Results of the cytochemical and immunological examinations are summarized in Table 1. Positivity with peroxidase, Sudan Black B, and

5 SAM cells were designated as such from the name of the patient and myeloid characteristics of the cells.
with GM-CSF. Although the duster/colony ratio was within the formation of SAM cells was observed when they were cultured gradually up to 14 days of culture. Colony formation of SAM the study (Table 2).

Colony Formation in Agar. Although addition of GM-CSF to their cultures could not sustain their growth for more than a month, in vitro colony formation of the cells was examined with or without GM-CSF. As shown in Chart 1, colony and cluster formation of SAM cells was observed when they were cultured with GM-CSF. Although the cluster/colony ratio was within the normal range, the maximum size of colonies was generally smaller than that derived from normal CFU-GMs at the same period of culture (100 to 200 cells and 500 to 2000 cells, respectively). Sensitivity of the cells to GM-CSF was examined 3 times and found not to be significantly different from that of normal CFU-GMs. Representative results are shown in Chart 2. Each colony stimulated with cystic fluid [2.5% (v/v)] was composed of cells with granulocytic characteristics when examined with regard to naphthol AS-D chloroacetate esterase and α-naphthyl acetate esterase activities simultaneously as described previously (17): pure granulocytic, 84.6%; mixed granulocytic and macrophage, 15.4%. When stained with Wright-Giemsa, most colonies were composed of immature myeloid cells with slight degenerative changes. This was in sharp contrast to the observation that polymorphonuclear granulocytes are observed frequently in normal granulocytic colonies. Increasing the concentration of the cells up to 1 x 10⁵/ml could not support the colony formation in the absence of exogenous GM-CSF. The size of each colony was observed, in most cases, to increase gradually up to 14 days of culture. Colony formation of SAM

cells was observed throughout the study (Table 2).

Ultrastructural Characterization. Ultrastructurally, proliferating SAM cells consisted of macrophages and round cells with either immature or mature appearances (Fig. 2A to D). Macrophages (Fig. 2A) were easily distinguished by their sizes (20 to 25 μm in diameter) with abundant phagocytosed materials in the cytoplasm. Round cells with immature appearance (9 to 12 μm in diameter) showed cytological characteristics resembling myeloblasts of normal bone marrow (Fig. 2B). They had abundant cytoplasm and contained a vesicular nucleus with a prominent nucleolus. In contrast to myeloblasts observed in normal bone marrow, they frequently showed cytoplasmic projections. Fat droplets were also observed frequently in the cytoplasm. Some immature cells showed cytoplasmic characteristics somewhat intermediate to monocytes, such as a developed tubular endoplasmic reticulum and indentation of the nucleus. Round cells with a more differentiated appearance showed general characteristics mimicking mature granulocytes (Fig. 2, C and D). Ultrastructurally, cytoplasmic granules of some mature granulocytes were hardly distinguished from those of normal granulocytes (Fig. 2D).

Chromosome Examination. When fresh leukemic cells in the bone marrow of the patient were analyzed cytogenetically, there was no structural rearrangement. The extra chromosome 8 was consistently the only abnormality on all occasions.

Table 1
Cytological and immunological examination of SAM cells
Examination was performed after 6 months of culture.

<table>
<thead>
<tr>
<th>Examination</th>
<th>Positive cells (%)</th>
<th>Degree of activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myeloperoxidase</td>
<td>10</td>
<td>+</td>
</tr>
<tr>
<td>Sudan Black B</td>
<td>20</td>
<td>++</td>
</tr>
<tr>
<td>Naphthol AS-D chloroacetate esterase</td>
<td>20</td>
<td>++</td>
</tr>
<tr>
<td>Periodic acid-Schiff</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>α-Naphthyl acetate esterase</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>0</td>
<td>±</td>
</tr>
<tr>
<td>EBNA ²</td>
<td>0</td>
<td>±</td>
</tr>
<tr>
<td>Surface immunoglobulin</td>
<td>0</td>
<td>±</td>
</tr>
</tbody>
</table>

* ++++, strong activity; ++, moderate activity; +, a little activity; ±, faint activity; —, no activity.
* Mainly positive in large cells.
* Epstein-Barr virus nuclear antigen.

Table 2
Differentials and colony-forming ability of SAM cells examined at separate periods of culture in vitro
Colony-forming cells were examined in semisolid agar with cystic fluid as a source of GM-CSF and counted on Day 10 of culture.

<table>
<thead>
<tr>
<th>Day of culture when examined</th>
<th>BL + PRO</th>
<th>MY + MET</th>
<th>ST + SEG</th>
<th>MO + MAC</th>
<th>Peridoxase-positive cells (%)</th>
<th>No. of colony-forming cells (10⁶ cells)</th>
<th>No. of cells in suspension</th>
</tr>
</thead>
<tbody>
<tr>
<td>143</td>
<td>94</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>14</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>296</td>
<td>53</td>
<td>17</td>
<td>20</td>
<td>25</td>
<td>24</td>
<td>3.5 x 10⁶</td>
<td>7.5 x 10⁶</td>
</tr>
<tr>
<td>471</td>
<td>ND</td>
<td>3</td>
<td>0</td>
<td>5</td>
<td>460</td>
<td>7.5 x 10⁶</td>
<td>7.5 x 10⁶</td>
</tr>
</tbody>
</table>

* Differentials were performed on at least 400 cells stained with Wright-Giemsa.
* BL, blast; PRO, promyelocyte; MY, myelocyte; MET, metamyelocyte; ST, stab; SEG, segmented form; MO, monocyte; MAC, macrophage; ND, not determined.

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Table 3
Proliferation of SAM cells cultured with BMPs

<table>
<thead>
<tr>
<th>BMP Treatment</th>
<th>Total no. of cells in suspension</th>
<th>Total colony-forming cells</th>
<th>Peroxidase-positive cells (%)</th>
<th>Abnormal karyotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAM + BMP</td>
<td>$4 \times 10^6$</td>
<td>$1.2 \times 10^6$</td>
<td>5.5</td>
<td>11/11</td>
</tr>
<tr>
<td>SAM alone</td>
<td>$5 \times 10^6$</td>
<td>$1.5 \times 10^6$</td>
<td>ND</td>
<td>9.5</td>
</tr>
<tr>
<td>BMP alone</td>
<td>$&lt;10^6$</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Original flask</td>
<td>$7.5 \times 10^6$</td>
<td>$3.5 \times 10^6$</td>
<td>4</td>
<td>25/25</td>
</tr>
</tbody>
</table>

- ND, not determined.
- Cells in the original flask at the time of examination were shown for reference.

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**DISCUSSION**

Prolonged proliferation with differentiation of human hemopoietic cells (SAM cells) was reported. Chromosomal examination of the cells at separate times of culture demonstrated that their growth was clonal with remarkable chromosomal rearrangements. Although definite evidence was not obtained, it is most likely that SAM cells had originated from the leukemic cells. (a) SAM cells had an abnormal karyotype which has rarely been observed in normal human hemopoietic cells cultured in vitro, especially at an early stage of cultivation (3, 19, 22). (b) They had an extra chromosome 8 which was also observed in the fresh leukemic cells. (c) A peripheral blood sample containing a number of leukemic cells from the patient was found to form numerous colonies (372 colonies/10⁵ cells) in response to GM-CSF. Most of these colonies were granulocytic when examined on specific esterase. The colony number was far more than that observed in normal subjects (usually less than one colony/10⁵ cells in our laboratory), suggesting that the leukemic cells might have the capacity to form colonies in agar. SAM cells were repeatedly demonstrated to have the same capacity (Table 2). In addition, cytochemical characteristics of colonies from SAM cells were almost the same as those from peripheral blood of the patient. Colony formation and the normal pattern of colony/cluster ratio shown in this study may make one doubt about the origin of the colonies from long-term cultures. However, these colonies were abnormal in that they could not grow into large colonies, and cells of these colonies had remained at the earlier stages of differentiation even after a sufficient period of culture. Moore et al. (21) reported that a normal pattern of colony formation by leukemic cells was observed in 17% of acute myeloid leukemia. Although there might be some contributions of remaining normal CFU-GMs to the results, the report suggests that some leukemic cells can form colonies in response to GM-CSF. Furthermore, KG-1 cells and leukemic cells reported by Brennan et al. (5) were shown to form colonies in semisolid media with the stimulation of GM-CSF. The results shown in this study are therefore not contrary to acute myeloid leukemia, but they may characterize the cells as an uncommon type of acute myeloid leukemia.
Characterization of SAM cells using morphological and cytochemical examinations disclosed that they were blastic cells and those at several stages of differentiation in the granulocytic and macrophage pathways. Ultrastructural examination also confirmed these observations. Although the percentages of the mature elements fluctuated during the observation periods, both the cytochemical characteristics and the ability to form colonies in agar were not lost throughout the study (Table 2). The ability of the immature cells to form colonies suggests that they might be at or near the level of CFU-GMs. Although most of the colonies and clusters formed by these cells were demonstrated to be granulocytic, it should be noted that some colonies were composed of both granulocytes and macrophages. The observation that granulocytes and macrophages were constantly found among the cells in suspension may be compatible with this result. Taken together, the clonal growth of haemopoietic cells involved bipotential immature cells and their possible descendants.

There seem to be several common features between SAM cells and each myeloid cell line (HL-60 and KG-1). However, the striking difference of the SAM cells from these cell lines is that SAM cells alone could not proliferate autonomously in vitro for a long time. Prolonged proliferation of the cells was confined to the original flasks. Their growth was found to start around the fibroblast-like cells. Fibroblast-like cells in the original flasks seemed to degenerate or die gradually through long-term cultures; this might be reflected in the observation that after 1 year of culture, SAM cells seemed to degenerate or die gradually through long-term cultures; this might be reflected in the observation that after 1 year of culture, SAM cells seemed to proliferate less actively than those proliferating before (data not shown). These findings would suggest that their growth was not autonomous. This was again supported by the observation that they grew only in the presence of BMPs and BMP-derived feeder layers.

Although we could not demonstrate the stimulatory effect of the medium conditioned by the SAM cells, it is probable that a factor produced by the adherent cells was important for their long-term growth. HL-60 cells were reported to be dependent initially on the fibroblast feeder layers (7). In addition, Brennan et al. (4) reported that the growth of HL-60 cells is still stimulated by the factor(s) produced by themselves. These reports and the findings described here may indicate the significance of stimulators for leukemic cell growth in vitro.

The growth pattern of SAM cells demonstrated in this study suggests that the addition of possible marrow helper cells to the primary cultures of leukemic cells may have, at least in some cases, a beneficial effect on their growth. Although our report was limited to one particular case, the helper effect of the adherent cells suggested here raises the possibility that they may also play some roles in vivo in the growth of leukemic cells. This possibility should be examined further.

ACKNOWLEDGMENTS

We are grateful to Dr. Yasukazu Tanaka, Department of Pathology, Tokyo Metropolitan Institute of Gerontology, Tokyo, for electron microscopic examination. We thank Setsuko Handa, Noniko Nakagami, and Mariko Sato for skilled technical assistance.

REFERENCES

Dependent Growth of Human Leukemic Cells

Fig. 1. Phase contrast and light microscopic appearances of the cells grown in vitro. A giant fibroblast-like cell and many fusiform or oval small cells attaching to its surface (A) and macrophages and large cells with cytoplasmic projections attaching to the bottom of the culture flask (B) are shown. Cytospin preparations of blastic cells (C) and a blastic cell with granulocytes at the levels of segmented form and metamyelocyte (D) are shown. A and B, × 200. C and D, Wright-Giemsa, × 1000.

Fig. 2. Electron micrograph of SAM cells. A macrophage (A) and a myeloblast (B) as well as mature cells (C and D) are shown. The bar shown in each photograph indicates 2 μm.

Fig. 3. A Q-banding karyotype showing a 47, XX, +8, t(12;13)(p13; q14) pattern. Arrows, rearranged chromosomes 12 and 13.
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