Human Malignancy-Associated Nucleolar Antigen as a Marker for Tumor Cells in Patients With Acute Leukemia

By Frances M. Davis, Walter N. Hittelman, Kenneth B. McCredie, Michael J. Keating, Lijda Vellekoop, and Potu N. Rao

Tumor burden in adult patients with acute leukemia is assessed using the percentage of blast cells in the bone marrow or blood. It is clear, however, that not all blast cells are leukemic cells, especially during rapid marrow regeneration. Similarly, some leukemia cell lines have been shown to differentiate in vitro, and the same process also occurs in vivo. Therefore, the leukemic burden may be due to more differentiated cells as well as to blast cells. The purpose of this study was to investigate whether the human malignancy-associated nucleolar antigen (HMNA) could be used as a marker for leukemic cells and to examine its potential as a diagnostic tool. The proportion of HMNA-positive cells in the bone marrow of patients with acute leukemia was determined by indirect immunofluorescence with antibodies to HMNA and was compared with the differential counts routinely made in the clinic laboratory. The percentages of HMNA-positive cells among the nucleated cells in the marrow of 72 patients with clinical evidence of leukemia were significantly higher (range 9%–98%, median 83%) than those observed for nonleukemic individuals (range <0.05%–2.5%, median 1%) or for fractions of marrow cells from normal volunteers enriched for normal early progenitor cells (<2%). Patients with leukemia in remission had a lower percentage of HMNA-positive cells (range 0%–83%, median 3%). The percentage of HMNA-positive cells increased as patients approached relapse. Although the percentage of HMNA-positive cells was related to the percentage of blast cells in the bone marrow of the patients with leukemia, some partially differentiated cells were also HMNA-positive in some specimens, and some blasts were HMNA-negative in other specimens. These studies indicate the potential usefulness of HMNA as a marker for leukemic cells.

Nucleoli in human tumor cells are usually distinguishable from those in normal cells on the basis of their size, number, structure, and irregular shape, and hence, have been useful in the diagnosis of malignancy since the 1930s.1 Antibodies to nucleoli, produced by immunization of animals with isolated nucleoli, have demonstrated nucleolar antigens in tumor cells that cannot be detected in normal cells.2,3 Rabbit antisera to nucleoli isolated from HeLa cells can be rendered functionally specific for nucleoli in human tumor cells by extensive absorption with normal cells and tissues. Such antisera stain a human malignancy-associated nucleolar antigen (HMNA) in nucleoli of tumor cells, but not in normal cells, as revealed by the indirect immunofluorescence test.4,5 The antigen has been detected in all types of human tumors tested, including carcinomas, sarcomas, and leukemias. These results suggest that this antigen could be useful for immunodiagnosis of malignant disease. Indeed, Busch and coworkers6 were able to classify correctly 94% of 80 unknown samples of breast tumors as benign or malignant.

Chan et al.7 have purified two proteins that react with the anti-HMNA antibodies. The major antigen has a molecular weight of approximately 68,000, with an isoelectric point (pI) of 6.3. The antibodies have a 20–30-fold higher specificity for tumor cells than normal cells.8 The localization of the antigen is mainly in the areas containing dense nucleolar ribonucleoprotein particles.9 HMNA has also been detected in some cell lines of fetal origin.9 It is a species-specific antigen which, like the cellular homologs of the oncogenes, is encoded in the genome of every cell, as its expression can be induced in somatic cell hybrids between transformed rodent cells and normal human cells that have lost some human chromosomes.10

As the determination of HMNA positivity or negativity is made on individual cells, it could be a useful immunodiagnostic aid for the management of patients with leukemia. The diseased tissues, the bone marrow and blood, are repeatedly sampled, and the tumor burden is assessed by the percentage of blast cells in the bone marrow and the marrow cellularity. However, it is clear that the morphology might not reflect the cell origin: not all blast cells are leukemic cells, particularly during periods of rapid marrow regeneration, such as following chemotherapy or bone marrow transplantation; there is evidence showing that leukemia cells can differentiate both in vivo11,12 and in vitro.13,14

Smetana et al.15,16 have reported that only blast cells were HMNA-positive in nine patients with acute myelogenous leukemia (AML), and blasts and a few promyelocytes were antigen positive in cells from five patients with chronic myeloid leukemia. Of the blast cells in the marrow specimens from these patients, 6%–80% were HMNA-positive. In contrast, the anti-
gen was not detected in peripheral blood cells from patients with infectious mononucleosis,\textsuperscript{15} mitogen-stimulated normal human lymphocytes,\textsuperscript{4} or agar colonies of cells from healthy donors.\textsuperscript{17}

The purpose of this study was to assess HMNA as a marker of leukemia cells: to quantitate the percentage of HMNA-positive cells in the bone marrows of a population of patients with acute leukemia at various stages of their therapy and to correlate the percentage of HMNA-positive cells with the cell differential counts routinely made in the clinic laboratory. It was of particular interest to determine whether all blast cells were HMNA-positive and whether some differentiated cells could be HMNA-positive. These studies were performed as baseline measurements to establish the usefulness of the technique in characterizing the stage of disease in patients with AML, acute lymphocytic leukemia (ALL), or acute undifferentiated leukemia (AUL).

**MATERIALS AND METHODS**

The studies described here involve bone marrow cells from patients with acute leukemia, who were under the care of the Leukemia Service of the Department of Developmental Therapeutics at the University of Texas M.D. Anderson Hospital, from normal healthy volunteers, and from patients with solid tumors undergoing marrow evaluation. Bone marrow aspirations from the iliac crest of patients are routinely obtained by special nurses for diagnostic procedures. These studies did not require bone marrow sampling beyond that required for normal patient care and have been approved by the Surveillance Committee of the University of Texas M. D. Anderson Hospital.

A small portion of the bone marrow aspirate (0.1 ml) was placed into a tube containing phosphate-buffered saline (PBS) and preservative-free heparin. Cells were collected by centrifugation at 800 g for 5 min, and red blood cells were lysed hypotonically by resuspension of the cells in 10 ml of 15 mM NaCl, 1 mM Na\textsubscript{2}HPO\textsubscript{4}–NaH\textsubscript{2}PO\textsubscript{4}, pH 7.3. Then, 1 ml of 1.3 M NaCl, 100 mM Na\textsubscript{2}HPO\textsubscript{4}, pH 7.3, was added immediately and the cells again collected by centrifugation at 800 g for 5 min. The cells were resuspended in Hanks' balanced salt solution with 10% fetal bovine serum and deposited on coded slides using a cytocentrifuge. For some studies, cells fractionated on discontinuous albumin gradients\textsuperscript{16,17} or Ficoll-Hypaque gradients, as previously described,\textsuperscript{2} were used. Cells were fixed in methanol for 10 min at 22°C and air dried.

Antibodies to HMNA were prepared as previously described.\textsuperscript{4} Briefly, nucleoli were isolated from HeLa cells using standard cell fractionation procedures, including hypotonic shock, NP-40 detergent treatment, Dounce homogenization, sonication, and differential sedimentation through sucrose. The nucleoli were mixed with complete Freund's adjuvant and injected intradermally and subcutaneously into New Zealand white rabbits. The IgG fraction was prepared by ammonium sulfate fractionation and chromatography on DEAE-Sephael. The IgG was absorbed with fetal calf serum and normal human cells and tissues, including serum and sonicated nuclei isolated from placenta and phytohemagglutinin-stimulated lymphocytes.

Indirect immunofluorescence was performed as follows. The cells were overlaid with 50 μl of anti-HMNA IgG at 20 μg/ml in 150 mM NaCl, 10 mM Na\textsubscript{2}HPO\textsubscript{4}–NaH\textsubscript{2}PO\textsubscript{4}, pH 7.3 (PBS), for 15 hr at 22°C. After thorough washing in PBS, fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit IgG (Miles Biochemicals, Elk hart, IN), at a 1:20 v/v dilution in PBS, was permitted to react with the bound rabbit IgG for 1 hr at 37°C. After being washed in PBS, the cells were either counterstained immediately in 0.06% Evan's Blue (Fisher Scientific, Pittsburgh, PA) in water or stained with Wright-Giemsa (Harleco, Gibbstown, NJ) after observation for fluorescence. Replicate slides from some samples were stained with toluidine blue,\textsuperscript{21} in order to determine the percentage of cells with nucleoli. Stained cells were observed using a Nikon Optiphot microscope equipped with epifluorescence and a "B" filter module and phase-contrast optics. For each determination, 200 cells were counted.

**RESULTS**

Detection of HMNA in Leukemic Cells

In order to use the human malignancy-associated nucleolar antigen (HMNA) as a marker for leukemia cells, this antigen had to be demonstrated in leukemia cells. Bone marrow cells from patients with acute leukemia, from normal healthy volunteers, and from patients with solid tumors undergoing bone marrow evaluation were deposited on slides using a cytocentrifuge and stained by indirect immunofluorescence with rabbit antibodies to HMNA. Photomicrographs of some bone marrow specimens stained in this way are shown in Fig. 1. Fluorescence over the nucleoli was observed in leukemia cells from patients with AML, ALL, or AUL, but not in marrow cells from healthy individuals or patients with nonleukemic disorders or solid tumors without bone marrow involvement.

The percentage of HMNA-positive cells in the bone marrow specimens was determined. The reproducibility of this determination was tested by dividing 52 specimens into 2 aliquots that were processed and scored independently. The correlation coefficient between these measurements was 0.97.

The percentages of HMNA-positive cells scored for patients with clinically evident leukemia (i.e., before treatment, during remission induction therapy, or at relapse), for patients with leukemia in complete remission, and for people without leukemia are shown in Fig. 2. HMNA-positive cells accounted for 9%-98% of the nucleated cells in the bone marrows of 72 patients with acute leukemia. The median percentage of HMNA-positive cells for the 47 patients with AML was 76% and that for the 25 patients with ALL or AUL was 88%. Only 2% or less of the cells in the marrows from 7 normal donors, 21 patients with solid tumors but no bone marrow involvement, and 3 patients with nonleukemic hematopoietic disorders (i.e., 2 patients with anemia and 1 patient with myelofibrosis) were scored as HMNA positive. A frequency of ≤2% positive cells is considered not significant in this assay, as such frequencies have been scored using control preimmune antibodies.

Patients with leukemia in remission generally had
lower proportions of HMNA-positive nucleated cells than patients with clinically evident disease (Fig. 2). However, 56% of the marrows from patients in remission had >2% HMNA-positive cells. For 43 patients with AML in complete remission, 0%–81% of the cells were HMNA-positive, the median value was 6%, and 6 patients (14%) had values >20%. Twenty-five patients with ALL or AUL in remission had 0%–83% HMNA-positive cells, with a median value of 2%.

The limits of detection of HMNA-positive cells are approximately at the level for blastic cells (0.5%–5%) observed in the marrows of healthy people. In order to determine whether normal, actively proliferating blast cells were HMNA-positive, proliferating marrows from patients with solid tumors recovering from chemotherapy or marrow transplantation were studied and found to be HMNA-negative (data not shown). In addition, bone marrow cells from two healthy volunteers and five untreated patients with acute leukemia were fractionated according to density on discontinuous albumin gradients. The lightest fractions from the normal healthy donors (fractions 1 and 2) contained less than 5% of the total nucleated cells, but consisted of approximately 50% myeloblasts. The proportion of HMNA-positive cells in these fractions was determined. No enrichment of HMNA-positive cells in any of the fractions from the healthy donors was observed (Table 1). Varying proportions of HMNA-positive cells were found in the fractions from the leukemia patients. It is of interest that fractions 1, 2, and 3 from the patients with AML, which contained early cells in the myelocytic series, were enriched in HMNA-positive cells. HMNA-positive cells were depleted in fraction 4, where lymphocytes were enriched. In contrast, the proportion of HMNA-positive cells in fraction 4 from the patients with ALL was high, while those in fractions 1, 2, and 3, which contained fewer lymphoid cells, were lower. Fraction 5 contained the most differentiated granulocytic and erythrocytic cells, as well as a variable proportion of mononuclear cells. Most of the HMNA-positive cells in this fraction were mononuclear cells, although some granulocytic cells in this fraction from the first AML patient were HMNA-positive.

Correlation Between HMNA-Positive Cells and Cells With Nucleoli

Cells of the myelocytic, monocytic, and erythrocytic series lose their nucleoli as they progress to their terminally differentiated state. It was expected that only cells with nucleoli, and perhaps only a fraction of the cells with nucleoli, would be HMNA-positive. Therefore, the percentage of cells with nucleoli in marrow specimens from five healthy people and five patients each with untreated or relapsed AML or
The percentage of HMNA-positive cells was compared with the percentage of blast cells in the bone marrow of patients with acute leukemia. Cells were stained for nucleoli using toluidine blue, and duplicate slides were processed for indirect immunofluorescence staining with anti-HMNA: (○) Samples from untreated patients with AML; (□) samples from untreated patients with ALL/AUL; (△) samples from normal volunteers. The theoretical line with slope = 1 is indicated by the dashed line. (O, ●) AML; (□, ■) ALL/AUL. Solid symbols (●, ■) indicate samples containing HMNA-positive cells that exhibited myelocytic or erythrocytic differentiation.

ALL/AUL was determined by staining with toluidine blue. The percentage of HMNA-positive cells in these samples was also determined. Although 20%-40% of the cells in the bone marrows of the healthy volunteers had recognizable nucleoli, these nucleoli were HMNA-negative (Fig. 3). In contrast, the percentages of HMNA-positive cells in the marrows from the patients usually were slightly greater than the percentages of cells with toluidine blue-stained nucleoli. These results indicate that the indirect immunofluorescence assay for HMNA is more sensitive for the detection of nucleoli in tumor cells than toluidine blue staining.

HMNA-Positive Differentiated Cells and HMNA-Negative Blasts

The percentage of HMNA-positive cells was compared with the percentage of blast cells in the bone marrows of patients with untreated acute leukemia (Fig. 4). The correlation coefficient was 0.78. The discordance between these two parameters is greater than would be expected on the basis of the variability of the individual measurements alone and suggests that the two parameters may be different, i.e., in some patients there are HMNA-negative blast cells, and in others, there are HMNA-positive, partially differentiated cells. Sequential determinations of the percentage of HMNA-positive cells and blast cells in samples from patients with acute leukemia at times prior to treatment showed that the ratios of HMNA-positive cells to blast cells were reproducible for individual patients, with a correlation coefficient of 0.92 (Fig. 5).

<table>
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<tr>
<th>Diagnosis</th>
<th>Percent HMNA-Positive Cells*</th>
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<tr>
<td></td>
<td>Fraction 1 (1.053 g/cu cm)</td>
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<tr>
<td>Normal</td>
<td>Total</td>
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<tr>
<td>1</td>
<td>1.9</td>
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<tr>
<td>2</td>
<td>0.5</td>
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<tr>
<td>AML</td>
<td>1</td>
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<td>2</td>
<td>17.1</td>
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<td>3</td>
<td>45.0</td>
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<tr>
<td>ALL</td>
<td>1</td>
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<td>2</td>
<td>40.9</td>
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Bone marrow cells from healthy normal persons or untreated patients with acute leukemia were fractionated in discontinuous albumin gradients as described in Materials and Methods. The percentage of HMNA-positive cells in each fraction was determined.

*A frequency of <2% positive cells is considered not significant in this assay, as such frequencies have been scored using control preimmune antibodies.

†ND, not determined: cells in the two lightest fractions were pooled, and data for the combined fractions are given in the 1.058 g/cu cm column.
These data indicate that a fraction of patients with untreated acute leukemia have some blast cells that are HMNA-negative. The ratio between the percentage of HMNA-positive cells and the percentage of blast cells was less than 0.8 for 21% of the 53 patients studied. As the presence of a nucleolus is a morphological characteristic of blast cells, it is unlikely that these blast cells were HMNA-negative because of a lack of nucleoli. Rather these HMNA-negative blast cells could represent normal cells or another population of abnormal cells that is HMNA-negative. There was some karyotypic evidence for two populations of blastic cells in the marrow of one patient with Philadelphia chromosome-positive ALL who had 92% blasts, but only 40% HMNA-positive cells. Cytogenetic analysis of marrow cells from this patient revealed that 60% of the cells had a normal karyotype (46,XY) and 40% had an abnormal one [20% 46,XY,t(9;22) and 20% 47,XY,t(9;22),+22q −].

These data also indicate that cells behave autonomously with regard to their HMNA phenotype and that HMNA-positive and HMNA-negative cells can coexist. Moreover, antigenicity is not transmitted from cell to cell through the plasma of the patient.

On the other hand, some patients exhibited higher proportions of HMNA-positive cells than blast cells. The ratio between the percentage of HMNA-positive cells and the percentage of blast cells was greater than 0.8 for 12% of the 53 patients studied. Two-thirds of this sample, as well as some specimens from patients at other stages of therapy, contained HMNA-positive cells that were at least partially differentiated along the myelocytic or erythrocytic series, as determined by phase-contrast microscopy and confirmed by subsequent Wright-Giemsa staining (closed symbols in Fig. 4). An example of such a cell in the granulocytic series is shown in Fig. 6. Cells of the human promyelocytic cell line HL-60 also remain HMNA-positive when induced by dimethyl sulfoxide to differentiate. Therefore, at least some differentiated leukemia cells retain enough of their nucleoli to remain HMNA-positive.

Correlation Between Increase in Percentage of HMNA-Positive Cells During Remission and Relapse

As discussed above, just over half of the bone marrow specimens from patients with acute leukemia in remission had >2% HMNA-positive cells. These nonblastic HMNA-positive cells may be partially differentiated or maturing leukemia cells. Because >80% of the patients who achieve complete remission will relapse within 5 yr, it was of interest to determine if the percentage of HMNA-positive cells was related to the proximity of relapse. Twenty-seven of the patients in this study have relapsed, and the mean percentage of HMNA-positive cells as a function of the time from the measurement to relapse is shown in Fig. 7. For a group of patients, the mean percentage of HMNA-positive cells was higher than normal during the year prior to relapse, but was within normal limits for measurements made more than 1 yr prior to relapse. Moreover, the higher the percentage of HMNA-positive cells, the sooner was the relapse. These data suggest that an increase in the percentage of HMNA-positive cells might be an indicator of residual disease and impending relapse.

**DISCUSSION**

These studies show that HMNA could be detected in significant proportions of cells in the bone marrow of patients with acute leukemia and that the proportions of HMNA-positive cells were higher in patients with clinical evidence of acute leukemia than in patients in complete remission. In contrast, the frequency of HMNA-positive cells in marrow from normal healthy volunteers, from patients with solid tumors with no marrow involvement, or from patients with nonleukemic hematopoietic disorders was 2% or less, which is considered to be the background level in this assay. Nevertheless, 20%–40% of the cells in these specimens had nucleoli detected using toluidine blue staining.

Enrichment of blast cells by fractionation of bone marrow cells from healthy volunteers on a discontinuous albumin gradient did not result in an increase above the background level in the percentage of HMNA-positive cells. Marrow cells in patients in the early phases of recovery from marrow aplasia are
HMNA-negative. Phytohemagglutinin-stimulated peripheral blood lymphocytes are HMNA-negative.\textsuperscript{4} Colonies of marrow cells from normal healthy donors, grown in agar or methylcellulose for 3–8 days, are HMNA-negative.\textsuperscript{17,24}

Most reportedly tumor-specific antigens, such as HMNA, have been observed in normal cell populations either as fetal or differentiation antigens. This is also the case for HMNA, as it has been detected in some cell lines of fetal origin.\textsuperscript{3} Nevertheless, such antigens may still be useful markers for tumor cells if they are not detectable in corresponding normal cells.

The studies reported here are baseline measurements to evaluate the HMNA in leukemia. The important finding in this study is that blastic and nonblastic HMNA-positive cells occur in the bone marrow of all patients with clinical evidence of acute leukemia, but not in the bone marrow of patients who have never had leukemia or involvement of the bone marrow by malignant disease. The data are consistent with the hypothesis that the HMNA-positive cells are leukemia cells, HMNA-negative cells with nucleoli are normal cells, and HMNA-negative cells without nucleoli could be either normal cells or leukemia cells.

In contrast to the observations of Smetana et al.,\textsuperscript{15,16} who reported that only blastic cells were HMNA-positive in nine patients with AML, we have observed that, in addition to blast cells, some of the differentiated cells were HMNA positive in the marrow of 20%–30% of patients with AML. It is probable that the antisera we used had a higher titer than that used by Smetana et al.,\textsuperscript{15,16} as we observed much stronger nucleolar fluorescence than indicated in their photographs. These authors did report, however, that a few promyelocytes in five patients with chronic myeloid leukemia were antigen positive. Moreover, the high percentages of HMNA-positive cells in the marrow of some patients in complete remission are consistent with the interpretation that differentiated leukemic cells can be HMNA-positive.

Although there was a good correlation between the percentage of HMNA-positive cells and the percentage of blast cells in the marrow, it was also clear that not all blast cells in marrows from patients with leukemia were HMNA-positive. The frequency of HMNA-positive blastic cells in our study ranged from 20%–100%. In 21% of the samples in our study, the percentage of HMNA-positive cells was 20% less than the percentage of blast cells (% HMNA-positive cells/\%blast cells <0.8). Smetana et al.\textsuperscript{16} reported that 6%, 10%, and 50% of the blastic cells in the bone marrow of three patients with AML exhibited bright nucleolar fluorescence. It is not clear whether these HMNA-negative blast cells are normal cells or are leukemia cells without detectable antigen. It is not likely that these cells had lost their nucleoli, as do more mature cells of the myelocytic and erythrocytic series,\textsuperscript{22} since they appeared to be immature. Moreover, the studies
showed that more cells were antigen-positive than those considered to be nucleolus-positive by toluidine blue staining. Karyotypic data from one patient showed that the percentage of HMNA-negative blast cells (65%) was similar to the proportion of normal diploid karyotypes observed (60%) in a sample initially containing 92% blasts.

It is of interest that three patients with AUL had some bone marrow cells that were HMNA-positive, yet had the morphology of immature cells of the myelocytic series. If confirmed, these observations might suggest the involvement of a multipotential stem cell, the presence of multiple leukemia clones, or the expression of an inappropriate differentiation pathway in AUL. Curtis et al.25 have reported a similar phenomenon in nine patients with AML who had blasts that expressed simultaneously the surface antigenic markers of two lineages.

The percentage of HMNA-positive cells in the bone marrow of patients with leukemia in remission is much lower (median 6% for AML, 2% for ALL) than for patients with clinical evidence of disease (median 76% for AML, 88% for ALL), but 56% of the samples contained a higher percentage (>2%) of HMNA-positive cells than that observed for normal healthy donors or patients with solid tumors with no marrow involvement. It is possible that these HMNA-positive cells represent residual leukemia cells that have differentiated. As ≥80% of adult patients with acute leukemia who achieve remission will relapse within 5 yr,23 a significant, but morphologically undetected, leukemic burden remains even during remission. Some evidence for this hypothesis is that the morphologically normal, differentiated cells in the marrows of some patients in remission demonstrate cytokinetic characteristics of transformed cells using the technique of premature chromosome condensation.19,26 This observation has been used to predict relapse. The detection of HMNA may also be a useful indicator of relapse.

Further studies will be needed to test HMNA as a marker for leukemia cells, especially in a wider variety of nonmalignant hematopoietic disorders, and to assess its utility in clinical situations suggested by the preliminary studies reported here. For example, prior to remission induction therapy, the presence of differentiated HMNA-positive cells would suggest that a patient’s leukemia cells were capable of maturation and that therapeutic modalities to enhance differentiation might be beneficial. Stability of marrow cellularity and the percentage of HMNA-positive cells concomitant with a decrease in the percentage of blast cells during remission induction therapy would suggest that the therapy had induced maturation of the leukemic blasts. The appearance during a course of therapy of a high fraction of blasts might be indicative either of persistent leukemia or regeneration of normal marrow elements, and the HMNA technique might be useful in distinguishing these possibilities. During remission, an increase in HMNA-positive cells may be a prognostic indicator for relapse. Relapse may be abrupt or smoldering, and an analysis of the presence and degree of differentiation of the HMNA-positive cells observed just prior to relapse might reflect the growth regulatory properties of the emerging leukemic clone. These proposed studies require multiple, sequential determinations, in a prospective fashion, but may result in an increased understanding of acute leukemia and may suggest therapeutic approaches based on the biology of the disease of an individual patient.

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Human malignancy-associated nucleolar antigen as a marker for tumor cells in patients with acute leukemia

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