The Human Homologue of Rat NG2, a Chondroitin Sulfate Proteoglycan, Is Not Expressed on the Cell Surface of Normal Hematopoietic Cells But Is Expressed by Acute Myeloid Leukemia Blasts From Poor-Prognosis Patients With Abnormalities of Chromosome Band 11q23


In our efforts to produce monoclonal antibodies that recognize cell-surface antigens expressed by hematopoietic precursor and stromal cells, we generated a monoclonal antibody, 7.1, which recognizes a 220- to 240-kD cell-surface protein whose N-terminal amino acid sequence is identical to the rat NG2 chondroitin sulfate proteoglycan molecule. This chondroitin sulfate proteoglycan, previously reported to be expressed by human melanoma cells, was not found to be expressed by normal hematopoietic cells, nor was it expressed on the cell surface of cell lines of hematopoietic origin including cell lines with 11q23 abnormalities. It was found on the cell surface of acute myeloid leukemia (AML) blasts and cell lines derived from nonhematopoietic tissues. Samples of leukemic marrow from 166 children with AML enrolled on Childrens Cancer Group protocol 213 were evaluated for cell-surface expression of this proteoglycan molecule. In 18 of 166 (11%) patient samples, greater than 25% of leukemic blasts expressed the NG2 molecule. These 18 patients had a poorer outcome with respect to survival (P = .002) and event-free survival (P = .035) with an actuarial survival at 4 years of 16.7%. Blast cell expression of the NG2 molecule was strongly associated with French-American-British M5 morphology (P < .0001) and abnormalities in chromosome band 11q23, site of the MLL gene. These results show that the NG2 molecule is expressed by malignant hematopoietic cells that have abnormalities in chromosome band 11q23, suggesting that antibody 7.1 may be useful in the rapid identification of this group of poor-prognosis patients.

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MATERIALS AND METHODS

Cells and cell lines. Samples of peripheral blood (PB) and bone marrow (BM) were obtained from healthy volunteers after informed consent and in accordance with institutional review board regula-
tions. Diagnostic marrow samples from 166 children with de novo AML enrolled on Childrens Cancer Group (CCG) protocol 213 were obtained, processed, and stored in the vapor phase of liquid nitrogen at the AML Reference Laboratory in Seattle, WA, as previously described. The SV-40 transformed human marrow stroma cell lines Bhasin, Readas, Stenburg, and Pinkney were kindly provided by Dr Jack Singer (Veterans Administration Hospital, University of Washington, Seattle). The cell lines KG1 and KG1a were the generous gift of Dr H. Koefler (University of California, Los Angeles) and HL-60 were the kind gift of Dr S. Collins (Fred Hutchinson Cancer Research Center, Seattle, WA). The cell lines Jurkat, HS62, K562, Raji, U937, Daudi, Nalm 6, and CEM were kindly provided by Dr J. Hansen (Fred Hutchinson Cancer Research Center). HEL cells were provided by Dr T. Papayannopoulou (University of Washington, Seattle) and the RS4;11 cell line was the generous gift of Dr J. Kersey (University of Minnesota, Minneapolis). COS cells and the human cervical carcinoma cell line HeLa were obtained from the American Type Culture Collection (ATCC, Rockville, MD).

Dr J. McDougall (Fred Hutchinson Cancer Research Center) kindly provided the untransformed and E6E7 transfected myome
trial smooth muscle cell lines MYSO;13 and ULTR;16, respectively, the E6E7 transformed adult aortic smooth muscle cell line AA LTR 16-2, the untransformed fetal aortic smooth muscle cell line AVSMC, and the Caski epithelial cell line.

Production of monoclonal antibody (MoAb) 7.1. Two Balb/c mice were immunized intraperitoneally with 0.6-3.0 × 10^6 Readas cells (an SV-40 transformed, human marrow stroma cell line) on days 0, 8, 15, 33, and 55 with 1.5 × 10^6 cells injected intravenously on day 62. On day 65 spleen cells were fused with SP2/0 myeloma cells using previously described techniques. IG-producing hybrids were selected on the basis of reactivity with another SV-40 transformed human marrow stromal cell line, Bhasin, which were grown on glass slides and fixed with 2% paraformaldehyde and 0.1% glutaraldehyde. IG-producing hybridomas were also selected based on nonreactivity with human PB monocytes and granulocytes as determined by flow microfluorimetry. Selected clones were expanded and used for ascites production in Balb/c mice as previously described. Anti

body 7.1 ascites was purified by high-pressure liquid chromatography (HPLC) and determined to be an IgG1 molecule using described immunodiffusion techniques.

Radiolabeling, immunoprecipitation, and gel analysis. Cells were surface labeled by lactoperoxidase iodination and immunolipo

nuprecipitation studies were performed as previously described. Briefly, cells were washed three times with phosphate-buffered sa

ti ne (PBS) pH 7.0 and suspended to 5 × 10^5 cells/mL in PBS pH 7.0 containing 10 mmol/L glucose (J.T. Baker, Phillipsburg, NJ) (90 mg/10 mL PBS), 1 M (mCI), glucose oxidase 20 μL (5 mg/mL) (Calbiochem, San Diego, CA), and lactoperoxidase 20 μL (70 IU/ mL) (Calbiochem) were added, in order, to 1 mL of cells at room temperature. The cells were incubated for 20 to 25 minutes at room temperature and then washed three times with PBS. The radiolabeled cells were lysed on ice for 30 minutes by resuspension in 50 μmol/L Tris-HCl pH 7.6 (Sigma, St Louis, MO) containing 150 μmol/L NaCl, 2% Triton X-100 (Sigma), 2 μmol/L phenylmethylsulfonylfluoride, and 0.1% (v/v) aprotinin. The lystate was centrifuged and the supernatant was pre
cleared with a myeloma cell line to remove IgM antibodies. The lystate was then incubated with MoAb 7.1-sepharose conjugate for 2 hours at 4°C. The beads were washed twice with lysis buffer (50 μmol/L Tris pH 8.0, 0.15 mol/L NaCl, 20 μmol/L EDTA, 2 μmol/L phenylmethylsulfonylfluoride, and 2% Triton X-100) and twice with 50 mmol/L Tris-HCl wash containing 0.5% NP-40 (Sigma) and 450 mmol/L NaCl.

The radilabeled protein was released by addition of 60 μL sample electrophoresis buffer (0.125 mol/L Tris-HCl, 2.5% sodium dodecyl sulfate [SDS], 25% glycerol, 0.002% bromophenol blue with or without 2.5% 2-mercaptoethanol) and was heated at 100°C for 5 minutes. Immunoprecipitated proteins were separated by electrophoresis in 8% polyacrylamide gels in the presence of SDS under reducing and nonreducing conditions. The gels were dried and radio
alabeled bands identified by radioautography.

Protein microsequencing. Due to the high level of expression of the NG2 molecule by the human cervical carcinoma cell line HeLa, this cell line was used for microsequencing studies. HeLa cells were grown in a bioreactor system (Applikon, Schiedam, Holland) with the preparation of 10, 35-mL cell pellets. Cell pellets were frozen at −80°C until use, then thawed at room temperature. Cell pellets were maintained at 4°C after thawing and washed with PBS without calcium or magnesium. After a 10-minute, 1,000-g spin, the cells were resuspended in 1% Triton X/PBS (Pierce, Rock

ford, IL) plus protease inhibitors 1 mmol/L phenylmethylsulfonyl

fluoride (PMSF) and 5 mmol/L EGTA. The cells were vortexed at 4°C and spun at 30 kg for 30 minutes at 4°C. The supernatant was removed and placed over an affinity column of 5.0 mg/mL 7.1 MoAb per mL Affi-I (Bio Rad, Hercules, CA). The column was washed with PBS/1% Triton, then 2 mol/L NaC/PBS followed by washes of PBS, 2 mol/L urea/PBS, then PBS. Protein was eluted from the column in 0.3-mL fractions with 100 mmol/L glycine, pH 2.0. Fractions with protein were determined by gel with fractions contain

ing protein concentrated by a 100 mwco Centricon (Amicon, Beverly, MA). The protein was boiled with reducing SDS sample buffer. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was prepared according to the method of Lammelli with samples run on a 7%, 0.5-mm thick gel. After electrophoresis the gel was stained with amido black in H2O. Stained gel was analyzed by eye and the 220- to 240-kDa band and blank bands were excised with a scalpel and digested according to the method of Rosenfeld et al. Briefly, the excised bands were washed twice with 150 μL 50% acetonitrile in 200 mmol/L ammonium carbonate, pH 8.9, for 20 minutes at 30°C and semidried at room temperature. The gel slices were partially rehydrated with 150 μL of 200 mmol/L ammonium carbonate, pH 8.9, containing 0.02% Tween 20. Then, 2 μL trypsin in 200 mmol/L ammonium carbonate, pH 8.9, was added (approximately 1:25). The gel slices were placed in an Eppendorf tube and digested for 24 hours at 37°C. The digestions were stopped by adding 1.5 μL of trifluoroacetic acid. Peptides were recovered by two 20-minute 37°C extractions using 60% acetonitrile and 0.1% trifluoroacetic acid. Before loading onto reverse phase, the extracts were combined and concentrated to a volume of approximately 20 μL in a SpeedVac (Savant, Farmingdale, NY). The eluted peptides were separated by reverse phase on a 500 μmol/L C18 dimension of 25 cm packed with Vydac C-18 material capillary column (Vydac, Hesperia, CA) and compared to the blank digest. Elution was monitored by MALDI-TOF/MS N-terminal sequence analysis performed on a 473 ABI sequencer (ABI, Foster City, CA) according to manufacturer's instructions.

MoAbs. MoAbs to cell-surface antigens associated with lymphoid and/or myeloid lineage differentiation (CD2, 3, 4, 5, 7, 8, 9, 10, 15, 19, 20, 33, 34, 36, glycoprotein lb) were used in the form

of ascites fluid at dilutions of 1:250 to 1:1,000 or as HPLC purified antibody. The specificity of the antibodies used was previously de

scribed. MoAb 7.1 was used in the form of HPLC purified antibody at a concentration of 10 μg/mL.

Indirect immunofluorescence studies. Diagnostic leukemia mar

row cells, normal PB and BM, and cells lines were stained using previously described indirect immunofluorescent antibody staining
AML BLAST NG2 EXPRESSION AND 11q23 ABNORMALITIES

Fig 1. Immunoprecipitation by antibody 7.1 of a 220- to 240-kD antigen from surface 125I-labeled COS and Pinkney cell lines (A); and marrow from one ALL patient and two AML patients (B). The antibodies used were 7.1 and isotype control antibody 21.A (IgG1, anti-Thy 1). The Pinkney cell line was derived from SV-40 transformed human marrow stroma. The immunoprecipitation samples were either reduced or not reduced and electrophoresed on 70/0 SDS polyacrylamide gel. Molecular weight markers are shown.

RESULTS

Amino acid sequence of the 220- to 240-kD protein immunoprecipitated by antibody 7.1. Internal sequence analysis of the peptides generated from the protein precipitated by MoAb 7.1 (Fig 1) showed: AQLSVVPDSDAPGEIEY-EVQR. Comparison of this sequence with that of rat NG2 shows 100% sequence homology over the 21 amino acids sequenced.

Cell-surface expression of the NG2 molecule by normal hematopoietic cells. Antibody 7.1 did not react with PB lymphocytes, monocytes, granulocytes, platelets, or red blood cells. Likewise multicolor flow cytometric analysis found no reactivity of this antibody with any population of cells within normal adult marrow (Fig 2), including the CD34 subpopulation of precursor cells (data not shown). T
cells that were activated by exposure to phytohemagglutinin (PHA, 2 μg/mL; Gibco) and 20% human interleukin-2 (IL-2) (Lymphocult-T; Biotest Diagnostics, Frankfurt, Germany) were also found to be nonreactive with the antibody.

**Cell-surface expression of NG2 by cell lines.** All cell lines of hematopoietic origin that were tested (KG1, KG1a, HEL, Jurkat, HSB2, K562, Raji, U937, HL-60, Daudi, Nalm6, and CEM) did not react with the 7.1 antibody (Table 1). In addition, the RS4;11 cell line, which has an abnormality in chromosome band 11q23, did not react with antibody 7.1. However, the NG2 molecule was detected on the cell surface of a variety of transformed and untransformed cell lines of nonhematopoietic origin. These cell lines were derived from smooth muscle (MYO-13, ULTR-16, AA LTR 16-2, AVSMC), cervical carcinoma (HeLa), and epithelial cells (Caski) (Table 1, Fig 2). Although the NG2 molecule was not expressed by the murine fibroblast cell line NIH 3T3, it was expressed by CV-1 and COS cell lines derived from nonhuman primate kidney cells.

**Cell-surface expression of NG2 by AML blast cells.** Samples of leukemic marrow from 166 children with AML were evaluated by flow microfluorimetry for cell-surface expression of the NG2 chondroitin sulfate proteoglycan. In 18 patient samples (11%), greater than 25% of leukemic blasts expressed the NG2 molecule (Fig 2).

**Study population.** To exclude a selection bias among patients who had marrow samples available for analysis, we compared the clinical characteristics and outcome in patients whose leukemia cells were immunophenotyped and all evaluable study patients. There was no statistical difference between the 166 patients tested and the full cohort of 591 eligible patients enrolled on study with respect to induction rate (P = .21), death during induction (P = .51), survival (P = .22), disease-free survival (DFS) (P = .31), age at diagnosis (P = .77), WBC at diagnosis (P = .43), FAB classification (P = .95), and percentage of blasts in marrow (P = .087).

**Clinical and laboratory characteristics of patients with NG2 molecule positive blast cells.** When we analyzed the clinical characteristics of the 18 patients whose marrow contained greater than 25% 7.1 positive blasts cells (Table 2), we found that blast cell surface expression of the NG2 molecule was associated with a younger age at diagnosis (P = .048) but was not associated with the WBC at diagnosis. Expression of NG2 was strongly associated with FAB M5 morphology (P < .001) with 12 of 18 samples being classified as FAB M5. In 4 patients, an FAB class could not be assigned by the central CCG pathologist who reviewed all marrows for enrolled study patients. The remaining two patient samples had FAB M4 morphology. We found no association between NG2 expression and the cell-surface expression of antigens associated with myeloid or lymphoid lineage differentiation (CD2, 3, 4, 5, 7, 8, 9, 10, 15, 19, 20, 33, 34, 36, or glycoprotein lb).

There was a strong association between blast cell expression of the NG2 molecule and abnormalities involving chromosome band 11q23. Review of the cytogenetic results by a central reviewer (D.A.) demonstrated that of the 166 patients in this study, 105 (63%) patients had cytogenetics available for central review. Cytogenetic abnormalities in chromosome band 11q23 were present in 13 patients. 27 patients had a normal karyotype, 65 patients had abnormalities other than band 11q23, and 61 patients did not have cytogenetic results available for review or the cytogenetic preparation was deemed unacceptable by the central reviewer cytogeneticist. Nine of the 13 patients whose blasts had an abnormality in chromosome band 11q23 were NG2 molecule positive (patients 7, 8, 9, 10, 11, 12, 16, 18) (Table 3) with 4 patients having blasts that were NG2 molecule negative but demonstrated 11q23 abnormalities (patients 19 through 22) (Table 4). In 5 patients (patients 1, 2, 4, 5, 14) whose blasts were 7.1 antigen positive but did not have cytogenetic evidence of 11q23 abnormalities, additional diagnostic leukemia samples were available in the AML Reference Laboratory. Southern blot analysis of these 5 patients’ leukemic

<table>
<thead>
<tr>
<th>Tissue Origin</th>
<th>Cells/Cell Lines</th>
<th>Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematopoietic:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PB</td>
<td>Activated Lymphocytes</td>
<td>Negative</td>
</tr>
<tr>
<td>BM</td>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td>KG1, KG1a, Jurkat, HSB2, Raji, U937, Daudi, Nalm6, CEM, HEL, HL-60; RS4;11</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>Nonhematopoietic:</td>
<td>Marrow stroma: Bhasin, Stenburg, Readus, Pinkey (Green Monkey) Kidney: CV-1, COS</td>
<td>Positive</td>
</tr>
<tr>
<td>Cervical Carcinoma: HeLa</td>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>Epithelial: Caski</td>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>Myometrial smooth muscle: MYO-13, ULTR-16</td>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>Adult aortic smooth muscle: AA LTR 16-2</td>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>Fetal aortic smooth muscle: AVSMC</td>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>(Mouse) fibroblasts: NIH 3T3</td>
<td></td>
<td>Negative</td>
</tr>
</tbody>
</table>
### Table 2. Clinical and Laboratory Characteristics of AML Patients Whose Blast Cells Express the NG2 Chondroitin Sulfate Proteoglycan Molecule

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age, Sex (mo)</th>
<th>WBC (× 10^9/L)</th>
<th>FAB</th>
<th>% Blasts</th>
<th>BMT</th>
<th>Outcome</th>
<th>Cause of Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7 F</td>
<td>416.0</td>
<td>*</td>
<td>90</td>
<td>No</td>
<td>Dead</td>
<td>Hemorrhage</td>
</tr>
<tr>
<td>2</td>
<td>12 M</td>
<td>116.6</td>
<td>M5</td>
<td>99</td>
<td>Yes</td>
<td>Alive</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>13 F</td>
<td>69.3</td>
<td>M4</td>
<td>94</td>
<td>No</td>
<td>Dead</td>
<td>Progressive disease</td>
</tr>
<tr>
<td>4</td>
<td>36 M</td>
<td>79.8</td>
<td>M5</td>
<td>68</td>
<td>No</td>
<td>Dead</td>
<td>Progressive disease</td>
</tr>
<tr>
<td>5</td>
<td>46 M</td>
<td>31.6</td>
<td>M5</td>
<td>97</td>
<td>Yes</td>
<td>Dead</td>
<td>Progressive disease</td>
</tr>
<tr>
<td>6</td>
<td>189 F</td>
<td>8.6</td>
<td>M6</td>
<td>47</td>
<td>Yes</td>
<td>Dead</td>
<td>Infection</td>
</tr>
<tr>
<td>7</td>
<td>79 F</td>
<td>2.9</td>
<td>M5</td>
<td>80</td>
<td>Yes</td>
<td>Dead</td>
<td>Progressive disease</td>
</tr>
<tr>
<td>8</td>
<td>53 F</td>
<td>21.2</td>
<td>M5</td>
<td>91</td>
<td>No</td>
<td>Alive</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>8 M</td>
<td>59.3</td>
<td>M4</td>
<td>64</td>
<td>No</td>
<td>Dead</td>
<td>Progressive disease</td>
</tr>
<tr>
<td>10</td>
<td>83 M</td>
<td>302.0</td>
<td>M5</td>
<td>81</td>
<td>No</td>
<td>Dead</td>
<td>Progressi ve disease</td>
</tr>
<tr>
<td>11</td>
<td>22 M</td>
<td>12.6</td>
<td>*</td>
<td>82</td>
<td>No</td>
<td>Dead</td>
<td>Infection</td>
</tr>
<tr>
<td>12</td>
<td>18 F</td>
<td>5.5</td>
<td>*</td>
<td>92</td>
<td>Yes</td>
<td>Dead</td>
<td>Progressive disease</td>
</tr>
<tr>
<td>13</td>
<td>157 F</td>
<td>1.3</td>
<td>*</td>
<td>87</td>
<td>No</td>
<td>Dead</td>
<td>Progressive disease</td>
</tr>
<tr>
<td>14</td>
<td>35 M</td>
<td>216.5</td>
<td>M5</td>
<td>98</td>
<td>No</td>
<td>Dead</td>
<td>Renal failure</td>
</tr>
<tr>
<td>15</td>
<td>38 M</td>
<td>324.5</td>
<td>M5</td>
<td>99</td>
<td>No</td>
<td>Alive</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>133 M</td>
<td>9.8</td>
<td>M5</td>
<td>98</td>
<td>No</td>
<td>Dead</td>
<td>Hemorrhage</td>
</tr>
<tr>
<td>17</td>
<td>146 M</td>
<td>36.4</td>
<td>M5</td>
<td>81</td>
<td>No</td>
<td>Dead</td>
<td>Infection</td>
</tr>
<tr>
<td>18</td>
<td>83 F</td>
<td>20.3</td>
<td>M5</td>
<td>92</td>
<td>No</td>
<td>Dead</td>
<td>Progressive disease</td>
</tr>
</tbody>
</table>

* CCG central review pathologist unable to assign an FAB morphologic classification.

### Table 3. Cytogenetic and Southern Blot Analysis for Rearrangements in the MLL Gene Located at Chromosome Band 11q23 for NG2 Molecule Positive Patient Samples

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Cytogenetics</th>
<th>Southern Blot Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>46,XX</td>
<td>Rearranged</td>
</tr>
<tr>
<td>2</td>
<td>46,XY</td>
<td>Rearranged</td>
</tr>
<tr>
<td>3</td>
<td>46,inv ins(X;11)(q24;q25)</td>
<td>Rearranged</td>
</tr>
<tr>
<td>4</td>
<td>46,XY, t(10;11)(p7;q3)*</td>
<td>Rearranged</td>
</tr>
<tr>
<td>5</td>
<td>46,XY,-2,+der(2)t(11;2)(q21;q37)/46,XY,-14,+der(14)t(1;14)(q23;q32)*</td>
<td>Rearranged</td>
</tr>
<tr>
<td>6</td>
<td>46,XX(17)XX,+8</td>
<td>NA</td>
</tr>
<tr>
<td>7</td>
<td>46,XX,+t(8;11)(q22;q23)/47,XX,+t(8;11)(q22;q23)</td>
<td>Rearranged</td>
</tr>
<tr>
<td>8</td>
<td>46,XX,-t(8;11)(q22;q23)/47,XX,-t(8;11)(q22;q23)</td>
<td>Rearranged</td>
</tr>
<tr>
<td>9</td>
<td>46,XY,t(9;11)(p22;q23)</td>
<td>NA</td>
</tr>
<tr>
<td>10</td>
<td>46,XX,t(9;11)(p22;q23)</td>
<td>NA</td>
</tr>
<tr>
<td>11</td>
<td>46,XY,t(9;11)(p22;q23)</td>
<td>NA</td>
</tr>
<tr>
<td>12</td>
<td>46,XX,t(9;11)(p22;q23),i(der8)(11q25–11q23::p22–cen::cen–p22::11q23–11q25)</td>
<td>Rearranged</td>
</tr>
<tr>
<td>13</td>
<td>47,XX,+8</td>
<td>NA</td>
</tr>
<tr>
<td>14</td>
<td>47,XY,+8</td>
<td>NA</td>
</tr>
<tr>
<td>15</td>
<td>46,XY/47,XY,+8</td>
<td>NA</td>
</tr>
<tr>
<td>16</td>
<td>46,XY,t(9;11)(q23;p13)</td>
<td>Rearranged</td>
</tr>
<tr>
<td>17</td>
<td>46,XY,t(10;11)(p22;q32p36.1)</td>
<td>NA</td>
</tr>
<tr>
<td>18</td>
<td>46,XX/46,XX,t(10;11)(p22;q32;p11.2)</td>
<td>NA</td>
</tr>
</tbody>
</table>

Abbreviation: NA, no sample available for analysis.

* Not available for central cytogenetics review.

Blasts showed that the MLL gene at 11q23 was rearranged in all 5 samples (Fig 3). Unfortunately, 4 additional patients (patients 6, 13, 15, 17) without cytogenetic evidence of 11q23 abnormalities did not have additional samples stored that were available for further analysis. Therefore, all 14 patients whose blasts expressed the NG2 molecule could be fully evaluated were found to have 11q23 abnormalities (Tables 5 and 6). As expected, Southern analysis was more sensitive at detecting abnormalities in 11q23 than conventional cytogenetics as evidenced by 5 patients (patients 1, 2, 4, 5, 14) (Table 3) without cytogenetic evidence of abnormalities in 11q23 but in whom the MLL gene was rearranged.

Conversely, 11q23 abnormalities detected by cytogenetic analysis (but not studied for MLL gene rearrangements) were found in 4 of 89 (4.5%) patients whose blasts did not express the NG2 molecule (Table 4). Only 1 of these 4 patients (patient 21) had additional diagnostic leukemic marrow samples available for further analysis. Cytogenetic analysis of patient 21 demonstrated 46XX,t(11;17)(q23;q25) and Southern blot analysis using the P/S4 probe confirmed that chromosome band 11q23 was rearranged within the MLL gene (data not shown). The laboratory and clinical characteristics of the 4 patients who had chromosome band 11q23

### Table 4. Cytogenetic and Southern Blot Analysis for Rearrangements in the MLL Gene of Patients Whose Blasts Cells Do Not Express the NG2 Molecule But Do Have Cytogenetic Abnormalities in Chromosome Band 11q23

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Cytogenetics</th>
<th>Southern Blot Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>46,XY,t(9;11)(p22;q23)</td>
<td>NA</td>
</tr>
<tr>
<td>20</td>
<td>46,XY,del(11)(q23)</td>
<td>NA</td>
</tr>
<tr>
<td>21</td>
<td>46,XY,t(11;17)(q23;q25)</td>
<td>Rearranged</td>
</tr>
<tr>
<td>22</td>
<td>46,XY,t(11;17)(p32;q23)</td>
<td>NA</td>
</tr>
</tbody>
</table>

Abbreviation: NA, no sample available for analysis.
AML BLAST NG2 EXPRESSION AND 11q23 ABNORMALITIES

Table 5. Correlation Between Blast Cell Surface Expression of the NG2 Molecule and Abnormalities of Chromosome Band 11q23 Detected by Cytogenetic Analysis and/or Southern Blot Analysis for Rearrangements in the MLL Gene Located at 11q23

<table>
<thead>
<tr>
<th>11q23 Abnormality and/or MLL gene rearranged</th>
<th>NG2 Molecule Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>14 4</td>
</tr>
<tr>
<td>No</td>
<td>0 85</td>
</tr>
<tr>
<td>Unknown</td>
<td>4 59*</td>
</tr>
</tbody>
</table>

*Neither cytogenetic analysis available or MLL gene rearrangement studies performed.

abnormalities but whose blasts did not express the NG2 molecule were not significantly different from the group of patients with NG2 positive blasts (Table 6). The age and WBC at diagnosis was similar in the two populations of patients. Two patients’ blasts had FAB M5 morphology, 1 patient had FAB M4 morphology, and 1 patient’s leukemia could not be assigned to an FAB subclass by the central review pathologist.

Outcome of patients with NG2 molecule positive blast cells. The outcome of the 18 patients with 7.1 positive blasts was significantly worse than the 148 patients whose blasts did not express the NG2 chondroitin sulfate proteoglycan (Fig 4). Patients with blasts that expressed this chondroitin sulfate proteoglycan molecule had a greater rate of death during induction (P = .037), poorer survival (P = .002), DFS (P = .035), and an actuarial survival rate at 4 years of 16.7% compared with an actuarial survival of 44.5% for patients whose blasts did not express this molecule.

The cause of death in the 15 patients whose blasts expressed the NG2 molecule was variable (Table 2). Nine patients died of progressive leukemia, 3 died of infection, 2 died of hemorrhage, and 1 patient died of renal failure. Although the number of patients is small, the causes of death are not different than those expected for children with AML.

DISCUSSION

In our efforts to identify antigens that may be shared by immature hematopoietic precursor and stroma cells, we generated an MoAb, 7.1, which recognizes a 220- to 240-kD cell-surface protein whose N-terminal amino acid sequence is identical to the rat NG2 chondroitin sulfate proteoglycan molecule. This chondroitin sulfate proteoglycan molecule was found to be expressed by childhood AML blasts and cell lines of nonhematopoietic origin, but was not demonstrated on the cell surface of normal hematopoietic cells or cell lines of hematopoietic origin, including the cell line RS4;11 which has abnormalities in chromosome band 11q23.

Proteoglycans are a large class of molecules that are found on the cell surface or the extracellular matrix and are thought to have important functions in promoting and inhibiting cell adhesion, transmembrane signaling, cellular migration, and cellular proliferation. The chondroitin sulfate proteoglycans class of molecules have been shown to be inhibitors of cell adhesion and migration and are thought to serve as barriers to cell migration in neural crest pathways. The rat NG2 molecule is a large 400- to 800-kD (core protein of 300 kD) membrane spanning proteoglycan whose primary structure shows a large extracellular domain, a single transmembrane domain, and a short cytoplasmic tail. The sequence of NG2 is unique and bears few similarities to other known proteins. Comparison of the amino-terminal sequences of the human melanoma proteoglycan, recognized by MoAb 9.2.27, with that of rat NG2 confirm that the melanoma-associated proteoglycan molecule is the human homologue. Additionally, preliminary experiments have shown that antibodies 9.2.27 and 7.1 are cross-reactive on cell lines that express the NG2 molecule (data not shown).

Unlike other cell-surface antigens expressed by childhood AML blasts, expression of this molecule was of strong prognostic significance. Unlike childhood acute lymphoblastic leukemia (ALL) where many clinical and laboratory parameters have been identified that predict for relapse of disease, few clinical or laboratory parameters have been identified in children with AML that are predictive of outcome. However, like childhood ALL, a high WBC at diagnosis is the strongest predictor of a poor response to therapy. Other previously described factors that have limited prognostic value in children with AML include extramedullary disease at diagnosis, age greater than 15 years at diagnosis, central nervous system disease at diagnosis, early response to therapy with less than 5% blasts on day 14 of treatment, Down syndrome, splenomegaly, coagulation abnormalities, and karyotypic abnormalities including -7, 7q-, and t(8;21). A univariate analysis of the sub-group of children with AML whose blasts expressed the chondroitin sulfate proteoglycan NG2 molecule clearly demonstrated that they have a worse outcome and respond poorly to the therapy that was more effective for children whose blasts did not express this molecule. The identification of this poor-prognosis group of patients suggests that therapeutic protocols may in the future be stratified for this group of patients as they may benefit from alternative types of therapy.

Although characteristic patterns of antigen expression have been identified for several FAB subclasses, overall, a clear correlation between immunophenotype and FAB classification has not been documented. Similarly, with rare ex-

Fig 3. Southern blot analysis of genomic DNA from patients 1, 2, 4, 5, 10, and 14, for MLL gene rearrangement. The case numbers are indicated above the lanes. RS (4;11), B1, and AN (4;11) are positive control cell lines, and “Cl” refers to the negative control cell line Nam-6. The restriction enzymes and probes indicated are: (a and b) Hind III/PS4, (c) EcoR1/PS4, and (d) EcoR1/4.2E.
exceptions such as an association between the co-expression of the natural killer (NK) cell antigen, CD56, and the B-lineage antigen, CD19, by FAB M2 leukemias with the t(8;21)(q22;q22), there are no clear correlations between cell surface antigen expression and specific cytogenetic abnormalities. Therefore, the strong association between cell-surface expression of the NG2 molecule and specific cytogenetic abnormalities involving chromosome band 11q23, but not all 11q23 abnormalities. The further identification and characterization of these subgroups is currently unknown. Furthermore, the exact relationship between NG2 expression and the presence of MLL gene rearrangements will require the study of a large cohort of patients in whom cytogenetic, molecular genetic, and flow cytometric data are all available.

Our findings suggest that the NG2 molecule detected by antibody 7.1 is aberrantly expressed within malignant hematopoietic cells, possibly related to expression of an important gene at, or regulated by, the 11q23 locus. Recent data indicate that the gene at chromosome band 11q23 (MLL, HTRX-1, ALL-1, HRX) is an evolutionarily, highly conserved gene homologous to the Drosophila trithorax gene, that is frequently involved in acute leukemia, particularly in those leukemias that arise during infancy.

In Drosophila, the zinc finger-like domains of the predicted amino acid sequence suggests that the trithorax protein binds to DNA. This transcription factor is thought to control homeotic genes and it has been suggested that the human homologue of the trithorax gene is involved in the control of genes which effect cellular differentiation, potentially suggesting that alterations in this gene may play a role in malignant transformation.

Nakamura et al have examined the reciprocal chromosome translocations involving the ALL-1 (MLL) gene at 11q23 and genes from chromosome 4 (AF-4), chromosome 9 (AF-9), and chromosome 19 (ENL), the commonly associated partner chromosome in translocations involving 11q23. Sequence analysis of the AF-4, AF-9, and ENL proteins demonstrated nuclear targeting sequences, serine- and proline rich regions, and stretches abundant in basic amino acids, suggesting that the different proteins fused to ALL-1 may provide similar functional domains. In a study of childhood ALL that accompanies this manuscript, Behm et al have clearly shown that the NG2 molecule is expressed only in ALL leukemic blasts that have reciprocal translocations involving the MLL gene [t(4;11)(q21;q23) and t(1;19)(q23;p13)]. Our results for childhood AML are less clear. Although the NG2 molecule was expressed on blasts with the reciprocal translocations t(11;19)(q23;p13) and t(9;11)(p22;q23), it was also expressed in blasts with the translocation t(11;17)(q23;q11.2) (patient 18) and t(10;11)(p7;q7) (patient 4). Although translocations involving chromosome band 11q23 and chromosome 19 occur in both ALL and AML, the t(11;19)(q23;p13.3) occurs primarily in ALL whereas t(11;19)(q23;p13.1) occurs only in AML. It is not known if the proteins encoded by the genes at chromosome 17q11.2 and the site on chromosome 10p3 contain functional domains such as those encoded on chrome-
mosomes 4, 9, and 19. Interestingly, in the 4 patients whose leukemic blasts had alterations in chromosome band 11q23 but were NG2 cell surface negative, only 1 patient had a reciprocal translocation involving chromosome 9 (patient 19). One patient had a translocation of chromosome 17, but in band q25 rather than q11.2 as seen in NG2 molecule positive patient 18. One patient had a reciprocal translocation involving chromosomes 11q23 and 1p32 (patient 22), and one had a deletion in chromosome band 11q23. Therefore, significant heterogeneity exist in the partner chromosome involved in translocations of chromosome band 11q23, making an association between a specific translocation and expression of NG2 difficult to discern. Therefore, we hypothesize that the gene which encodes the NG2 molecule is controlled by a transcription factor that is encoded by the trithorax-like MLL gene located on chromosome band 11q23 and that certain types of alterations in this gene result in the aberrant expression of the NG2 molecule.

It is also possible that the NG2 molecule is normally expressed at some stage of normal human embryonic hematopoietic development. If this is the case, cellular transformation that disrupts the 11q23 locus may result in the aberrant, continued expression of the NG2 molecule. This hypothesis is supported by data in the rat embryo which suggests that the expression of NG2 is developmentally regulated with its highest expression in neural and extraneural tissues on primitive, proliferating cells with decreased expression on more differentiated cells.

Because the NG2 molecule has not been detected on any normal hematopoietic tissue, the NG2 molecule may prove to be useful in the diagnosis of the subpopulation of childhood AML patients whose blasts have abnormalities in chromosome band 11q23, the detection of minimal disease, and could potentially serve as a target for novel leukemia-specific therapeutic modalities.

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