NRP-1/CD304 Expression in Acute Leukemia

A Potential Marker for Minimal Residual Disease Detection in Precursor B-Cell Acute Lymphoblastic Leukemia

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Key Words: CD304; Neuropilin-1; Minimal residual disease; Acute myeloid leukemia; Precursor B-cell acute lymphoblastic leukemia

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Upon completion of this activity you will be able to:
• list 3 human hematopoietic cell types that express neuropilin-1 (NRP-1)/CD304.
• compare the expression of NRP-1/CD304 on B-cell precursor acute lymphoblastic leukemia with that on acute myeloid leukemia.
• discuss the use of NRP-1/CD304 for minimal residual disease detection in B-cell precursor acute lymphoblastic leukemia.

Abstract

Neuropilin-1 (NRP-1)/CD304 is a marker for plasmacytoid dendritic cells. We determined the distribution of NRP-1/CD304 expression on normal hematopoietic cells and in 167 acute leukemias by flow cytometry. NRP-1/CD304 surface expression was frequent in precursor B-cell acute lymphoblastic leukemia (36/51 [71%]) and uncommon in acute myeloid leukemia (22.9%). In acute myeloid leukemia, expression was noted in all (4/4) acute myeloid leukemias with the M4eo subtype and in 50% of specimens (6/12) with complex cytogenetics. On hematopoietic cells, NRP-1/CD304 was expressed on normal erythrocyte progenitors, plasma cells, and B-cell progenitors, as well as plasmacytoid dendritic cells. Expression was not consistently detected on other hematopoietic cell types. Owing to this distribution of expression, the detection of NRP-1/CD304 alone on a hematopoietic cell cannot be used to determine plasmacytoid dendritic cell differentiation. Finally, we show that NRP-1/CD304 is overexpressed in 30% of precursor B-cell acute lymphoblastic leukemia samples compared with normal B-cell progenitors, allowing for its potential use as a marker for the detection of minimal residual disease.

CD304, or neuropilin-1 (NRP-1), is a transmembrane C-type lectin found on plasmacytoid dendritic cells (PDCs).1 It has been known to have an important role in angiogenesis as a vascular endothelial growth factor receptor and is involved in neuronal guidance during embryogenesis.2-4 NRP-1/CD304 on dendritic cells is involved in initiating immune responses and guides entry of dendritic cells into lymphatics.5,6

NRP-1/CD304 expression is reported to be specific for PDCs in humans.1,7 However, studies in mice have indicated NRP-1/CD304 expression on cells other than PDCs, including RBC precursors, B lymphoblasts, and regulatory T cells (Tregs).8,9 Expression of NRP-1/CD304 on human Tregs has not been detected, however.10 NRP-1/CD304 expression has not been thoroughly evaluated on normal human bone marrow cells.

The vascular effects of NRP-1 have led to its investigation in acute leukemia.11-15 Karjalainen et al11 screened human leukemia cell lines with a combinatorial phage display library for internalizeable peptides and identified NRP-1/CD304 as a widely expressed antigen on human leukemia cells. This group and others demonstrated expression of NRP-1/CD304 in most cases of acute leukemia, including acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL), although only a small number of ALL samples have been evaluated.11,15 These studies have relied on suboptimal methods to detect NRP-1/CD304, including reverse transcription–polymerase chain reaction (RT-PCR) for NRP-1/CD304 messenger RNA on unfractionated marrow cells and immunohistochemical staining.

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of formalin-fixed, paraffin-embedded tissue sections. A rigorous flow cytometric evaluation of NRP-1/CD304 expression in acute leukemia has not been performed.

Rare leukemias arise from PDCs and express NRP-1/CD304. These neoplasms account for 0.2% to 0.4% of all acute leukemias and can be difficult to identify owing to their lack of lineage-specific marker expression. Given the potential importance of NRP-1/CD304 in acute leukemia and the use of NRP-1/CD304 as a marker of PDC neoplasms, we sought to determine its distribution in acute leukemia and normal bone marrow by flow cytometry.

Materials and Methods

Patient Information

We evaluated the expression of NRP-1/CD304 on blasts by flow cytometry in 167 patients diagnosed with acute leukemia at University Hospitals Case Medical Center, Cleveland, OH, from 2006 to 2010. NRP-1/CD304 expression was also evaluated in 25 selected normal bone marrow and 10 peripheral blood specimens from discarded samples after institutional review board approval. Peripheral blood analysis was carried out using EDTA-anticoagulated blood initially drawn for CBC analysis from subjects with normal cell counts and Wintrobe indices. Normal bone marrow samples were from patients (age range, 2-73 years; median, 31.9 years) with normal bone marrow morphologic findings and no evidence of disease by flow cytometry. The diagnoses from these normal samples were as follows: AML in remission, 8; ALL in remission, 6; chronic myelogenous leukemia in remission, 1; chronic myelogenous leukemia with lymphoid blast crisis in remission, 1; B-cell prolymphocytic leukemia in remission, 1; neuroblastoma staging, 2; myeloid sarcoma in remission, 1; idiopathic thrombocytopenic purpura, 1; and anemia or thrombocytopenia with normal marrow morphologic features, 5.

Leukemias were classified using the French-American-British (FAB) criteria since complete clinical and cytogenetic information was lacking in many cases to properly classify all cases using the World Health Organization 2008 diagnostic system. In addition, we were interested in the differentiation state of the blasts provided by the use of the FAB system. Leukemias consisted of 51 precursor B-cell ALLs, 5 precursor T-cell ALLs, 105 AMLs, 2 biphenotypic acute leukemias (B-lymphoblastic/monocytic), 1 juvenile myelomonocytic leukemia, 2 transient myeloproliferative disorders of Down syndrome (TMDs), and 1 PDC leukemia. Patient data, including cytogenetic information, was obtained by review of the medical record when available, after institutional review board approval.

Flow Cytometry

Samples were submitted in accordance with routine clinical protocols. Four-color direct immunofluorescent staining was performed on 200 μL of whole blood or bone marrow as described by the manufacturer. The following antibodies were used: CD3–fluorescein isothiocyanate (FITC) and allophycocyanin (APC), CD5–phycoerythrin (PE), CD9–FITC, CD10–PE and FITC, CD11c–APC, CD13–PE, CD14–APC, CD16/56–PE, CD19–APC and peridinin chlorophyll protein (PerCP), CD20–FITC and PE, CD22–PE, CD23–PE, CD25–FITC and PE, CD34–APC, CD38–APC, CD45–PerCP, CD56–FITC, CD71–FITC, CD117–PE, HLA-DR-PerCP and APC, CD303–APC, NRP-1/CD304–PE and APC, Lin– cocktail, anti-κ-FITC and PE, and anti-λ-PE. All antibodies were purchased from BD Biosciences (San Jose, CA) except for NRP-1/CD304 and CD303, which were obtained from Miltenyi Biotech (Auburn, CA).

Samples procured from 2006 to March 2009 (n = 90) were analyzed on a FACSCalibur flow cytometer (BD Biosciences) using CellQuest software (BD Biosciences) and samples obtained from March 2009 to 2010 (n = 77) were evaluated on a FACSCanto flow cytometer (BD Biosciences) using Diva software (BD Biosciences). We collected and analyzed 100,000 total cells on a routine basis; 500,000 total events were collected for clinical evaluation for minimal residual disease (MRD). Alignment of forward scatter (FSC), side scatter (SSC), FL-1, FL-2, FL-3, and FL-4 channels on the flow cytometers was maintained within the coefficients of variation recommended by the manufacturer. Compensation was performed daily using calibrate beads (BD Biosciences) with compensation rechecked with CD45-stained lymphocytes every other day.

The level of NRP-1/CD304 expression was determined on specific cell populations based primarily on CD45, SSC and FSC back-gating after first identifying the cell population by its antigen profile, as indicated subsequently. Cells with the desired phenotype represented more than 90% of the events in the back-gated region before determining NRP-1/CD304 expression. NRP-1/CD304 expression was then compared with an antibody control within the same sample. Specific absolute NRP-1/CD304 fluorescence was determined by subtracting the control antibody fluorescence from the NRP-1/CD304 fluorescence of the gated population.

CD19-moderate/CD10-dim/CD20-moderate to bright (transitional B cells, n = 6), CD45-moderate to bright/CD19-moderate/CD10−/CD20-moderate (mature B cells, n = 25), CD45/ moderate to dim/CD22-moderate/HLA-DR− (basophils, n = 9), CD45-dim to moderate/SSC-bright (eosinophils, n = 10), and CD45-dim to negative/CD9-moderate/SSC-low/FSC-low blood cells (platelets, n = 9). The following cell types were identified without back-gating since greater than 90% purity could not be obtained using CD45, FSC, and SSC back-gating, and CD304/NRP-1 was assessed directly on the phenotypically defined population: PDCs identified as lin−/HLA-DR-moderate to bright/CD11c− cells (n = 3) or CD303-moderate/CD45-moderate cells (n = 3),16,18 Tregs defined as CD3-moderate/CD4-moderate to slightly dim/CD25-bright cells within the FSC and SSC defined lymphocyte gate (n = 3), myeloblasts defined as CD34-moderate/CD33-moderate/CD19− cells (n = 3), and plasma cells defined as CD38-bright/CD45-dim to moderate cells (n = 13). All hematopoietic cell data were collected on the FACSCanto flow cytometer.

Statistical Analysis

The difference of frequencies of positive expression of NRP-1/CD304 among leukemias was examined by using the χ² test. The difference of quantitative expression of NRP-1/CD304 between 2 groups was examined by using the t test if the populations were normally distributed. The Anderson-Darling test was used to verify the normality assumption. The distribution of expression of NRP-1/CD304 in ALL and AML was non-normal using this test, and, therefore, a comparison between these groups was performed using the Mann-Whitney test. The association between 2 biomarkers was estimated by the Pearson correlation coefficient. All tests were 2-sided, and a P value of .05 or less was considered statistically significant. Statistical analysis was performed using Excel software with installed QI macros (Microsoft, Redmond, WA).

Results

NRP-1/CD304 Expression on Leukemic Blasts

We evaluated 167 acute leukemias (51 B-ALLs, 5 T-ALLs, 105 AMLs, 2 TMD, 1 juvenile myelomonocytic leukemia, 2 bilineal leukemias, and 1 PDC leukemia) for the expression of NRP-1/CD304 Table II. NRP-1/CD304 expression was detectable on blasts in 64 cases overall (38.3%). NRP-1/CD304 was expressed in 71% of B-ALLs (36/51), in which its expression was significantly more common than in AML (24/105 [22.9%]; P < 1 × 10⁻⁶) and all other acute leukemia types combined (28/116 [24.1%]; P < 1 × 10⁻⁷). Examples of NRP-1/CD304 expression in acute leukemia are shown in Image II. Partial expression was common in AML, occurring in about 40% of the NRP-1/CD304+ cases. Expression was partial in 14% of the B-ALLs (5/36). Blasts in juvenile myelomonocytic leukemia (1 case) and TMD (2 cases) did not express NRP-1/CD304. Of the 5 T-ALL cases, 1 was NRP-1/CD304+, and blasts in both bilineal leukemias were NRP-1/CD304+. Both of these cases were B lymphoblastic-monoblastic leukemias. NRP-1/CD304 was expressed in the 1 PDC neoplasm in the study.

Expression of NRP-1/CD304 was examined in leukemia subtypes based on genetics as data permitted (Table 1). For the B-ALLs, cytogenetic information was available in 35 cases; 10 were Philadelphia chromosome positive (Ph+). Of 10 Ph+ B-ALLs, 9 (90%) expressed NRP-1/CD304, whereas 17 (68%) of the 25 non-Ph+ B-ALLs were NRP-1/CD304+. This difference did not reach statistical significance (P = .09).

Cytogenetic information was available for 67 AMLs. All 4 AML M4eo cases expressed NRP-1/CD304, whereas none of the AMLs with t(15;17) (n = 7) or t(8;21) (n = 6) were NRP-1/CD304+. AMLs with complex cytogenetics (3 or more cytogenetic abnormalities) were more commonly NRP-1/CD304+ (6/12 [50%]) than other AMLs (P = .03). Conversely, AMLs with normal cytogenetics were more commonly NRP-1/CD304− (2/19 [11%]) than other AMLs (P = .02). Of the 4 cases with an 11q23 translocation, 1 was CD304+. This case was accompanied by additional cytogenetic abnormalities leading to a complex karyotype. NRP-1/CD304 was expressed in the single Ph+ AML that was evaluated. AMLs were also evaluated for their NRP-1/CD304 expression by FAB subtype. Data are shown in Table 1. NRP-1/CD304 expression was not statistically correlated with a specific FAB subtype.

Table II

<table>
<thead>
<tr>
<th>Leukemia</th>
<th>No. of Cases</th>
<th>Median (Range)</th>
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ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; JMML, juvenile myelomonocytic leukemia; PDC, plasmacytoid dendritic cell; Ph, Philadelphia chromosome; TMD, transient myeloproliferative disorder of Down syndrome.
Expression Level of NRP-1/CD304 on Blasts

Quantitative data were assessed on a single platform in a subset of cases since the study spanned a period in which we migrated from examining clinical samples on a BD FACSCalibur to a BD FACSCanto. Therefore, the level of NRP-1/CD304 expression on cases positive for NRP-1/CD304 was assessed in 23 B-ALLs (of 36 positive) and 14 AMLs (of 24 positive) only on the FACSCanto flow cytometer. Strong NRP-1/CD304 expression was uncommon. Rare cases had very strong expression of NRP-1/CD304 (>5,000 fluorescent units). Although there was no significant overall difference in the mean or median absolute fluorescence between NRP-1/CD304+ AML and B-ALL cases, more B-ALL cases demonstrated moderate to strong expression of NRP-1/CD304 (>1,000 absolute fluorescent units) compared with AML (10/23 [43%] vs 2/14 [14%]; P = .03).

NRP-1/CD304 Expression in Normal Hematopoietic Cells

Expression of NRP-1/CD304 on leukemic blasts, especially B-ALL, spurred us to examine the distribution of expression of NRP-1/CD304 on normal hematopoietic cells in the blood and bone marrow. NRP-1/CD304 was consistently absent on peripheral blood lymphocytes, granulocytes, basophils, eosinophils, and platelets. Very low-level expression of NRP-1/CD304 was discernible on monocytes in some samples. Moderate to strong expression of NRP-1/CD304 was detected on cells consistent with PDCs defined as Lin−/HLA-DR+/CD11c− cells, as expected (mean ± SD absolute fluorescence, 3,825 ± 1,497; Figure 2). Similar results were seen using PDCs identified by expression of CD303. NRP-1/CD304 expression was not detected on Tregs. We examined 3 samples each with 5% to 8% Tregs (of the CD4+ T cells) defined as CD3-moderate/CD4-moderate/CD25-bright cells. NRP-1/CD304 was not
Absolute neurolipin-1 (NRP-1)/CD304 fluorescence was determined in a subset of B-cell acute lymphoblastic leukemia (B-ALL) and acute myeloid leukemia (AML) cases and compared with the expression on early and late normal B-cell progenitors. Moderate to strong expression of NRP-1/CD304 (>1,000 absolute fluorescent units) was present in 10 (43%) of 23 B-ALL compared with 2 (14%) of 14 AML specimens ($P = .03$).

In bone marrow, we detected expression of NRP-1/CD304 on B-cell progenitors, plasma cells, and erythroid precursors. NRP-1/CD304 was weakly and partially expressed on early (CD34-moderate/CD20−/CD10-moderate/CD45-very dim; mean ± SD absolute fluorescence, 221 ± 188) and late (CD34−/CD20-dim/CD10-moderate/CD45-dim; mean ± SD absolute fluorescence, 142 ± 113) B-cell progenitors (Image 2). Transitional B lymphocytes (CD34−/CD20-bright/CD10-moderate/CD45-dim to moderate) had very weak variable expression of NRP-1/CD304 (mean ± SD absolute fluorescence 120 ± 100). Mature B cells (CD20-moderate/CD10−/CD45-moderate to bright) were NRP-1/CD304−. Therefore, it seems that NRP-1/CD304 expression is lost at the later stages of B-cell maturation. The expression of NRP-1/CD304 on B-cell progenitors may explain its frequent expression in B-ALL. Marrow plasma cells (CD38-bright/CD45-dim to moderate) demonstrated reactivity for NRP-1/CD304 with mean ± SD absolute fluorescence of 1,113 ± 692. Expression of NRP-1/CD304 was detected on erythroid progenitors defined as CD71-moderate/CD45− cells (absolute fluorescence, 555 ± 420), although variable (Image 3). The expression of NRP-1/CD304 correlated with CD38 on RBC precursors ($R^2 = 0.64$; data not shown). NRP-1/CD304 was essentially undetectable above isotype control on these cells. The findings are in agreement with those of others.

In peripheral blood, we detected expression of NRP-1/CD304 on B-progenitors, plasma cells, and erythroid precursors. NRP-1/CD304 was weakly and partially expressed on early (CD34-moderate/CD20−/CD10-moderate/CD45-very dim; mean ± SD absolute fluorescence, 221 ± 188) and late (CD34−/CD20-dim/CD10-moderate/CD45-dim; mean ± SD absolute fluorescence, 142 ± 113) B-cell progenitors (Image 2). Transitional B lymphocytes (CD34−/CD20-bright/CD10-moderate/CD45-dim to moderate) had very weak variable expression of NRP-1/CD304 (mean ± SD absolute fluorescence 120 ± 100). Mature B cells (CD20-moderate/CD10−/CD45-moderate to bright) were NRP-1/CD304−. Therefore, it seems that NRP-1/CD304 expression is lost at the later stages of B-cell maturation. The expression of NRP-1/CD304 on B-cell progenitors may explain its frequent expression in B-ALL. Marrow plasma cells (CD38-bright/CD45-dim to moderate) demonstrated reactivity for NRP-1/CD304 with mean ± SD absolute fluorescence of 1,113 ± 692. Expression of NRP-1/CD304 was detected on erythroid progenitors defined as CD71-moderate/CD45− cells (absolute fluorescence, 555 ± 420), although variable (Image 3). The expression of NRP-1/CD304 correlated with CD38 on RBC precursors ($R^2 = 0.64$; data not shown). NRP-1/CD304 was essentially undetectable above isotype control on these cells. The findings are in agreement with those of others.

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NRP-1/CD304 as a Potential Marker for MRD in B-ALL

Owing to the moderate to strong expression of NRP-1/CD304 in some cases of B-ALL, we evaluated the usefulness of NRP-1/CD304 as a marker for MRD in B-ALL.

NRP-1/CD304 Expression in B-ALL Compared With Normal B Lymphoblasts

To be useful for an MRD marker, the expression level of NRP-1/CD304 should differ on B-ALL cells compared with normal B lymphoblasts. Therefore, we compared the level of expression of NRP-1/CD304 on normal B lymphoblasts with its expression in B-ALL (Figure 1). B lymphoblast expression of NRP-1/CD304 was weak and variable as shown previously. Of the 23 NRP-1/CD304+ B-ALL samples, 10 (43%) had levels of expression significantly greater than that of normal B-cell progenitors, indicating that blasts in about 30% of B-ALLs (43% of 70.5% NRP-1/CD304+ B-ALL cases) could be distinguished from normal B-cell precursors using NRP-1/CD304 in bone marrow specimens.

Correlation of NRP-1/CD304 Expression With Other Antigens in B-ALL

To use NRP-1/CD304 for MRD detection, the marker should have expression that does not correlate with other antigens used for detecting B-ALL cells. Therefore, we compared...
the expression of NRP-1/CD304 with that of other antigens commonly expressed on blasts in B-ALL (CD10, CD19, CD20, CD22, CD34, CD38, CD45, and HLA-DR) using absolute fluorescence. There was no correlation of CD304 expression with CD19, CD20, CD22, CD34, CD45, or HLA-DR Figure 3. However, NRP-1/CD304 expression in B-ALL was inversely correlated with CD38 ($R^2 = 0.4127$) with a very weak direct correlation with CD10 ($R^2 = 0.1932$; Figure 3). The data demonstrate that NRP-1/CD304 has limited correlation with most surface markers typically used to detect MRD in B-ALL.

Detection of NRP-1/CD304+ B-ALL Cells in Bone Marrow

To demonstrate the potential value of NRP-1/CD304 to detect B-ALL cells in an MRD setting, we performed a mixing experiment, spiking cells from a patient with known NRP-1/CD304+ B-ALL into a bone marrow sample containing normal B-cell progenitors from a child with neuroblastoma. B-ALL cells were introduced at levels of 1/1,000, 1/10,000, and 1/100,000 total marrow cell levels; $1 \times 10^6$ cells were acquired in a tube stained with CD10-FITC, NRP-1/CD304-PE, CD45-PerCP, and CD19-APC, and CD19+ low SSC cells were gated Figure 4. As can be seen, blasts were detectable down to a 1/10,000 cell level using NRP-1/CD304 in conjunction with CD10. B-ALL cells were not clearly discernable at a 1/100,000 cell level, although only 10 cells would be expected to be recovered at that level.

Use of NRP-1/CD304 for MRD Detection at Day 28 After Induction in a Patient With B-ALL

To further illustrate the usefulness of NRP-1/CD304 for the detection of MRD, we present an example of MRD at day 28 after induction in a 7-year-old girl with relapsed B-ALL. At the time of relapse, the patient’s blasts were moderately to strongly positive for CD304 (top). The blasts were also CD10-moderate and CD19-moderate with slight overexpression of these antigens compared with normal B-cell progenitors. The patient received cyclophosphamide, etoposide, and high-dose methotrexate as reinduction chemotherapy, and a bone marrow examination with 4-color flow cytometry was performed at day 28 after initiation of therapy. Representative flow cytometric histograms are shown (Image 5B) using low SSC gating. Blasts are highlighted in blue. Although evaluation of these plots is subjective, blasts are clearly not discernible from other cells in the CD45 × SSC plot and show only a modest degree of separation from other cells in the CD10 × CD19 plot. Use of CD304 enhances discrimination of the blasts from other cells in this case, as
Figure 3. The absolute fluorescence of neurolipin-1 (NRP-1)/CD304 was compared with the expression of CD10 (A), CD19 (B), CD20 (C), CD22 (D), CD34 (E), CD38 (F), CD45 (G), and HLA-DR (H) to determine its usefulness for minimal residual disease detection in B-cell acute lymphoblastic leukemia. NRP-1/CD304 expression was not correlated with the expression of CD19, CD20, CD22, CD34, CD45, or HLA-DR; however, NRP-1/CD304 expression was inversely correlated with CD38 (F, $R^2 = 0.4127$) and very weakly correlated with CD10 (A, $R^2 = 0.1932$). B, $R^2 = 0.0008$; C, $R^2 = 0.0481$; D, $R^2 = 0.0096$; E, $R^2 = 0.042$; G, $R^2 = 0.0067$; H, $R^2 = 0.0069$. 
is evident in the CD304 × CD19 plot (Image 5B). Note the CD45 × CD304 plot fails to clearly identify the leukemic blasts owing to the presence of erythroid progenitors and PDCs that express CD304. The MRD level in this sample was estimated at 0.2%.

Discussion

Our data show that NRP-1/CD304 is frequently expressed on B-ALL blasts (71%), whereas the protein is detected only infrequently on blasts in AML (22.9%). Previous studies have indicated that most if not all acute leukemias express NRP-1/CD304. Both studies were published evaluating the expression and did not examine other acute leukemias. After that report, NRP-1/CD304 expressed with AML subtype or karyotype was overexpressed in all cases compared with normal bone marrow. The authors found no correlation of the level of NRP-1/CD304 with FAB type in either study. More recently, Karjalainen et al examined NRP-1/CD304 in AML by immunohistochemical analysis as a means to assess the role of angiogenesis factors in AML. These investigators examined 76 cases and determined that NRP-1/CD304 was overexpressed in all cases compared with normal bone marrow. The authors found no correlation of the level of NRP-1/CD304 expressed with AML subtype by karyotype and did not examine other acute leukemias. After that report, 2 additional studies were published evaluating the expression of NRP-1/CD304 in AML by RT-PCR. Both studies were small (<30 patients with AML) and used the mononuclear cell fraction of bone marrow and/or blood to evaluate for the NRP-1/CD304 message. Both groups detected NRP-1/CD304 messenger RNA in all samples with message levels greater than control marrow. The expression level was not related to FAB type in either study.

Kreuter et al were the first to report on the expression of NRP-1/CD304 in AML. They examined NRP-1/CD304 in AML by immunohistochemical analysis to detect the usefulness of flow cytometry compared with immunohistochemical analysis. Our data show that NRP-1/CD304 is frequently expressed on B-ALL blasts (71%), whereas the protein is detected only infrequently on blasts in AML (22.9%). Previous studies have indicated that most if not all acute leukemias express NRP-1/CD304. Both studies were published evaluating the expression and did not examine other acute leukemias. After that report, NRP-1/CD304 was overexpressed in all cases compared with normal bone marrow. The authors found no correlation of the level of NRP-1/CD304 with FAB type in either study. More recently, Karjalainen et al examined NRP-1/CD304 in a series of patients with leukemia. This group was screening a peptide library for peptides that bound and became internalized by leukemia cells to use as a potential drug delivery system in leukemia or lymphoma. One of the peptides identified serendipitously had sequence similarity to NRP-1/CD304 ligands, prompting the investigators to examine the distribution of NRP-1/CD304 on a limited number of leukemia samples (5 B-ALL and 24 AML) by immunohistochemical analysis. They demonstrated expression above baseline bone marrow levels in all of the B-ALL samples and two thirds of the AML samples. Expression in the B-ALL samples was stronger than that in AML. The findings are similar to ours, although the percentage of cases expressing NRP-1/CD304 was significantly higher than reported herein.

The differences observed between the previous studies and ours could be due to the enhanced sensitivity and specificity of flow cytometry compared with immunohistochemical analysis and RT-PCR. The RT-PCR studies were particularly problematic since mononuclear fractions were used and NRP-1/CD304 is expressed in plasma cells and normal RBC and B-cell progenitors in bone marrow, as shown in this study. Contaminating normal elements could have easily given a positive signal in these studies. Alternatively, soluble or cytoplasmic NRP-1/CD304 could be expressed in AML. Several alternatively spliced forms of NRP-1/CD304 exist, leading to proteins that are predicted to be soluble. We evaluated only the surface expression of NRP-1/CD304 by flow cytometry and would not have detected intracellular NRP-1/CD304 in leukemia cells. A soluble protein could be stained using an immunohistochemical approach, however. Additional studies using intracellular staining methods would need to be performed to address this possibility.

We attempted to determine whether there was a relationship between NRP-1/CD304 expression and a specific AML FAB subtype or karyotype. For B-ALL, 10 Ph+ B-ALL cases were included in the study; 9 (90%) were NRP-1/CD304+
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compared with 68% of the other B-ALLs. Although this difference suggests that Ph+ B-ALL cases may express NRP-1/CD304 more frequently than other types of B-ALL, the results did not reach statistical significance. For AML, there was no association of NRP-1/CD304 expression with an AML FAB subtype; however, NRP-1/CD304 was expressed on blasts in all 4 AMLs with the inv(16) cytogenetic abnormality. The small number of cases with this karyotype limited our ability to determine statistical significance, but it is noteworthy that NRP-1/CD304 was not detected in cases with the t(8;21) or t(15;17) recurrent cytogenetic abnormalities, was uncommon in cases with normal cytogenetics (11%), and was uncommon overall in AML (22.9%). Expression of NRP-1/CD304 was also observed more commonly in AMLs with complex cytogenetics (50%) than other AML subtypes. It is tempting to speculate that the expression of NRP-1/CD304 in some cases of AML may indicate some form of PDC differentiation.

The frequent expression of NRP-1/CD304 in B-ALL led us to investigate the potential use of NRP-1/CD304 as a marker for MRD in this disorder. NRP-1/CD304 is overexpressed compared with normal B lymphoblasts in roughly 40% of the NRP-1/CD304+ cases and could potentially be useful as an MRD marker in 30% of patients with B-ALL overall. It is important to note that NRP-1/CD304 expression was not correlated with the expression of other antigens commonly used to detect leukemic B lymphoblasts except for a weak correlation with CD10. It is interesting that there was a significant correlation of NRP-1/CD304 with CD38 in B-ALL. As such, it might be of interest to examine the usefulness of CD38 for differentiating normal B lymphoblasts from leukemic B lymphoblasts.

By mixing cells from a patient with a known NRP-1/CD304+ B-ALL into a marrow sample from a patient with neuroblastoma with a high percentage of hematogones, we demonstrated that normal B lymphoblasts can be distinguished from B-ALL. The sensitivity of NRP-1/CD304 (in conjunction with CD19 and CD10) was roughly 1/10,000, the level necessary for therapeutic decision making. In addition,
we showed a clinical example in which B-ALL blasts were easier to identify using CD304 in an MRD setting. On a cautionary note, however, there is partial expression of NRP-1/CD304 in some B-ALL cases, which can limit the usefulness of NRP-1/CD304 for precisely quantifying leukemic B lymphoblasts in MRD.

Our NRP-1/CD304 data in B-ALL are similar to those in a recent study by Coustan-Smith et al. These authors also identified NRP-1/CD304 as a marker potentially useful for MRD in B-ALL from a genome-wide gene expression screen comparing B-ALL blasts with normal B lymphoblasts. Candidate genes were then examined by flow cytometry. They observed 28.7% of B-ALL cases had overexpression of NRP-1/CD304 compared with normal B lymphoblasts, which is very close to the 30% value we identified in this study.

The expression of NRP-1/CD304 in leukemia also led us to examine the distribution of NRP-1/CD304 in normal human hematopoietic cells. In mice, NRP-1/CD304 has been detected on bone marrow RBC precursors, B-cell progenitors, and Tregs, in addition to PDCs. However, in humans, NRP-1/CD304 has consistently been identified only in human PDCs in peripheral blood. A thorough examination of bone marrow cells for the expression of NRP-1/CD304 had not been previously undertaken. We show here that similar to mice, humans express NRP-1/CD304 on RBC progenitors and B lymphoblasts in the bone marrow. Early (CD34-moderate/CD20−) and late (CD34−/CD20-dim) B-cell progenitors were NRP-1/CD304+, although the expression was weak and variable from person to person. We tried to identify samples in which there was a relatively prominent hematogone population to generate the best possible data for this study. Transitional B lymphocytes also seem to express NRP-1/CD304, although the expression seemed less than that on the other progenitor B-cell populations. Here again, the expression was variable from patient to patient. We did not detect NRP-1/CD304 on mature B lymphocytes, however. Weak NRP-1/CD304 expression was also detected on bone marrow plasma cells (CD38-bright cells).

We did not identify NRP-1/CD304 expression on peripheral blood cell types other than PDCs, except for occasional, very weak expression on monocytes. This latter finding is not surprising since Ji et al. showed that macrophage colony-stimulating factor stimulation can induce NRP-1/CD304 expression on monocydic cells as they differentiate into macrophages. We did not detect NRP-1/CD304 on resting Tregs, confirming the findings of others that NRP-1/CD304 is absent from resting Tregs in humans, unlike in mice. Finally, in contrast with the findings of de Paulis et al., we did not detect surface expression of NRP-1/CD304 on basophils. The reason for this discrepancy is uncertain.

We define the distribution of NRP-1/CD304 on normal human hematopoietic cells and its expression in acute leukemia. Owing to its expression on erythroid progenitors, plasma cells, and B-cell progenitors, as well as on PDCs, NRP-1/CD304 expression alone cannot be used to determine PDC differentiation. NRP-1/CD304 surface expression is frequent in B-ALL and is less often encountered in AML. NRP-1/CD304 expression seems rather common in some AML subsets, including AML M4eo (4/4 [100%]) and AML cases with complex cytogenetics (6/12 [50%]). The protein is overexpressed in roughly 30% of B-ALL samples when compared with normal B-cell progenitors, allowing for its potential use as a marker of MRD in a subset of B-ALL cases.

References
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