RAPID COMMUNICATION

Molecular Detection of the (2;5) Translocation of Non-Hodgkin’s Lymphoma by Reverse Transcriptase-Polymerase Chain Reaction

By James R. Downing, Sheila A. Shurtleff, Maria Zielińska, Anita M. Curcio-Brìnt, Frederick G. Behm, David R. Head, John T. Sandlund, Dennis D. Weisenburger, A.E. Kosakowska, Paul Thorner, Adonis Lorenzana, Marc Ladanyi, and Stephan W. Morris

The t(2;5)(p23;q35) translocation was initially identified in cases of anaplastic large-cell lymphoma (ALCL) that expressed the Ki-1 (CD30) antigen. We have recently cloned this translocation and shown it to encode a chimeric product consisting of the N-terminal portion of a nonribosomal nucleolar phosphoprotein, nucleophosmin (NPM), from chromosome 5, fused to the kinase domain of a novel transmembrane tyrosine-specific protein kinase, anaplastic lymphoma kinase (ALK), from chromosome 2. To better define the spectrum of lymphomas that contain this translocation, we have analyzed 70 cases of non-Hodgkin’s lymphoma (NHL) for expression of the t(2;5)-derived NPM/ALK chimeric message by reverse transcriptase-polymerase chain reaction (RT-PCR). Using a previously described set of oligonucleotide primers, NPM/ALK chimeric transcripts were detected in 21 of 22 cases that contained the t(2;5) by cytogenetic analysis and in 10 of 48 cases that either lacked evidence of the t(2;5) or had unsuccessful cytogenetics. In all but 1 case, the NPM/ALK PCR products were of identical size and sequence, suggesting that the genomic chromosome breaks are clustered in a single intron in both NPM and ALK. The NPM/ALK-expressing cases were not confined to NHLs with anaplastic morphology and included 15 ALCLs, 6 immunoblastic lymphomas, and 10 diffuse large-cell lymphomas. Moreover, only slightly greater than half of the cases with anaplastic morphology and 59% of CD30-expressing cases were NPM/ALK positive. Thus, neither anaplastic morphology nor the expression of CD30 accurately predicted the presence of this molecular genetic subtype of lymphoma.

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Therapy for non-Hodgkin’s large-cell lymphoma has greatly improved over the last 10 years, with disease-free survival rates of greater than 50% being achieved with current chemotherapy protocols. Unfortunately, a significant number of patients continue to fail to respond to therapy. The identification of biologic subtypes of large-cell lymphomas that have either an inherently higher risk of failing therapy or that comprise a disease subgroup amenable to a more directed form of therapy remains a necessary goal to further improve survival rates for this disease. A recently described subgroup of large-cell lymphomas are the so-called Ki-1⁺ lymphomas (also called anaplastic large-cell lymphoma [ALCL] or large-cell anaplastic lymphoma, and in this report referred to as Ki-1⁺ lymphomas/ALCL), which account for up to 30% to 40% of all pediatric large-cell lymphomas. These cases are characterized by expression of the CD30 antigen (recognized by the Ki-1 and Ber-H2 monoclonal antibodies [MoAbs]), are primarily T cell in lineage, and typically have anaplastic morphologic features. Results of cytogenetic studies on Ki-1⁺ lymphomas/ALCL have shown the presence of a (t(2;5)(p23;q35) translocation in a high proportion of these cases.

Clinically, Ki-1⁺ lymphomas/ALCL are aggressive, high-grade lymphomas that exhibit a bimodal age distribution similar to that observed in Hodgkin’s disease. Patients present with peripheral lymphadenopathy and frequent extranodal disease involving skin, bone, soft tissue, gastrointestinal tract, and/or lung. Several studies have suggested that Ki-1⁺ lymphomas/ALCL may have a better overall survival rate compared with other large-cell lymphoma subtypes.

Although Ki-1⁺ lymphomas/ALCL have been suggested to constitute a distinct clinicopathologic entity, the morphologic and immunophenotypic criteria required for this diagnosis have not been clearly defined. There is no uniformity in cell lineage, expression of the CD30 antigen, presence of the t(2;5), or morphologic appearance among cases diagnosed as Ki-1⁺ lymphomas/ALCL. Cases have been classified as ALCL that lack CD30 antigen expression and/or the t(2;5); conversely, cases that lacked anaplastic morphology have been shown to express Ki-1 and/or contain the t(2;5) translocation. In addition, morphologic variants of Ki-1⁺ lymphomas have been recently described, including a small-cell variant with exceedingly rare anaplastic cells.

Thus, the exact relationship between CD30 expression, the presence of the t(2;5), and the histologic subtype of lymphoma remains to be established. Because of the variable criteria used to make a diagnosis of Ki-1⁺ lymphomas/
ALCL, it has become difficult in practice to accurately define this entity. The presence of the t(2;5) translocation in a significant proportion of these cases suggests that it might serve as a useful genetic abnormality to define a distinct lymphoma subtype. Once defined, the clinical relevance of this subgroup could be determined through prospective treatment protocols.

We have recently cloned the t(2;5) and have shown it to involve the genes encoding nucleophosmin (NPM; also known as B23 or numatrin), a nonribosomal nucleolar phosphoprotein on chromosome 5, and the novel transmembrane tyrosine-specific protein kinase, anaplastic lymphoma kinase (ALK) on chromosome 2.22 The translocation results in the fusion of these genes on the der(5) chromosome, producing a chimeric NPM/ALK gene and message. The resulting fusion product consists of the N-terminal end of NPM fused in-frame to the intracellular kinase domain of ALK.22,23 NPM is an ubiquitously expressed 38-kD phosphoprotein that is involved in the late stages of preribosomal particle assembly and plays a role in shuttling ribosomal ribonucleoproteins between the nucleolus and the cytoplasm.24-26 Currently, little is known about the normal function of the ALK-encoded tyrosine-specific protein kinase receptor or the nature of the ALK ligand(s). ALK is a member of the insulin receptor subfamily, with greatest homology to the leukocyte tyrosine kinase (LTK), and is expressed in the small intestine, brain, prostate, and testes.22 However, no expression of ALK has been detected within the hematopoietic system. The (2;5) translocation results in transcription of the ALK tyrosine kinase domain driven off the strong NPM gene promoter and leads to its inappropriate expression in lymphoid cells.

Using primers derived from NPM and ALK sequences bracketing the breakpoint, we have developed a nested reverse transcriptase-polymerase chain reaction (RT-PCR) assay that allows the rapid, accurate, and sensitive detection of the fusion mRNA resulting from this translocation.22,27-29 In the present study, we have used this assay to analyze 70 pediatric and adult cases of non-Hodgkin's lymphoma (NHL) for the presence of the t(2;5)-derived NPM/ALK chimeric message. Our data show that neither anaplastic morphology nor the expression of CD30 accurately predict the presence of this molecular genetic subtype of lymphoma.

**MATERIALS AND METHODS**

**Patient samples.** Representative portions of diagnostic tissue from patients with NHL were obtained with informed consent. Samples consisted of either dispersed mononuclear cells frozen as viable cell suspensions or tissue blocks frozen in OCT embedding media (Miles Inc, Elkhart, IN). Samples were obtained from St Jude Children's Research Hospital (SJCRH; 19 cases); Memorial Sloan-Kettering Cancer Center (15 cases); The Hospital for Sick Children, Toronto (14 cases); The Foothill Hospital, Calgary (12 cases); and The University of Nebraska Medical Center (10 cases). Cases were selected based on a diagnosis of NHL and the presence of cryopreserved material but were selectively biased toward cases containing a (2;5) translocation and/or expression of CD30, and thus do not represent a random sampling of this malignancy. The morphology of each case was reviewed by an experienced hematopathologist at the participating institution and 64% of the cases were also centrally reviewed by three hematopathologists, (J.R.D., D.R.H., and F.G.B.).

In addition, all difficult cases were centrally reviewed and a consensus diagnosis was reached. Cytogenetic analysis was performed successfully on 70% of cases using standard techniques at the participating institution. Control cell lines included the leukemic cell lines HL6028,29 and K56222 and the t(2;5)-containing lymphoma cell lines SU-DHL-1 and SUP-M2.33

**Immunophenotyping.** Immunophenotypic analysis was performed at the participating institutions and the reported results are those obtained by chart review. A variety of different standard techniques were used including immunoperoxidase on flash-frozen tissue or formalin-fixed, paraffin-embedded sections and immunofluorescence microscopy or flow cytometry. The CD30 antigen was detected with either the Ber-H2 MoAb (Dako, Santa Barbara, CA) or the Ki-1 MoAb (Dako). Samples were determined to be positive if the majority of the neoplastic cells in adequately fixed portions of the specimen displayed a membrane and/or Golgi pattern of staining or were positive by immunofluorescence analysis. Cell lineage was determined by assessing the reactivity of the neoplastic cells with antibodies specific for various T- and B-cell-associated antigens including CD45 (leukocyte common antigen; Dako), CD3 (Dako), CD43 (MT1; Biotest, Danville, NJ), CD45RO (UCHL-1; Dako), CD20 (L26; Dako), MB-2 (Biotest), and CD45RA (4KB5; Dako). A case was defined as T lineage if it reacted with one or more of the antibodies directed against the T-cell-associated antigens (UCHL-1, CD3, and MT-1) and lacked reactivity with antibodies to the B-cell-associated antigens (L26, 4KB5, and MB-2). A B-cell lineage was assigned if the opposite pattern of reactivity was observed.

**RT-PCR.** Total RNA was extracted and cDNA was synthesized as previously described.34 Amplification of the NPM/ALK chimeric message was performed using Taq polymerase (Perkin-Elmer-Cetus, Norwalk, CT) and the oligonucleotide primers 5'A and 3'A (Fig 1B), which amplify a 177-bp product. To increase the sensitivity of detection, nested primer PCR analysis was performed using 5% of the original PCR products and the oligonucleotide primers, 5'B and 3'B (Fig 1B), which amplify a 123-bp product. The presence of amplifiable RNA was confirmed by RT-PCR amplification of either β-actin transcripts, as previously described,29 or by amplification of the ubiquitously expressed normal NPM mRNA using the 5' oligonucleotide primers 5'A (Fig 1B) and a 3' oligonucleotide (3'NPM) having the sequence 5'-GCTACCACTTCCAGGGGCAGA-3' that is homologous to normal NPM coding sequences located 3' to the point at which the NPM/ALK fusion occurs. To prevent sample cross-contamination, extreme care was taken in handling samples and negative controls were included in both the RNA extraction and amplification reactions.35 After amplification, PCR products were size fractionated by electrophoresis through a 1.2% agarose gel in the presence of ethidium bromide and either directly visualized under UV light or transferred to nylon membranes and hybridized with specific probes. The NPM/ALK chimeric message was detected using a 32P-end-labeled junction-specific probe (Fig 1B), and negative cases were probed with the 3'B (B) nested oligonucleotide radiolabeled with 32P to identify products that had variant junction sequences. The normal amplified NPM product was detected in Southern blots with an NPM specific probe having the sequence 5'-GTGCTGTCCACTAATATGCAC-3'.36

**RESULTS**

The (2;5) translocation leads to the fusion of the ALK receptor tyrosine kinase gene on chromosome 2 to the NPM gene on chromosome 5 and results in the generation of a der(5)-encoded NPM/ALK chimeric mRNA and protein (Fig 1A). The RT-PCR assay used in this study for the detection of the NPM/ALK chimeric mRNA has been previously described33 and results in the specific amplification of a 177-
ALK demonstrated an ability to detect the presence of the bp the leukemic cell line K562, which lacks this translocation, further IO-fold by use of the described nested PCR approach. A chimeric transcript in all cases with cytogenetic evidence of 70 cases of NHL collected from both pediatric and general patients was highly selected with two goals in mind: (1) to first determine whether this assay detects the (2;5) translocation (Fig IB). Mixing experiments between and the t(2;5)-containing lymphoma cell line SU-DHL-l the t(2;5) or whether variant mRNAs exist that are unamplifiable RNA. Figure 2 shows representative results of these cases from these 70 patients and analyzed by RT-PCR for both the der(5)-encoded NPM/ALK chimeric product consists of the N-terminal portion of NPM fused inframe to the cytoplasmic domain of ALK. The position of the oligonucleotide primers and probe used for RT-PCR analysis are shown. (B) Sequence of the amplification oligonucleotide primers for standard [5'(A) and 3'(A)] and nested [5'(B) and 3'(B)] RT-PCR analysis and the detection probe.

bp NPM/ALK chimeric product from cases containing the (2;5) translocation (Fig 1B). Mixing experiments between the leukemic cell line K562, which lacks this translocation, and the t(2;5)-containing lymphoma cell line SU-DHL-l demonstrated an ability to detect the presence of the NPM/ALK chimeric transcript at a sensitivity of 1 in 1 × 10^5 cells (data not shown). This level of sensitivity was increased a further 10-fold by use of the described nested PCR approach.

In this study, we have applied this assay to a group of 70 cases of NHL collected from both pediatric and general hospitals in the United States and Canada. This group of patients was highly selected with two goals in mind: (1) to first determine whether this assay detects the NPM/ALK chimeric transcript in all cases with cytogenetic evidence of the t(2;5) or whether variant mRNAs exist that are unamplifiable with these oligonucleotide primers; and (2) to assess the correlation of anaplastic morphology and Ki-1 expression with the presence of the NPM/ALK chimeric message. The patients analyzed ranged in age from 2 to 86 years, with a mean of 25.6 years. The morphologic spectrum of cases included 28 ALCL (15 T-cell, 7 B-cell, 5 non-T/non-B, and 1 lineage undefined), 21 immunoblastic lymphomas (7 T-cell, 6 B-cell, 1 non-T/non-B, and 7 lineage undefined), 19 diffuse mixed or diffuse large-cell lymphomas (9 T-cell, 6 B-cell, and 4 non-T/non-B), and 2 cases of Burkitt's lymphoma that were initially diagnosed as diffuse large-cell lymphoma but on re-review were classified as small, noncleaved cell lymphomas. Cytogenetic analysis was successfully performed on 49 of these cases and showed the presence of the t(2;5) in 22 patients.

Total RNA was isolated from diagnostic lymph node biopsies from these 70 patients and analyzed by RT-PCR for both the der(5)-encoded NPM/ALK message and the ubiquitously expressed NPM transcript as a control for the presence of amplifiable RNA. Figure 2 shows representative results of the analysis of 7 patients with cytogenetically proven t(2;5)-containing lymphomas, including examples of anaplastic and nonanaplastic cases, and 2 patients whose tumors lacked cytogenetic evidence of this translocation. As shown, a standard RT-PCR reaction was able to detect the NPM/ALK fusion messages in the majority of patients; however, in rare cases, a nested RT-PCR approach was required for detection of the chimeric product (Fig 2, lane 10).

Samples from 22 patients were found to contain a (2;5) translocation by cytogenetic analysis. RT-PCR analysis of these cases resulted in the amplification of a specific NPM/ALK fusion transcript in all but 1 case. The single t(2;5)-containing case that lacked a detectable NPM/ALK chimeric transcript was from a 63-year-old patient with a B-lineage, Ki-1+ ALCL. In addition, 10 of 48 cases that either had no evidence of the t(2;5) by cytogenetics (27) or were not analyzed by cytogenetics (21) were found to express the NPM/ALK fusion transcript. The identification of the NPM/ALK chimeric mRNA in cases analyzed by cytogenetics but lack-
DETECTION OF t(2;5) BY RT-PCR

Fig 2. Representative RT-PCR analysis for the NPM/ALK fusion transcript. Total RNA was extracted from the t(2;5)-containing cell line SU-DHL-1 and 9 diagnostic tissue samples from patients with large-cell NHL. Seven of these cases had cytogenetically proven t(2;5), whereas 2 cases lacked this translocation. The extracted RNA was reverse transcribed and then amplified for the NPM/ALK chimeric transcript in standard (Std) and nested PCR assays. PCR products were separated by electrophoresis, transferred to a nylon membrane, and hybridized with either an NPM/ALK junction-specific probe (Std and Nested) or an NPM-specific probe (NPM-control) and detected by autoradiography. RT-PCR amplification was performed for the ubiquitously expressed NPM message as a control for the presence of amplifiable RNA.

Table 1. Histology of 70 Cases of NHL Analyzed by RT-PCR for NPM/ALK Expression

<table>
<thead>
<tr>
<th>Histology</th>
<th>Results of RT-PCR Analysis</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>NPM/ALK Positive</td>
</tr>
<tr>
<td>ALCL</td>
<td>15</td>
</tr>
<tr>
<td>Immunoblastic</td>
<td>6</td>
</tr>
<tr>
<td>Diffuse mixed and diffuse large-cell</td>
<td>10</td>
</tr>
<tr>
<td>Burkitt’s lymphoma</td>
<td>0</td>
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* This number includes 4 follicular large-cell lymphomas.

DISCUSSION

The diagnosis of Ki-1+ lymphoma/ALCL appears to identify a somewhat heterogenous group of NHLs with variable clinical, morphologic, and immunophenotypic features. Cytogenetic analysis suggests that a proportion of these cases contain a (2;5) translocation. To explore the possibility that
detection of this genetic lesion might identify a more uniform clinicopathologic subgroup of lymphomas, we have used an RT-PCR assay for the t(2;5)-derived NPM/ALK chimeric message to examine 70 cases of NHL. Our results showed that the RT-PCR assay used in this study was able to detect the NPM/ALK chimeric message in 21 of 22 t(2;5)-containing cases and in 10 of 48 cases that either lacked this cytogenetic lesion or had unsuccessful cytogenetics. In all
DETECTION OF t(2;5) BY RT-PCR

Table 2. Immunophenotype of Cases Analyzed by RT-PCR for NPM/ALK Expression

<table>
<thead>
<tr>
<th>Results of RT-PCR for NPM/ALK</th>
<th>Ki-1*</th>
<th>Lineage</th>
</tr>
</thead>
<tbody>
<tr>
<td>+NPM/ALK</td>
<td>27</td>
<td>2</td>
</tr>
<tr>
<td>-NPM/ALK</td>
<td>19</td>
<td>11</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not determined.

The methods used for detection of Ki-1 antigen expression and lineage assignment are described in the Materials and Methods.

but 1 NPM/ALK-expressing case, identically sized RT-PCR products were observed, suggesting that breakpoints were clustered in a single intron in both genes. The 1 exception expressed a variant product that resulted from a more 3' fusion point in ALK. In addition, 1 case was identified that had cytogenetic evidence of the t(2;5) but lacked RT-PCR evidence of NPM/ALK expression. The absence of amplifiable NPM/ALK in this case most likely reflects additional breakpoint heterogeneity, resulting in the inability to amplify the chimeric transcript with the oligonucleotide primers used. Despite these 2 exceptions, the RT-PCR assay described here should allow the amplification of the NPM/ALK chimeric product in the vast majority of cases containing this translocation and thus appears to be a highly effective method for detecting this genetic lesion.

Analysis of the clinicopathologic characteristics of the NPM/ALK-expressing cases showed that this genetically defined subgroup of lymphomas only partially overlapped with classically defined Ki-1+ lymphoma/ALCL. All but 2 of the NPM/ALK-expressing cases for which immunophenotypic data were available expressed the Ki-1 antigen and, with 1 exception, were either T cell or non-T/non-B cell in lineage. However, the morphologic spectrum of these cases was quite variable and included 15 ALCLs, 10 diffuse mixed or diffuse large-cell lymphomas, and 6 immunoblastic lymphomas. Moreover, only slightly greater than half of the lymphomas with anaplastic morphology were found to express NPM/ALK, and only 59% of Ki-1+ cases expressed this chimeric message. In addition, within the ALCL histologic subgroup the mean age of the NPM/ALK-expressing cases was 18.4 years, whereas in the NPM/ALK-negative cases it was 46 years. These data suggest that classically defined Ki-1+ ALCL in young patients is more frequently associated with molecular evidence of t(2;5) than in the adult patient population. However, this latter result should be interpreted with caution because this patient cohort was highly selected and, although nearly half of the patients were more than 21 years of age, the analyzed cases do not represent a random sampling of NHL.

In addition to aiding in the identification of this molecular genetic subtype of NHL, the described RT-PCR assay should also help in differentiating this neoplasm from several morphologically related malignancies. The pleomorphic nature of the malignant cells in ALCL has frequently led to the misdiagnosis of these cases as metastatic carcinoma, melanoma, malignant fibrous histiocytoma, or malignant histiocytosis. In fact, nearly one third of the Ki-1+ ALCLs reported in a recent clinical study were initially misdiagnosed. Because the t(2;5) translocation has not been identified by cytogenetics in any nonhematopoietic malignancy, the identification of this genetic lesion should be of value in differentiating NPM/ALK-expressing lymphomas from these other tumors. In addition, differentiation of Hodgkin's disease and Ki-1+ lymphomas/ALCL can occasionally be difficult; however, we and others have failed to detect expression of the t(2;5)-derived NPM/ALK chimeric transcript in more than 100 cases of Hodgkin's disease. Thus, this RT-PCR assay should also be of aid in differentiating NPM/ALK-expressing cases from Hodgkin's disease. Because of the high level of sensitivity and specificity of the RT-PCR assay for NPM/ALK, as few as 200 cells obtained from a fine needle aspirate are sufficient to perform this analysis. Thus, application of this assay in certain clinical settings may eliminate the need for an open biopsy. Moreover, this assay has been used successfully on formalin-fixed and paraffin-embedded material; therefore, this analysis is possible in cases in which frozen tissue is unavailable.

In summary, t(2;5)-containing large-cell lymphomas appear to comprise a distinct genetic subgroup of NHLs that can have variable histologic and immunophenotypic characteristics. Whether this genetic subgroup identifies a clinically relevant subset of NHLs remains to be defined. However, classification of this group of lymphomas based on a putative etiologic mechanism holds promise for the ultimate development and use of specifically targeted therapy.

ACKNOWLEDGMENT

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Molecular detection of the (2;5) translocation of non-Hodgkin's lymphoma by reverse transcriptase-polymerase chain reaction

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