Transcripts of the npm-alk Fusion Gene in Anaplastic Large Cell Lymphoma, Hodgkin’s Disease, and Reactive Lymphoid Lesions

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Anaplastic large cell lymphoma (ALCL) and Hodgkin’s disease (HD) have some pathologic and immunohistochemical similarities, and a histogenetic relationship between them has been suggested by some investigators. By cytogenetic study, the t(2;5)(p23;q35) translocation appears to be unique for ALCL. The breakpoints of the t(2;5)(p23;q35) have recently been cloned and are reported to involve a novel tyrosine kinase gene, anaplastic lymphoma kinase (alk), on chromosome 2 and the nucleophosmin gene (npm) on chromosome 5. Therefore, we studied the frequency of npm-alk translocation in ALCL using a reverse transcriptase-polymerase chain reaction (RT-PCR) assay. We also studied HD and a variety of reactive lymphoid lesions since there is contradictory information in the literature on the occurrence of the npm-alk rearrangement in HD. We detected npm-alk hybrid mRNA in 8 of 22 cases of ALCL (36%), but none of the 21 cases of HD or the 11 cases with reactive lesions contained amplifiable template. All positive ALCL had the T or indeterminate phenotype and occurred in young adults or children. There was very good correlation between a cytogenetically detectable t(2;5) and a positive signal by RT-PCR. Our results indicate a selective but relatively infrequent association between the t(2;5) and ALCL of T or indeterminate phenotype, not shared with HD or reactive hyperplasia.

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Anaplastic large-cell lymphoma (ALCL) is a recently recognized category of non-Hodgkin’s lymphoma (NHL). Histologically, this lymphoma in its classical form consists of large anaplastic cells that preferentially occupy the sinuses and T-cell areas of lymph nodes. The tumor cells often exhibit a cohesive pattern of growth and strongly express CD30 (Ki-1), which is an activation-associated marker belonging to the nerve growth factor/tumor necrosis factor receptor family and expressed by subsets of activated T and B cells, as well as by Reed-Sternberg cells in Hodgkin’s disease (HD).

The histologic diagnosis of ALCL can be difficult and many cases have previously been classified as lymphocyte-depleted HD, malignant histiocytosis, melanoma, or anaplastic carcinoma. Today, most misdiagnoses can be avoided with the application of modern analytical techniques including immunohistochemistry, electron microscopy, and molecular analysis of T- and B-cell antigen receptor gene rearrangements. However, ALCL can still be confused with HD due to the presence of cells resembling Reed-Sternberg cells and an overlapping immunohistochemical and genotypic profile. Although the reciprocal translocation t(2;5)(p23;q35) has been associated with ALCL, it has not been described in HD. However, cytogenetic analysis is hampered in HD by the generally small proportion of neoplastic cells that are not very active mitotically, resulting in poor yield of tumor cell metaphases in chromosome preparations.

Recently, Morris et al have shown that the t(2;5)(p23;q35) results in the fusion of the nucleophosmin gene (npm) on chromosome 5 with the anaplastic lymphoma kinase gene (alk) on chromosome 2. The alk gene shows sequence similarity to the insulin receptor subfamily of tyrosine kinases. The abnormal fusion protein resulting from the translocation retains the tyrosine kinase domain and likely contributes to the malignant transformation of ALCL. Southern blot analysis using probes close to the npm-alk breakpoints or a reverse transcriptase-polymerase chain reaction (RT-PCR) using npm and alk primers have consistently detected a fusion product in cases of ALCL or cell lines with a cytogenetically detected t(2;5). The RT-PCR assay is an extremely sensitive technique that should be able to detect the presence of a small number of neoplastic cells with t(2;5), independent of cell division. As such, RT-PCR appears ideally suited to investigate the possible relationship between the t(2;5) and HD.

Therefore, we have undertaken a study of the utility of a RT-PCR assay in the detection of the t(2;5) in ALCL, and compared the results with available cytogenetic data. A series of classical HD was also studied to determine whether the t(2;5) with the same breakpoints was detectable in this condition. Tissues showing reactive lymphoid hyperplasia were also examined for the presence of rare cells with the translocation.

Materials and Methods

Patient samples. Cases of primary ALCL were identified in the Nebraska Lymphoma Study Group (NLSG) Registry. All the cases were reviewed and those showing the typical histopathologic features of ALCL, ie, large anaplastic cells with strong expression of CD30 on tumor cells, along with snap-frozen tissue were selected for further study. None of the morphologic variants of ALCL was included in this study. Three of the tumors occurred in extranodal sites including two skin lesions and a thigh mass, but one of the skin lesions represented a recurrent tumor after a nodal primary and the other skin tumor was associated with axillary lymphadenopathy. Human immunodeficiency virus infection was not diagnosed in any of the patients studied. Two cases without anaplastic morphology were included.
identified from our cytogenetic database, because of the presence of the t(2;5)(p23;q35). These two cases were analyzed for the correlation of cytogenetic findings with the RT-PCR assay. The most recent 21 cases of HD, including 15 cases of the nodular sclerosis and six cases of the mixed cellularity subtypes, with classical features and snap-frozen tissue were also identified in the NLSG database.

Control tissue. Frozen placental tissue and K562 cells, an erythroleukemia cell line, were used as negative controls for the npm-alk rearrangement assay. The JB6 cell line (kindly provided by Dr M. Kadin, Beth Israel Hospital, Harvard Medical School, Boston, MA) derived from a case of ALCL and carrying a t(2;5) was used as a positive control.

RNA isolation and cDNA preparation. Tissue samples that had been either snap-frozen or embedded in OCT medium and snap-frozen were used. Intact tissue (3 mm) was minced with a fresh razor blade, transferred to a prelabeled Eppendorf tube with 1 mL TRIzol (GIBCO BRL, Gaithersburg, MD) containing 5 µg glycogen carrier (Boehringer Mannheim, Indianapolis, IN) and immediately vortexed. The OCT-embedded tissue was sectioned on a cryostat with disposable knife blades, and three 20-µm sections with approximately 1 cm² area were collected in 1 mL TRIzol. Every step was taken to avoid the possibility of cross contamination. Tissue was minced in a laminar flow hood with new towels over the cutting area and UV irradiation between each sample. An extensive cleaning procedure was applied between the cutting of each sample in the cryostat, including dismantling of the cutting knife holder and five cleansing cycles with ethanol and 0.1 mol/L HCl. A new blade was used for each sample. Gloves were changed between each step in the procedure. To monitor the efficacy of the contamination prevention procedures, negative control placental tissue was introduced as every fifth sample.

Total RNA extraction, precipitation, and washings were performed according to the TRIzol protocol. Total RNA was quantitated using spectrophotometric absorbance at 260/280 nm, diluted to 0.2 µg/µL, and stored in 10 µL fractions at −70°C for further analysis. Contaminating DNA was removed by DNAase treatment (GIBCO BRL). cDNA was prepared using random hexamer primers and reverse transcriptase (GIBCO BRL) according to the manufacturer’s instruction.

RT-PCR. The presence of amplifiable cDNA was confirmed by performing PCR for glyceraldehyde 3 phosphate dehydrogenase (G3PDH) mRNA with a PCR product of 497 bp.17 The 5’ npm and 3’ alk primers and the junction oligonucleotide used for probing have been previously described,20 and their sequences are 5’-CCC-TTGGGCGGTITGGAAATAACCC-3’, 5’-GAGGTTGGCAGCT-TGCTCACG-3’, and 5’-AGCATTGTAGTGTACCCGGGA-3’, respectively. Amplification was performed with addition of 10 pmol of each primer, 1 U of Taq polymerase, 125 mmol MgCl₂, 10 mmol deoxynucleotide triphosphates in 1× PCR buffer (50 mmol/L KCl, 10 mmol/L Tris HCl at pH 8.3, 0.1 mg/mL gelatin, 0.45% Nonidet P40, and 0.45% Tween 20) in a total volume of 45 µL. Hot start technique was used and the reaction started with the addition of 5 µL of cDNA when the reaction mixture reached 94°C. The cycling parameters were 40 cycles at 94°C for 30 seconds, 62°C for 1 minute, and 72°C for 30 seconds, followed by a final extension at 72°C for 10 minutes. The RT-PCR products were electrophoresed on 1.5% NuSieve agarose gels and visualized with ethidium bromide staining. The PCR products were then blotted by capillary transfer onto nylon membranes, which were hybridized with end-labeled npm-alk junctional oligonucleotide, washed at the appropriate stringency, and then autoradiographed was performed.

Immunologic studies. Immunophenotyping was performed on paraffin and/or frozen sections using a panel of monoclonal and polyclonal antibodies against leukocyte-associated antigens. The cases were classified as being of T-cell type when the neoplastic cells in frozen sections were positive with one or more of the antibodies to CD2, CD3, or CD5 and negative for CD19, CD20, and CD22 and/or paraffin sections were positive with at least two antibodies to CD45RO, CD43, or CD3 in the absence of CD20 positivity. The cases were classified as being of B-cell type when frozen sections were positive with one or more antibodies to CD19, CD20, or CD22 in the absence of CD2, CD3, and CD5, and/or when paraffin sections showed CD20 negativity with no more than one of either CD43 or CD45RO being positive. When a case did not fit any of the above criteria, it was considered to have an indeterminate phenotype.

RESULTS

Performance of RT-PCR for npm-alk. Using the control cell line JB6, the sensitivity of the optimized npm-alk RT-PCR assay was determined. cDNA was prepared from 1 µg of RNA (equivalent to approximately 10⁵ cells) and serial 10-fold dilutions of the RT-reaction product from JB6 were made in the RT-reaction product from 1 µg of K562 RNA to simulate a progressively decreasing amount of tumor cells with npm-alk transcripts in irrelevant cells. The expected 175-bp npm-alk product was clearly visible on agarose gels when a dilution of 10⁻⁵ JB6 cDNA was used as template (Fig 1).

RT-PCR in ALCL, HD, and reactive conditions. RNA samples from 22 cases of CD30-positive ALCL, 21 cases of classical HD, and 11 cases of reactive lymphoid hyperplasia were suitable for analysis. Only cases with amplifiable products after the RT reaction using G3PDH primers were subjected to the npm-alk RT-PCR assay. All results were confirmed by probing the membranes for the desired 175-bp

![Fig 1. Sensitivity of the RT-PCR assay. Serial dilutions of cDNA from JB6 cell line were amplified and the products electrophoresed as follows: lanes 1 through 6 at 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵, 10⁻⁶, and 10⁻⁷ dilutions, respectively. Lane 7, reaction performed with no template DNA. M, molecular-weight markers at 100-bp interval. A signal is detectable down to 10⁻⁵ dilution.](image-url)
Table 1. *npm-alk* RT-PCR Analysis in CD30-Positive ALCL, Hodgkin’s Disease, and Reactive Lymphoid Hyperplasia

<table>
<thead>
<tr>
<th>Samples (n)</th>
<th>RT-PCR for t(2;5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALCL (22)</td>
<td>8</td>
</tr>
<tr>
<td>HD</td>
<td>0</td>
</tr>
<tr>
<td>Nodular sclerosis (15)</td>
<td>0</td>
</tr>
<tr>
<td>Mixed cellularity (6)</td>
<td>0</td>
</tr>
<tr>
<td>Reactive hyperplasia (11)</td>
<td>0</td>
</tr>
</tbody>
</table>

hybrid product using the breakpoint specific probe. Of the 22 ALCL cases, 8 (36%) were positive by the RT-PCR assay (Fig 2). None of the 21 cases of HD or the 11 cases of reactive hyperplasia showed the expected 175 bp product (Table 1). All HD and reactive cases remained negative following transfer and probing with the labeled *npm-alk* junction oligonucleotide.

None of 10 placental negative control samples interspersed among the test samples, the negative control cell line RNA and cDNA, or the reaction mixtures without DNA template added to the assay procedure was positive.

Clinical features. The cases of ALCL with the t(2;5) differed from the negative cases with respect to age and tumor cell phenotype. The positive cases were younger and were only of T-cell or indeterminant phenotype (Table 2).

Correlations with cytogenetic data. Of the 22 cases of ALCL, 10 had been previously analyzed cytogenetically. Among these, the classical t(2;5)(p23;q35) was found in six cases, and in one additional case, the origin of a der 5q35 was uncertain. The six cases with the classical t(2;5) were all positive in the RT-PCR analysis.

Two additional cases of NHL with the t(2;5), but not included in our analysis of ALCL because of atypical morphologic features or the absence of CD30, were also studied. These tissue samples were tested with the RT-PCR assay and a non-anaplastic, CD30⁺ peripheral T-cell lymphoma from a 7-year-old boy was found to be positive, whereas a B-large-cell lymphoma with monoclonal kappa expression from a 73-year-old man was negative. Thus, of the eight cases with a t(2;5)(p23;q35) demonstrated cytogenetically, seven (88%) were positive with the RT-PCR assay. None of the four cyogenetically negative ALCL cases showed the *npm-alk* product with RT-PCR.

DISCUSSION

While the majority of cases of ALCL can be recognized by a constellation of morphologic and immunohistochemical features, there is no single characteristic feature that is pathognomonic of this lymphoma. The discovery of the t(2;5) in cases of ALCL was significant since it was thought to be a specific marker for this form of lymphoma, and to provide a clue as to its pathogenesis. However, the reported incidence of the t(2;5) is highly variable in ALCL, probably due to variable methods of case selection in the various studies. The cloning of the breakpoint in the t(2;5)(p23;q35) was a significant step toward the understanding of the pathogenesis of ALCL, and it opened the way to the demonstration of the t(2;5) by molecular techniques. The new assays have allowed the study of a larger number of cases of ALCL since tissues with adequately preserved DNA and/or RNA can now be examined. Several such studies of ALCL have been published or presented at recent meetings and the results are summarized in Table 3. Bullrich et al. studied 16 cases of ALCL by Southern blot analysis and found two positive cases (12.5%). A similar percentage of positives (16%) was found by Lopategui et al. when 37 paraffin-embedded tissues were studied by RT-PCR, while Orscheschek et al. reported that four of five cases (80%) they examined were positive. Variable percentages of ALCL have been reported to be positive (13% to 43%) in abstracts by other groups. Some of the differences in these reported series may be due to case selection with regard to age, phenotype, cytogenetic findings, and the diagnostic criteria for inclusion in the ALCL category. Our finding of 36% is close to the median of the reported series. We have been very strict in the pathologic criteria for case selection, so our results should be representative of classic cases of ALCL. Morphologic variants, CD30⁺ cases, primary cutaneous ALCL, and cases associated with the acquired immunodeficiency syndrome have not been included. Our patients spanned the entire age spectrum, with four of the patients under 18 years of age. It is interesting that the t(2;5)-positive
cases of ALCL with t(2;5), the breakpoints seem to occur wherein 11 of 13 cases of HD (85%) were found to be negative for the t(2;5) by molecular assays or only a low percentage of positive cases have been found. A striking exception is the report by Orscheschek et al., where B-cell ALCL is associated with breakpoints that are outside of these introns requires further investigation. The B-cell ALCL with der 5q35 in our series may indeed not have the breakpoint from the one commonly found in ALCL. In most clinicopathologic subset of ALCL deserves further investigation.

In conclusion, the RT-PCR assay is a useful alternative to cytogenetic studies and has the advantages of high sensitivity, less labor intensiveness, and does not require cell culture. For CD30+ ALCL with classical morphology, about 36% of the cases had detectable t(2;5)(p23;q35) by this

### Table 2. Age, Sex, and Tumor Phenotype of Cases of CD30+ ALCL

<table>
<thead>
<tr>
<th>ALCL</th>
<th>No. Cases</th>
<th>Male/Female (%)</th>
<th>Median Age (yrs)</th>
<th>Phenotype T/B</th>
</tr>
</thead>
<tbody>
<tr>
<td>All cases</td>
<td>22</td>
<td>13/9</td>
<td>35 (3-86)</td>
<td>8/4/10</td>
</tr>
<tr>
<td>RT-PCR(+)</td>
<td>8</td>
<td>4/4</td>
<td>18 (3-32)</td>
<td>4/0/4</td>
</tr>
<tr>
<td>RT-PCR(−)</td>
<td>14</td>
<td>9/5</td>
<td>58 (9/86)</td>
<td>4/4/6</td>
</tr>
</tbody>
</table>

### Table 3. Review of the Literature: t(2;5) in ALCL, HD, and Benign Conditions

<table>
<thead>
<tr>
<th>Studies</th>
<th>Method</th>
<th>No. Studied</th>
<th>No. Positive (%)</th>
<th>No. Studied</th>
<th>No. Positive (%)</th>
<th>No. Studied</th>
<th>No. Positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Current study</td>
<td>RT-PCR</td>
<td>22</td>
<td>8 (36)</td>
<td>21</td>
<td>0 (0)</td>
<td>11</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Bullrich et al</td>
<td>Southern blot</td>
<td>16</td>
<td>2 (13)</td>
<td>9</td>
<td>2 (22)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Bedanyi et al</td>
<td>RT-PCR</td>
<td>—</td>
<td>— (—)</td>
<td>40</td>
<td>0 (0)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Lopategui et al</td>
<td>RT-PCR</td>
<td>37</td>
<td>6 (16)</td>
<td>—</td>
<td>— (—)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Orscheschek et al</td>
<td>RT-PCR</td>
<td>5</td>
<td>4 (80)</td>
<td>13</td>
<td>11 (85)</td>
<td>4</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Downing et al†</td>
<td>RT-PCR</td>
<td>49</td>
<td>21 (43)</td>
<td>—</td>
<td>— (—)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>McBride et al‡</td>
<td>Southern blot</td>
<td>15</td>
<td>2 (13)</td>
<td>—</td>
<td>— (—)</td>
<td>6*</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Ngan et al</td>
<td>RT-PCR</td>
<td>15</td>
<td>2 (13)</td>
<td>24</td>
<td>5 (21)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Wellmann et al</td>
<td>RT-PCR</td>
<td>22</td>
<td>8 (36)</td>
<td>—</td>
<td>— (—)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Yee et al‡</td>
<td>RT-PCR</td>
<td>17</td>
<td>5 (30)</td>
<td>41</td>
<td>2 (5)</td>
<td>20</td>
<td>0 (0)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td>198</td>
<td>58 (29)</td>
<td>149</td>
<td>20 (14)</td>
<td>41</td>
<td>0 (0)</td>
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</tbody>
</table>

* Lymphomatoid papulosis in all six cases.
† Cases classified as ALCL and immunoblastic included for analysis.
assay with a higher incidence in childhood cases and cases with non-B phenotype. This translocation appears undetectable in reactive hyperplasia and also negative in the majority of HD. The positive HD cases may be due to inclusion of some overlapping ALCL cases or the presence of rare Reed-Sternberg cells with t(2;5) as an expression of cytogenetic instability in the tumor cell population.

ACKNOWLEDGMENT

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REFERENCES

22. Ngan B: The presence of transcripts of the fusion of kinase gene ALK to nucleophosmin gene NPM in the t(2;5)(p23;q35) translocation defines subsets of non-Hodgkin's lymphoma with or without CD30 (Ki-1) expression and Hodgkin's disease. Mod Pathol 8:118A, 1995 (abstr)
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