Lack of the t(2;5) or Other Mutations Resulting in Expression of Anaplastic Lymphoma Kinase Catalytic Domain in CD30+ Primary Cutaneous Lymphoproliferative Disorders and Hodgkin’s Disease

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The t(2;5) (p23;q35) chromosomal translocation has been found in a high proportion of lymph node-based CD30+ large cell lymphomas of T-cell lineage. This translocation is believed to result in the expression of a fusion protein containing the catalytic domain of anaplastic lymphoma kinase (ALK) under the control of the promoter for nucleophosmin, a nucleolar phosphoprotein. Expression of ALK activity, which does not normally occur in lymphocytes, is postulated to be involved in the pathogenesis of lymphomas bearing the t(2;5) translocation. Several primary cutaneous lymphoproliferative disorders and Hodgkin’s disease are also known to contain CD30+ large lymphoid cells. To determine the role of the t(2;5) translocation in these diseases, we developed a DNA-based polymerase chain reaction (PCR)/Southern blot assay to detect this translocation at the genomic level in lymphomatoid papulosis (14 cases), primary cutaneous CD30+ large cell lymphoma of T-lineage (10 cases) and Hodgkin’s disease (13 cases). Two cases of pityriasis lichenoides were also studied. The t(2;5) translocation was not present in any of these specimens. To determine if some other somatic mutation might have resulted in inappropriate expression of ALK catalytic domain, we devised an RNA-based reverse transcriptase-PCR assay to detect transcripts encoded by this ALK region. None were found in the six additional cases of lymphomatoid papulosis that were studied. In aggregate, these results strongly suggest that inappropriate expression of ALK is not involved in the pathogenesis of these CD30+ lymphoproliferative disorders, and that lymph node–based CD30+ large cell lymphoma is a disease that is biologically distinct from skin-based CD30+ lymphoproliferative disorders and Hodgkin’s disease. Using methods developed for this report, we also cloned and sequenced the t(2;5) genomic junctional sequences present in the SUP-M2 and SU-DHL-1 cell lines. These intron sequences will be useful for mapping t(2;5) breakpoint clusters.

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Materials and Methods

Patient samples. Genomic DNA for t(2;5) and NPM studies was extracted from 39 tissue samples from 37 patients diagnosed with one or more of the following: lymphomatoid papulosis, primary cutaneous CD30+ large cell lymphoma of T-lineage, HD, or pityriasis lichenoides. Standard clinico-pathologic criteria were used to establish the diagnosis in each case. Tables 1 and 2 summarize important clinicopathologic features including the cytologic subtypes of the large cell lymphomas and HD cases, the types of tissues analyzed, and the associated lymphoproliferative disorders that were present in six cases. All specimens were primary untreated lesions obtained from adults at least 25 years old. All large cell lymphomas had a primary cutaneous presentation. Expression of CD30 and T-lineage antigens (CD2, CD3, and/or CD5) by large cell lymphomas and a variable subset of cells in the lymphomatoid papulosis cases was confirmed in all cases using immunoperoxidase techniques as described previously.

RNA for ALK studies was extracted from

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human tissue samples of patients with lymphomatoid papulosis, DNA and RNA extraction were performed using standard methods.3

Controls. The lymphoma cell lines SUP-M2 and SUP-DHL-1, kindly provided by Dr Stephen D. Smith (University of Chicago, Chicago, IL), were used as positive controls for t(2;5) translocations and ALK catalytic domain transcript expression. The Jurkat cell line (clone E6-1, human acute T-cell leukemia) was obtained from the American Type Culture Collection (Rockville, MD) and was used as a positive control for NPM studies and as a negative control for t(2;5) and ALK transcript studies. RNA samples extracted from normal breast skin and tonsil also served as negative controls for ALK transcript studies.

Primers and probes. All primers were synthesized by the Molecular Biology Core Laboratory at Case Western Reserve University (Cleveland, OH). For genomic t(2;5) amplification, the following primers specific for the NPM-ALK translocation were used: 5' NPM: 'ACTTAGTAGTGTACCGCCGGA-3', containing the NPM/ALK domain, and 3' ALK: 5'-GCACTCCAAAGGACCATGTGTCTG-GAA-3' and 3' ALK: 5'-GCACTCCAAAGGACCATGTGTCTG-GAA-3'.

The NPM was used with the primer 3' NPM: 5'- GCTACCACCTCCAGGGGCAGA-3' to amplify the ubiquitously expressed NPM gene as a control for genomic DNA integrity. The nested oligonucleotide-5'- AGC-ACCTTAGTAGTGTACCGCCGGA-3', containing the NPM/ALK fusion protein junction region, was used as a probe for confirmatory Southern blot analysis. The primers 5'- ALK: 5'-CATGGCGCTTGGGGAGTTGTATG-3' and 3' ALK: 5'-GCACTCCAAAGGACCATGTGTCTGGT-TTATAGA-3' were designed by us to amplify the catalytic domain of ALK transcripts from RNA. The nested oligonucleotide-5' -AAAATCCAGTTCGCTCTGTCTTGACAGGACACTT-3', specific for an internal region of this ALK transcript, was used as a probe for confirmatory Southern blot analysis.

Genomic polymerase chain reaction (PCR) analysis of t(2;5) and NPM. The conditions of PCR analysis were as follows: 94°C for 1 minute, 62°C for 1 minute, 72°C for 2 minutes 30 seconds (34 cycles); 72°C extension for 7 minutes (1 cycle). Each PCR reaction used 2 μg genomic DNA, 1× PCR buffer (Perkin-Elmer, Norwalk, CT), 200 μmol/L each deoxynucleoside-5'-triphosphate (dNTP), 10 pmol each primer, 2 mmol/L MgCl₂, 2.5 U AmpliTaq DNA polymerase (Perkin-Elmer) in a final volume of 100 μL. Applications were performed in a Perkin-Elmer 480 Thermocycler. Amplification of t(2;5) product from the SUP-M2 and SUP-DHL-1 cell lines were used as positive controls. DNA from the Jurkat cell line was used as a negative control.

PCR amplification of human NPM gene was used as a control for DNA integrity and produced a PCR product 185 bp long. The specificity of genomic PCR products was confirmed by Southern analysis using nested oligonucleotide probes labeled with α-32P-deoxyadenosine-5'-triphosphate (dATP) (previously described). The SUP-M2 and SU-DHL-1 genomic DNA yielded t(2;5) PCR products of approximately 1,600 bp and 1,700 bp, respectively.

Reverse transcriptase-PCR (RT-PCR) analysis of ALK catalytic domain. The conditions of the RT reaction were as follows: 25°C for 10 minutes, then 37°C for 75 minutes. The RT reaction contained 2 μg or 0.5 μg RNA, 1× Taq Extender buffer (Stratagene, La Jolla, CA), 416 μmol/L each dNTP, 125 μmol/L each dNTP (in the subsequent PCR reaction, this concentration becomes diluted to 125 μmol/L), 5 pmol 3' primer, 5 pmol 5' primer, 10 pmol 5' primer, 5 pmol of the 3' primer, 25 pmol of ALK transcript, and 2.5 U AmpliTaq DNA polymerase (Perkin-Elmer), and 2.5 U Taq Extender PCR additive (Stratagene) in a final volume of 100 μL. Amplifications were carried out in a Perkin-Elmer’s 480 Thermocycler. The SUP-M2 and SU-DHL-1 cell lines were used as positive controls for amplification of t(2;5) product. RNA from the Jurkat cell line, normal skin and tonsil were used as negative controls. RT-PCR amplification of human NPM gene was used as a control for RNA integrity. PCR products were analyzed by Southern analysis. An ALK nested oligonucleotide, labeled with α-32P-dATP, was used as a probe (previously described).

Southern blot analysis. Ten microliters of the PCR reaction products was electrophoresed in a 1% agarose gel and then vacuum transferred to a nylon membrane (GeneScreen 45; Pharmacia, Piscataway, NJ). Membranes were prehybridized and then hybridized at 50°C overnight in a solution of 5X SSPE, 5X Denhardt's, 0.5% sodium dodecyl sulfate (SDS), and the appropriate 5'α-32P end-
labeled oligonucleotide probe. Blots were washed twice in 2× SSC, 0.1% SDS, first for 5 minutes at room temperature, then for 20 minutes at room temperature. A third wash was performed in 0.5× SSC, 0.1% SDS for 30 minutes at 37°C. Blots were exposed to x-ray film with intensifying screens at ~80°C.

**Nucleotide sequence analysis.** Ligation of PCR products and transformations were performed using the TA Cloning Kit (Invitrogen). Sequencing reactions were performed using the AutoRead Sequencing Kit (Pharmacia). Seven microliters of the reactions were run on a 6% polyacrylamide gel (Gel-Mix 6; GIBCO-BRL) at 45°C. Sequences were then analyzed using the ALF Automated Nucleotide Sequencer (Pharmacia).

**RESULTS**

Analysis of SUP-M2 and SU-DHL-1 cell lines using a genomic PCR assay for the t(2;5). Prior studies of the t(2;5) in CD30⁺ lymphomas relied on an RT-PCR assay which necessitated the use of RNA targets extracted from lesional tissues. This approach poses special problems for skin biopsies, which are usually too small to yield both the RNA needed for t(2;5) RT-PCR analysis and the DNA needed for clonality analysis of antigen receptor gene rearrangements. To allow analysis of DNA samples already in our specimen library, to avoid the need for additional biopsies, and to avoid the need to process more labile RNA samples, we developed a PCR-based method for detecting the t(2;5) in genomic DNA extracts as detailed in Materials and Methods. Using this DNA-based assay, we successfully amplified the genomic DNA segments containing the t(2;5) breakpoint from two cell lines, SUP-M2 and SU-DHL-1, known to contain this translocation.¹ The identity of the PCR products was confirmed by Southern blot analysis using a nested oligonucleotide probe. Typical results are shown in Fig 1. Although these genomic t(2;5) PCR products are relatively large, they are clearly within the amplifiable range. We cloned and sequenced these PCR products. The sequences are detailed in Fig 2. The genomic nucleotide sequences of t(2;5) in mycosis fungoides (MF)-associ-
ated diseases. Using the genomic PCR/Southern blot assay previously described, we analyzed a variety of MF-associated diseases for the presence of the t(2;5) translocation. These cases are detailed in Table 1 and included 14 cases of lymphomatoid papulosis, 13 cases of HD, 9 cases of primary cutaneous CD30+ large cell lymphoma and 2 cases of pityriasis lichenoides. None of these cases contained a detectable t(2;5). Representative findings are shown in Fig 1.

Analysis of lymphomatoid papulosis using a RT-PCR assay for ALK catalytic domain. To determine if some somatic mutation other than the t(2;5) occurred in MF-associated diseases and thereby resulted in inappropriate expression of ALK functional activity via some novel mechanism, we designed an RT-PCR assay for the detection of transcripts containing the ALK catalytic domain as detailed in Materials and Methods. The identity of the ALK RT-PCR product was confirmed by Southern blot analysis using a nested oligonucleotide probe. Six additional cases of lymphomatoid papulosis had RNA available for this type of RT-PCR analysis. None of them contained detectable message for the ALK catalytic domain. Representative findings are shown in Fig 3.

DISCUSSION

Using a genomic PCR assay, we have shown that the t(2;5) (p23; q35) chromosomal translocation was not detectable in primary cutaneous CD30+ large cell lymphoma of T lineage, lymphomatoid papulosis, pityriasis lichenoides, or HD. False-negative results due to DNA degradation were excluded by successful amplification of a 185-bp genomic NPM sequence in all DNA samples. Successful amplification and sequencing of distinct t(2;5) genomic PCR products from two different cell lines confirmed the specificity of our assay. It could be argued that genomic PCR assays for the t(2;5) might be less sensitive than RT-PCR assays because the former targets only one copy per cell whereas the latter targets multiple transcripts. On the other hand, RNA targets are more susceptible to degradation during tissue processing and the level of t(2;5) transcripts might vary depending on the condition of the cells at the time of biopsy. In any event, genomic PCR assays of other translocations, such as the t(14;18) in follicular lymphomas, have been shown to be able to detect small numbers of tumor cells. Similarly, our own genomic PCR/Southern blot assay had a sensitivity threshold of at least 10^-2, making it suitable for analysis of our cases. In all likelihood, detection of genomic t(2;5) PCR products is enhanced by the ability of each amplified genomic t(2;5) fragment to hybridize with two copies of the nested NPM/ALK junctional cDNA probe because the genomic t(2;5) PCR product has its NPM and ALK sequences separated by a large intron region. Furthermore, the validity of our genomic PCR results was supported by the results of our RT-PCR assay, which we used to analyze an additional group of lymphomatoid papulosis cases in which RNA was available for study. This assay showed that there was no detectable expression of ALK catalytic domain, such as might occur as the result of the t(2;5) or some other somatic mutation involving the ALK gene. In aggregate, these studies strongly suggest that ALK gene mutations are not typically involved in the development of any of the lymphoproliferative disorders that we analyzed.

Our findings are consistent with a prior study in which none of four primary cutaneous CD30+ lymphomas were t(2;5)-; however, another study found 2 out of 6 cases to be t(2;5)'. Considering our 10 additional cases, it would appear that the prevalence of the t(2;5) among primary cutaneous CD30+ lymphomas is low, ie, 2 out of 20 (10%). These findings contrast with the relatively common occurrence of the t(2;5) translocation in primary extracutaneous CD30+ large cell lymphomas, especially pediatric cases.'1.3.10.11 However, it is apparent that many differences exist between these tumors and those that are primary in the skin. For example, lymph node–based CD30+ large cell lymphomas occur more often in pediatric patients, generally express epithelial membrane antigen, generally lack cutaneous lymphocyte-associated antigen, and have a worse prognosis.12 These differences strongly suggest that despite similar histopathologic features, t(2;5)' and t(2;5) CD30+ large cell lymphomas are distinct biological entities that arise through different pathogenetic mechanisms. In addition, our current findings indicate that primary cutaneous CD30+ large cell lymphomas are typically t(2;5) and that this distinction is also shared by lymphomatoid papulosis, pityriasis lichenoides, and HD.

Our results are in agreement with prior studies of more than 100 cases of American HD, which found no evidence of the t(2;5) using RT-PCR10.13.15 and only 2 out of 9 cases positive for NPM translocations using genomic Southern blotting. In contrast, one study of European HD found evidence of the t(2;5) translocation by RT-PCR in 11 out of 13 cases.16 The resolution of this discrepancy will require analysis of additional European cases.
Recent studies of T-cell receptor gene rearrangements have shown a common clonal origin for multiple cutaneous lymphoproliferative disorders arising in individual patients. This includes mycosis fungoides and lymphomatoid papulosis; lymphomatoid papulosis and large cell lymphoma; lymphomatoid papulosis, large cell lymphoma, and HD. The association among these disorders is not uncommon. For example, there is a 5% to 20% association between lymphomatoid papulosis and these other conditions, and large cell transformation has been noted in as many as 18% of mycosis fungoides cases in some series.6

The phenomenon of associated diseases, including transformation to more clinically aggressive forms of neoplasia, is not unique to cutaneous lymphoproliferative disorders. Peripheral T-cell lymphomas, including small cell and lymphoepithelioid variants, may transform to large cell lymphoma.21 About 25% of follicular B-cell lymphomas transform to diffuse large cell lymphoma or rarely to acute lymphoblastic leukemia.22 Approximately 10% of B-cell chronic lymphocytic leukemia transform to large cell lymphoma, a phenomenon known as Richter’s syndrome.23 Similarly, chronic myelogenous leukemia (CML) routinely eventuates in “blast crisis.”24 As with cutaneous lymphoproliferative disorders, it has been shown for follicular lymphomas, Richter’s syndrome,25,26 and CML27 that the transformed tumor shares the same clonal origin as the original neoplasm.

Although this clonal relationship helps to explain the clinical association among these diseases, it does not provide a pathogenetic mechanism for the occurrence of more than one of these disorders in the same patient. Sequential somatic mutations have been shown in certain lymphoid neoplasms arising sequentially in individual patients. For example, studies of the transformation of follicular B-cell lymphoma to acute lymphoblastic leukemia have shown the sequential acquisition of genetic abnormalities such as t(14;18) and t(8;14) chromosomal translocations.28 It remains to be determined whether the same series of somatic mutations are involved in the development of multiple cutaneous lymphoproliferative disorders. However, if such a series exists, our findings strongly suggest that mutations involving the ALK gene are not involved because none were detected in patients with multiple disorders including transformation of mycosis fungoides to large cell lymphoma (Table 2).

In addition to the findings noted earlier, our report also describes novel molecular biologic assays for DNA-based testing for the t(2;5) translocation and RNA-based testing for expression of ALK catalytic domain. A DNA-based, nested PCR assay has also been described for detection of the t(2;5); however, it lacked confirmatory Southern blot analysis and theoretically could be more prone to false-positive results secondary to contamination.29 The assays developed in our current study should prove useful for the assessment of possible ALK gene mutations in other diseases. We used our DNA-based method to determine the genomic DNA sequence of the t(2;5) translocations present in two tumor cell lines, SUP-M2, and SU-DHL-1. Once the germline sequence of either the NPM or ALK gene is known, it will be possible to use these and other t(2;5) translocation sequences to develop a map of the genomic breakpoint cluster(s) involved in this mutation. Usage of genomic PCR methods such as the one developed during this report should facilitate this work. The t(2;5) has been detected by RT-PCR in RNA extracted from paraffin sections (10,11); however, frozen tissue is generally considered superior for RT-PCR analysis. The design of new primers complementary to intron sequences flanking the genomic t(2;5) breakpoints could allow the analysis of much shorter DNA regions than the 1,600 to 1,700 bp described in this study. This would create a t(2;5) genomic PCR assay more suitable for analysis of the fragmented DNA present in formalin-fixed, paraffin-embedded tissues, thereby obviating the need for fresh tissue and allowing DNA-based analysis of the t(2;5) in archival specimens.

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