Amplification of Genomic DNA Demonstrates the Presence of the t(2;5)(p23;q35) in Anaplastic Large Cell Lymphoma, But Not in Other Non-Hodgkin's Lymphomas, Hodgkin's Disease, or Lymphomatoid Papulosis

By Andreas H. Sarris, Rajyalakshmi Luthra, Valli Papadimitracopoulou, Mimi Waasdorp, Meletios A. Dimopoulos, J.A. McBride, Fernando Cabanillas, Madeleine Duvic, Albert Deisseroth, Stephan W. Morris, and William C. Pugh

Anaplastic large cell lymphoma (ALCL) is a distinct clinicopathologic variant of intermediate grade non-Hodgkin's lymphomas (NHL) composed of large pleomorphic cells that usually express the CD30 antigen and inter leukin (IL)-2 receptors, and is characterized by frequent cutaneous and extranodal involvement. With variable frequency ALCL bear the t(2;5)(p23;q35) chromosomal translocation that fuses the nucleophosmin (NPM) gene on chromosome 5q35 to a novel protein kinase gene, Anaplastic Lymphoma Kinase (ALK), on chromosome 2p23. We determined the frequency of this translocation with a novel DNA polymerase chain reaction (PCR) technique using 0.5 μg of genomic DNA, 5'-primers derived from the NPM gene and 3'-primers derived from the ALK gene and hybridization with internal probes. The presence of amplifiable DNA in the samples was tested in the inclusion PCR reaction of oligonucleotide primers designed to amplify a 3016-bp fragment from the β-globin locus. NMP-ALK fusion amplicons were detected using DNA isolated either from all three ALCL cell lines tested, or from all four primary ALCL tumors known to contain the (2;5)(p23;q35) translocation. Nested amplicons were detected by hybridization in 100% of specimens diluted 104-fold and in 20% of those diluted 105-fold. We subsequently examined archival genomic DNA from 20 patients with ALCL, 39 with diffuse large cell, 2 with mantle cell, 20 with peripheral T cell, 13 with low-grade NHL, 31 with Hodgkin's disease (HD), and 6 with lymphomatoid papulosis. Fusion of the NPM and ALK genes was detected in three of 18 patients with ALCL who had amplifiable DNA (17%, 95% confidence intervals 4% to 41%), but not in any patients with other NHL, HD, or lymphomatoid papulosis. The amplicon sizes were different in all cell lines and patients reflecting unique genomic DNA breakpoints. We conclude that with genomic DNA-PCR the rearrangement of the NPM and ALK loci is restricted to patients with ALCL. Further studies are needed to determine the prognostic significance of the NPM-ALK rearrangement, to determine whether its detection can aid in the differential diagnosis between ALCL, Hodgkin's disease, and lymphomatoid papulosis, and to establish the usefulness of the genomic DNA PCR in the monitoring of minimal residual disease in those patients whose tumors bear the t(2;5).

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and has led some to consider them extremes on a continuous spectrum of malignant lymphoid neoplasms. This morphologic overlap, the clinical and morphologic similarity between lymphomatoid papulosis and ALCL, especially the primary cutaneous form, and the report that a common T-cell clone was involved in cutaneous T-cell lymphoma, HD, and LP that developed over time in one patient has suggested the existence of a biologic relationship between these disorders.

Cytogenetic analysis showed the presence of the t(2;5)(p23;q35) chromosomal translocation with variable frequency in ALCL. With cell lines derived from patients with ALCL, Morris et al. at St Jude Children's Research Hospital demonstrated that the t(2;5)(p23;q35) chromosomal translocation fuses sequences from a nuclear phosphoprotein gene, called nucleophosmin (NPM), that is located on chromosome 5q35 to sequences of a novel gene, designated Anaplastic Lymphoma Kinase (ALK), that is located on chromosome 2p23. The NPM gene is highly conserved and its protein product is involved in the late stages of ribosomal assembly. The ALK gene codes for a novel transmembrane protein kinase that has sequence homology to the β-chain of the insulin receptor, the α-chain of the insulin-like growth factor-1 receptor, the leukocyte tyrosine kinase, and the Drosophila homologue Sevenless. The t(2;5)(p23;q35) translocation results in the formation of a fusion protein consisting of amino-terminal sequences derived from the NPM gene fused to carboxy-terminal cytoplasmic sequences from the ALK gene, including the consensus protein tyrosine kinase residues. The breakpoint locations predicted that this fusion protein would lack the extracellular and transmembrane domains of ALK and would thus be expected to be located intracellularly. In fact, such a cytoplasmic protein kinase was independently isolated as a hyperphosphorylated phosphotyrosine containing protein of Mr 80,000 daltons from a cell line bearing the t(2;5)(p23;q35) translocation and was subsequently demonstrated to be the product of the NPM-ALK gene fusion. Antibodies raised against the kinase domain of the ALK reacted with lymphoma cells in biopsies of all three patients with ALCL bearing a t(2;5)(p23;q35) translocation.

We now report the frequency of the t(2;5)(p23;q35) translocation among adult patients with non-Hodgkin's lymphoma (NHL), HD, and lymphomatoid papulosis seen at the University of Texas M.D. Anderson Cancer Center (MDACC). We used a newly developed polymerase chain reaction (PCR) assay that uses genomic DNA, 5'-primers derived from the NPM gene, and 3'-primers derived from the ALK gene.

MATERIALS AND METHODS

Reagents and enzymes. All chemicals were reagent grade or better. Restriction endonucleases were obtained from Gibco-BRL (Gaithersburg, MD) and AmpliTaq polymerase from Perkin Elmer-Cetus (Foster City, CA). Antibodies for immunostaining were obtained from DAKO (Carpinteria, CA) and included monoclonal antibodies against CD15 (Leu M-1), CD20 (L26), CD30 (Ber-H2), CD45 (leukocyte common antigen), CD45RO (UCHL-1), epithelial membrane antigen (EMA), and polyclonal anti-CD3.

Cells and tissues. The t(2;5)(p23;q35)-positive cell lines SUP-M2, SU-DHL-1 and UCONN-L29,31,32 were obtained from St Jude Children's Research Hospital and were the source of the high molecular weight genomic DNA that was used as a positive control in DNA-PCR amplification experiments. The genomic DNA of four additional patients with ALCL known to contain the t(2;5)(p23;q35) gene rearrangement were obtained from St Jude Children's Research Hospital (S.M.). Patients referred to the University of Texas MDACC for diagnosis and treatment of malignant lymphoma were the subjects for this study if archival genomic DNA was available for analysis. Surgical biopsies were obtained for routine diagnostic studies after signed informed patient consent was obtained according to standard institutional guidelines and high molecular weight genomic DNA was extracted from tissue not used for standard diagnostic tests. Immunohistochemical studies were performed on paraffin sections using conventional avidin-biotin-peroxidase methodology. Pathologic classification of NHL was performed according to the REAL classification, of ALCL according to the criteria summarized by Pileri et al., and of HD according to Lukes and Butler. The diagnosis of lymphomatoid papulosis was based on previously described combined clinicopathologic criteria.

Restriction analysis. DNA aliquots were digested with the appropriate restriction enzymes (Hind III, BamHI, Bgl II), blotted onto nylon filters, and hybridized with radioactive probes using standard methods.12 Because genomic DNA fragments located immediately adjacent to the chromosome 2 breakpoint within the ALK gene were not available at the time of the study, filters were probed with p16-3/1.25, which is a 1.2 kb Sac I fragment of the NPM gene within 7 kb centromeric of the NPM breakpoint in cell lines SU-DHL-1 and SUP-M2. This fragment detects NPM gene rearrangements in genomic DNA digested with many restriction enzymes.12 We also used probe p21-3/3E, which is a 3-kb EcoR I genomic fragment of the NPM gene located approximately 15 kb telomeric of the breakpoint in cell lines SU-DHL-1 and SUP-M2. This probe detects rearranged NPM fragments in genomic DNA restricted with Bgl II.12 As positive control, we used DNA from the SUP-M2 cell line derived from a patient with ALCL with the t(2;5)(p23;q35) chromosomal translocation, and as control normal human genomic DNA. Because both hybridization probes contained human genomic repetitive DNA sequences, the probes and filters were preannealed with cold competitor DNA.

Genomic DNA PCR (DNA-PCR). This was performed using a modification of a previously described method.17 We used as outer 5'-end primer oligonucleotide NPM-A, derived from the NPM gene and as 3'-end primer oligonucleotide ALK-A derived from the ALK gene (Fig 1). As a control for the presence of amplifiable DNA able to generate large amplicons, we included a second primer pair that would generate 3016 fragment from the β-globin region. The 5'-prime oligonucleotide was 5'-GAAGAGCCAAGGACAGGTAC-3', and the 3'-prime oligonucleotide was 5'-GTTTGATGCTAGCTCC-AC TTC-3'. The final reaction mix contained 0.5 μg of genomic DNA, 10 mmol/L Tris-Cl, pH8.3, 250 μmol/L of each deoxyribonucleotide triphosphate, 50 μmol/L KCl, 1.5 mmol/L MgCl2, and 50 ng of each primer, in a total volume of 50 μL, overlaid with 35 μL of mineral oil. The reaction tubes were heated at 80°C for 5 minutes before the addition in each tube of 1.5 U of AmpliTaq polymerase. After hot start with the enzyme, the samples were denatured for an additional minute at 94°C, and then were subjected to 35 cycles of PCR by denaturing at 94°C for 40 seconds, annealing at 64°C for 1 minute, and extending at 72°C for 4 minutes. The last elongation step lasted for 10 minutes. One microliter of the initial PCR product was used as a template for the nested PCR reaction that was prepared as before, but used as nested 5'-end primer oligonucleotide NPM-B, derived from the NPM gene, and as nested 3'-end primer oligonucleotide ALK-B, derived from the ALK gene (Fig 1). Following a 1-minute denaturation at 92°C, the reaction mix was...
subjected to 20 cycles of amplification, each consisting of a 40-second denaturation step at 92°C, a 1-minute annealing step at 54°C, and a 3-minute elongation step at 72°C. The last elongation step was 10 minutes. The amplified products (18 μL) were analyzed by electrophoresis on a 0.8% agarose gel, stained with ethidium bromide, visualized, and photographed under ultraviolet light. The gels were subsequently transferred manually to Sure Blot membranes (Oncor, Gaithersburg, MD) in alkaline conditions according to the instructions provided by the manufacturer. The membranes were hybridized with a T4 nucleotide kinase end-labeled NPM-ALK junctional oligonucleotide probe, 5'-AGCACTTAGTAGTGTACCGCC-3', and labeled fragments were detected by autoradiography.

Clinical information. The records of all patients were retrieved and reviewed to establish the clinical presentation, laboratory and immunologic characteristics of the tumors, the Ann Arbor stage51 and the International Prognostic Index score.52 Final stage was determined in a multidisciplinary conference where all clinical, radiologic, and laboratory data were reviewed by participating hematologists, medical oncologists, radiotherapists, and radiologists. The 95% confidence intervals were calculated with the BICSI software (Department of Biostatistics, University of Texas MDACC, Houston, TX).

RESULTS

To establish the PCR assay, we first amplified genomic DNA isolated from three cell lines derived from patients with ALCL shown to carry the t(2;5)(p23;q35) by classical cytogenetics, restriction enzyme analysis, and reverse transcriptase PCR.28 Amplification of 0.5 μg of genomic DNA gave rise to amplicons of 1,603, 1,677, and 1,715 bases (Fig 2A, lanes 9 to 14), with comparable yield by either outer primer or nested PCR. The different sizes of these amplicons were confirmed by DNA sequencing that demonstrated different molecular breakpoints in each (data not shown). With serial dilution of 0.5 μg of genomic DNA (corresponding approximately to 60,000 to 80,000 cells) from the UCONN-L2 cell line into normal genomic DNA, it was demonstrated that nested amplicons were consistently detected by autoradi-
Table 1. Molecular Detection of Rearrangement Involving the NPM and ALK Genes in Patients With Lymphoma

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Abbreviation: CI, confidence interval.

* Includes 39 patients with diffuse large cell, 20 with peripheral T cell, 2 with mantle cell, 5 with follicular, 4 with mucosa-associated, and 4 with small lymphocytic lymphomas. Three patients with diffuse large cell lymphoma reflected Richter's transformation of chronic lymphocytic leukemia.

Table 2. Clinical and Laboratory Characteristics of Patients With ALCL and Detection of NPM-ALK Rearrangements

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Abbreviations: U, unstageable; NA, not available; ND, not done; R, rearranged; G, germline; TCR, T-cell receptor; IG, immunoglobulin heavy chain; CLL, chronic lymphocytic leukemia; IPI, International Prognostic Index; EMA, epithelial membrane antigen; IP, immunophenotype.
with the classical nodal ALCL, and two developed ALCL in the background of treated small lymphocytic lymphoma and chronic lymphocytic leukemia, both of B-cell phenotype. The lymphoma cells were CD30-positive in 17 (85%), and were of T-cell lineage in 12 (60%), B-cell lineage in five (25%), and null in three patients (15%). In the two patients with preexistent low-grade B-cell lymphoproliferative disorders, the ALCL were null cell in one and T cell in the other (patients 8 and 10, respectively). Five patients (25%) were initially diagnosed as HD (patients 1, 4, 5, 14, and 19) and four (patients 1, 4, 5, and 14) were actually treated as HD before referral to MDACC where the diagnosis was changed to ALCL based on morphology and immunophenotype. Patient 2 was initially diagnosed and treated as seminoma with platinum, etoposide, and bleomycin without response. The diagnosis was subsequently changed to HD and he was treated with mechlorethamine, vincristine, procarbazine, and prednisone and he achieved a complete remission that lasted 6 months. He was then referred to MDACC where the diagnosis was changed to ALCL. Patient 19 was initially diagnosed as HD, but was referred for an opinion to MDACC where the diagnosis was changed to ALCL, Hodgkin’s-related variant, before any therapy was given. The changes in diagnosis were also supported in three of these five patients by clonal rearrangement of the immunoglobulin genes in one (Table 2, patient 4), of the β-chain of the T-cell receptor in two (patients 5 and 19). All diagnostic revisions were completed before to the analysis of NPM-ALK rearrangement.

The rearrangement of the NPM locus was detectable with restriction analysis of genomic DNA using probes derived from the NPM gene in two patients of 17 and 37 years of age (Table 2, patients 2 and 7). Nested amplification of genomic DNA generated amplicons of 1,190 and 2,200 bases, confirming the NPM-ALK rearrangement (Table 2, patients 2 and 7 respectively, and Fig 3, lanes 2 and 7). One 9-year-old patient without detectable NPM rearrangement by restriction analysis exhibited an NPM-ALK amplicon of 900 bases by nested DNA PCR (Table 2 patient 5, and Fig 3, lane 5). The sizes of these amplicons were clearly distinguishable from each other and from the amplicons generated from the cell lines or from the four validation patients (Fig 1A). All three patients with the NPM-ALK rearrangement presented with nodal disease, two were both T cell and CD30-positive (patients 2 and 5), whereas the third was null cell and CD30-negative (patient 7). Pathologically two patients had classical and one monomorphic ALCL. Overall, in this group of 18 patients with ALCL and amplifiable DNA, the NPM-ALK rearrangement was observed in 17% (95% CI of 4% to 41%). If the two patients with the primary cutaneous ALCL and the one with preexisting low grade lymphoma are excluded, the translocation was detected in three of 15 patients with nodal ALCL for an overall frequency of 20% and 95% CI extending from 4% to 48%. Similarly, among these 15 patients with primary nodal ALCL, the translocation was seen in three of nine who were younger than 40 years (33%, 95% CI 7% to 70%). The diagnosis of ALCL was correctly established at presentation in all three patients bearing the NPM-ALK gene rearrangement. One of them relapsed after 5 months and expired after 11 months from disseminated lymphoma despite appropriate initial and salvage therapy (Table 2, patient 2). The other two remain in unmaintained complete remission at 27 and 50 months after presentation (Table 2, patients 5 and 7).

**DISCUSSION**

We report the frequency of the NPM-ALK chromosomal translocation in patients with lymphoma and lymphomatoid papulosis with a nested PCR technique using genomic DNA, S'-primers derived from the NPM gene and 3'-primers derived from the ALK gene. This technique detects the NPM-
ALK rearrangement without the requirement for gene transcrip
tion implicit in reverse transcriptase-based PCR tech
iques and can be performed using genomic DNA that is 
routinely prepared in most pathology laboratories without 
the need for special tissue processing for extraction of 
DNA. Almost all cases of ALK that have been analyzed so 
the need for special tissue processing for extraction of 
techniques and can be performed using genomic DNA that is 
DNA-PCR generates amplicons of different size for each 
patient. This is a reflection of different molecular 
introns. As restriction analysis has demonstrated tight clus-
terings in the laboratory.

We did not detect the rearrangement in nonanaplastic large 
cell lymphomas, other NHLs, HD, or lymphomatoid pa-

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Abbreviations: C, cytogenetic analysis; RT, reverse transcriptase; ISH, in-situ hybridization; IM, immunocytochemistry.
* Only cases that were morphologically ALCL.
† Only ALCL with karyotypic t(2;5)(p23;q35) were studied.
‡ Excludes the five cases of Waggon et al because this was not a prevalence study.

One small study of patients with ALCL has re-
ported a frequency of 80% for NPM-ALK rearrangements,15-27 which is much higher than the frequency reported in pediatric 
or adult ALCL by either cytogenetics or by molecular meth-
ods by other investigators (Table 3). However, because of 
the small number of patients examined, the 95% CI ranged 
from 28% to 99% and it is not clear whether this higher 
apparent frequency is statistically higher than most results 
reported in Table 3. The frequency of the NPM-ALK gene 
rearrangement among all 425 reported ALCL patients is 
33%, with 95% CI extending from 29% to 38% (Table 3). 
The presence of the t(2;5) has also been reported in pediatric 
peripheral T-cell lymphoma,54 T-cell lymphomas of small 
irregular lymphocytes with few CD30 positive cells,7 and 
in nonanaplastic CD30-positive or negative pediatric NHL.27 
However, it is not yet clear whether this truly indicates the 
presence of the rearrangement outside the spectrum of ALCL 
or simply variations in the pathologic subclassification of 
NHL and ALCL, especially its monomorphic variant.47

A Japanese series of patients with ALCL has suggested that 
the NPM-ALK rearrangement (detected immunocyto-
chemically) was associated with better 5-year survival.52 
However, positive patients were younger and had lower rates 
of extranodal involvement than negative patients. These 
considerations, coupled with the absence of data on initial 
and postrelapse therapy, on the distribution of other prognostic 
factors, and on the presence of human T-lymphotropic virus-
I (HTLV-I) antibodies among the patients analyzed, makes 
the prognostic significance of the NPM-ALK rearrangement 
an issue that requires further investigation. The exact role 
of the t(2;5)(p23;q35) translocation in the pathogenesis of 
ALCL remains to be fully defined and larger numbers of 
patients are needed to determine its prognostic significance.

The NPM-ALK gene rearrangement was not detected in 
patients with HD by us (95% confidence interval 0% to 13%) 
and seven other series (see Table 4), but has been detected in 
four series with frequencies ranging from 5% to 85%.55-59,60,61,62 
Orscheschek et al62 reported the highest frequency of 85%,
DETECTION OF t(2;5) WITH GENOMIC DNA PCR

which was similar to the frequency of 80% reported by that group in unselected cases of ALCL.49 This extraordinarily high frequency is clearly outside the CI reported by most investigators for HD (Table 4) and remains unexplained. The frequency of the NPM-ALK rearrangement among all 368 screened cases of HD is 5%, with 95% CIs extending from 3% to 8%, and is much lower than the frequency of 33% for all reported ALCL patients (Tables 3 and 4). However, it is unclear whether all the reported HD patients bearing the NPM-ALK rearrangement had in fact HD, because the distinction of ALCL from HD can be difficult at times.3,11,22,37 This was evident in our series, because 25% of our patients with ALCL were initially diagnosed as having HD. Pathology review at MDACC resulted in revision of these diagnoses to ALCL based on morphologic criteria, immunophenotyping, and the detection of clonal rearrangements of the immunoglobulin and/or the T-cell receptor β-chain loci.

We conclude that the nested genomic DNA-PCR technique is useful for the rapid detection of the NPM-ALK rearrangement, that this rearrangement is confined to patients with ALCL, and is absent in other NHL, HD, and lymphomatoid papulosis. Further studies are needed to determine the prognostic significance of NPM-ALK rearrangement, to resolve whether its detection can aid in the differentiation of ALCL from HD or lymphomatoid papulosis, and to establish the usefulness of NPM-ALK DNA-PCR in monitoring minimal residual disease in those patients whose tumors possess the t(2;5).

ACKNOWLEDGMENT

We thank Jackie Creeks for assistance with the manuscript, Michael Cook for assistance with illustrations, and Dr Maureen Goode for scientific editing. We also express our gratitude to the physicians, nurses, social workers, and fellows of the Lymphoma Section who over the years cared for the patients included in this study.

REFERENCES


Table 4. Frequency of NPM-ALK Rearrangement in HD

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Abbreviations: RT, reverse transcriptase; ISH, in situ hybridization; IM, immunocytochemistry; C, cytogenetic analysis.


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Amplification of genomic DNA demonstrates the presence of the t(2;5) (p23;q35) in anaplastic large cell lymphoma, but not in other non-Hodgkin's lymphomas, Hodgkin's disease, or lymphomatoid papulosis

AH Sarris, R Luthra, V Papadimitracopoulou, M Waasdorp, MA Dimopoulos, JA McBride, F Cabanillas, M Duvic, A Deisseroth, SW Morris and WC Pugh