Chromosomal Abnormalities in Nodal and Extranodal CD30+ Anaplastic Large Cell Lymphomas: Infrequent Detection of the t(2;5) in Extranodal Lymphomas

German Ott,[1]* Tiemo Katzenberger,[1] Reiner Siebert,[2] John F. DeCoteau,[3] Jonathan A. Fletcher,[4] Joan H.M. Knoll,[5] Jörg Kalla,[1] Andreas Rosenwald,[1] M. Michaela Ott,[1] Klaus Weber-Matthiesen,[2] Marshall E. Kadin,[3] and Hans Konrad Müller-Hermelink[1]

[1]Institute of Pathology, University of Würzburg, Würzburg, Germany
[2]Department of Human Genetics, University of Kiel, Germany
[3]Department of Pathology, Beth Israel Hospital and Harvard Medical School, Boston, Massachusetts
[4]Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts
[5]Division of Genetics, Children's Hospital, Boston, Massachusetts

To determine the significance of the t(2;5)(p23;q35) translocation in nodal and extranodal anaplastic large cell lymphoma (ALCL), we performed cytogenetic, molecular genetic, and immunohistochemical analyses of tumor tissues from 11 patients with CD30+ ALCL. Three of five patients with nodal ALCL had additional infiltration of the skin. Six patients had extranodal ALCL, two had primary intestinal ALCL, three had a primary cutaneous ALCL, and one had osseous ALCL. Cytogenetic investigation detected the t(2;5) in all patients with nodal ALCL but not extranodal ALCL. Tumor cells in t(2;5)+ lesions also stained immunohistochemically for $p80^{NPM/ALK}$, whereas no staining for $p80^{NPM/ALK}$ was detected in extranodal ALCL. Two extranodal lesions had *NPM/ALK* fusion transcripts detected by nested reverse transcriptase–polymerase chain reaction. Fluorescence in situ hybridization analysis of these two lymphomas showed in one case a significant number (4%) of cells with a split hybridization signal, indicative of disruption of the *NPM* gene. Additional recurrent breakpoints observed in extranodal ALCL were 1p36, 6p25, and 8q24. Loss of genetic material occurred at 6q in one extranodal ALCL. Our results suggest that the t(2;5) more frequently plays a pathogenetic role in primary nodal than in extranodal ALCL and that this translocation may not be the primary event in some CD30+ ALCL. *Genes Chromosomes Cancer 22:114–121, 1998.*

INTRODUCTION

The translocation t(2;5)(p23;q35) has been shown to be strongly associated with CD30+ anaplastic large cell lymphomas (ALCL) of T-cell and null cell phenotype (Le Beau et al., 1989; Bitter et al., 1990; Ebrahim et al., 1990; Mason et al., 1990). Cloning of the breakpoints of the t(2;5) revealed the translocation to juxtapose the tyrosine kinase gene, *ALK*, at 2p23 with the nucleophosmin gene, *NPM*, at 5q35. By using the reverse transcriptase–polymerase chain reaction (RT-PCR) technique with *NPM* and *ALK* primers, it has been possible to detect the *NPM/ALK* fusion transcript in CD30+ ALCL carrying this particular translocation (Morris et al., 1994). A polyclonal antibody has been generated that specifically detects the chimeric protein $p80^{NPM/ALK}$ in cells positive for the t(2;5) or the *NPM/ALK* fusion mRNA (Shiota et al., 1994, 1995). Recently, two-color fluorescence in situ hybridization (FISH) assays have been developed for the detection of the t(2;5) or *NPM* disruption (Lu-Kuo et al., 1994; Weber-Matthiesen et al., 1996; Mathew et al., 1997).

Anaplastic large cell lymphomas may occur as de novo tumors or can evolve from other types of T-cell malignancies (Lennert and Feller, 1992). The most common primary site is the lymph node, but a morphologically identical variant has been described in the skin as the most common extranodal site (Kaudewitz et al., 1989). In addition, occasional cases of primary intestinal lymphomas with anaplastic large cell morphology and CD30 expression have been reported. However, to the best of our knowledge, no data have been published concerning chromosome banding analysis of cutaneous or intestinal CD30+ ALC lymphomas.

In the present paper, we report on chromosome banding findings in 11 patients with ALCL of both

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*Correspondence to: Dr. G. Ott, Institute of Pathology, Josef-Schneider-Str. 2, D-97080 Würzburg, Germany.

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TABLE 1. Clinical Findings

Case	Age/sex	Symptoms
1	9/m	Lymphadenopathy: jugular, mediastinal, abdominal
2	29/m	Lymphadenopathy: cervical, abdominal, inguinal
3	3/m	Axillary lymphadenopathy, reddish skin papules
4	24/f	Cervical and axillar lymphadenopathy, skin papules
5	12/m	Fever, erythematous maculopapular skin rash, generalized lymphadenopathy, hepatosplenomegaly
6	66/m	Known enteropathy for years, gastric ulcer, ascites
7	51/f	Primary large cell lymphoma in the small intestine in 1989; no clinical or morphological signs of enteropathy
8	67/f	Large tumor in the proximal humerus with extensive bone destruction; no extraosseous manifestations
9	20/m	Isolated nodule on the back; no signs of extracutaneous dissemination
10	54/m	Isolated skin nodules on arm
11	45/m	History of lymphomatoid papulosis for 6 years; no lymphadenopathy

nodal and extranodal origin and on additional molecular genetic, molecular cytogenetic, and immunohistochemical analyses for the detection of the t(2;5).

MATERIALS AND METHODS

Clinical Data

Fresh tumor tissues suitable for cytogenetic and molecular genetic analyses were obtained from 11 patients (8 men, 3 women) aged 3–67 years. A summary of clinical data and site of tumor origin is given in Table 1.

Histological Examinations and Immunophenotyping

Formalin-fixed and paraffin-embedded material was examined by routine histology using hematoxylin and eosin, Giemsa, periodic acid-Schiff (PAS) and Gordon-Sweet silver stains. Diagnostic immunophenotyping was carried out on paraffin and frozen sections with a panel of antibodies including B- and T-cell markers, CD15 (LeuM1), and CD30 (BerH2). In addition, stains were performed with the p80 antibody (Shiota et al., 1994; DeCoteau et al., 1996).

Cytogenetic Evaluations

Lymphoma specimens were disaggregated mechanically by pressure through 100-μm nylon gauze, and cell cultures were set up according to standard techniques. Harvesting was performed directly or after 24 h of culture. Metaphases from case 5 were studied on a cell line (JB6) established from peripheral blood (Kadin et al., 1990). Metaphases from cases 10 and 11 were obtained from unstimulated cell cultures at 2 weeks and 4 weeks, respectively. Slides were prepared by using conventional methods, and chromosomal abnormalities were described according to the International System for Human Cytogenetic Nomenclature (Mitelman, 1995).

RT-PCR Analyses

Reverse transcriptase-PCR was performed essentially as described by DeCoteau et al. (1996). Briefly, total cellular RNA was extracted and single-stranded cDNA was prepared from total cellular RNA by RT according to the manufacturer's recommendations (Perkin-Elmer, Branchburg, NJ). The cDNA was amplified in the presence of 1 U of *Taq* polymerase (Cetus, Emeryville, CA) and 5′*NPM* and 3′*ALK* primers in a total volume of 40 μl. The amplification conditions were denaturation at 95°C for 1 min, annealing at 58°C for 45 s, and extension at 72°C for 2 min. At the end of 35 cycles, PCR products were electrophoresed through 1.6% agarose gels containing ethidium bromide in Tris-Borate-EDTA buffer and visualized with ultraviolet light. The PCR products were then transferred to nylon membranes (GeneScreen Plus™, NEN Research Products, Boston, MA) and hybridized with an ^{32}P-end-labeled *NPM/ALK* junction oligonucleotide. Membranes were washed at high stringency and exposed to X-ray film. All negative cases were also analyzed by seminested PCR using 2 μl of the first-round amplification products and the 3′*ALK* and *NPM-ALK* oligonucleotides as primers. The primer sequences have been published by Morris et al. (1994): 5′*NPM*, 5′-TCCCTTGGGGGGCTTTGAAATAACACCC-3′, 3′*ALK*, 5′-CGAGGTGCGGAGCTTGCTCAGC-3′ and *NPM-ALK*, 5′-AGCACTTAGTAGTGTACCGCCGGA-3′. In all cases, amplification of human beta-actin mRNA was performed in parallel with commercially obtained primers (Clontech, Palo Alto, CA) to control for the integrity of the cDNA and PCR reactions. Negative controls included cases of follicular lymphomas and reactive lymph node hyperplasia.

FISH Analyses

Four cases (3, 6, 8, and 9) were investigated by FISH using two different YAC probes mapping to either side of the breakpoint region in the *NPM*

gene at 5q35 (Weber-Matthiesen et al., 1996). YAC clones 939F4 and 756A7 (CEPH, Paris, France) were prepared as previously described and labeled with biotin and digoxigenin, respectively. The hybridization procedure was performed on pepsin-digested cytospin slides obtained from cell suspensions in Carnoy's fixative. Cells were hybridized overnight with 1.5 µl of hybridization mixture (50% formamide, 10% dextran sulfate, 2× SSC, pH 7.5, 50 ng of each YAC probe, 100 µg of unlabeled human Cot-1 DNA in a total volume of 10 µl) after simultaneous denaturation at 75°C for 7 min. Posthybridization washes were performed three times in 0.1× SSC at 60°C for 5 min. The biotin-labeled YAC probe (756A7) was detected with a cascade using Cy3-conjugated avidin (Jackson/Dianova, Hamburg, Germany), biotinylated mouse anti-avidin antibody (Vector, Burlingame, CA), and again with Cy3-conjugated avidin (red fluorescence). The digoxigenin-labeled YAC probe (939F4) was visualized with a cascade using mouse anti-digoxigenin antibody, digoxigenin-conjugated sheep anti-mouse antibody, and Fluorescein isothiocyamate (FITC)-conjugated sheep anti-digoxigenin antibody (Boehringer Mannheim, Germany; green fluorescence). Cells were counterstained with Diamidine-phenylindole dihydrochloride (DAPI) and mounted in an antifade solution. Four hundred to six hundred cells per case were evaluated on a Zeiss Axiophot microscope. Illustrations were documented using the ISIS imaging system (MetaSystems, Altlussheim, Germany). A previous study has demonstrated that cases positive for the t(2;5) in cytogenetic analysis have a split of one signal pair in a significant number of cells. The percentage of cells with this derivative signal constellation in normal control cells (false positive cells) is less than 1% (Weber-Matthiesen et al., 1996).

RESULTS

Histopathology and Immunophenotyping

On the basis of morphology and immunophenotyping, all 11 cases were classified as CD30+ ALCL. Five lymphomas originated in lymph nodes, with three of them (cases 3–5) also involving the skin. Two cases were primary intestinal tumors. One additional lymphoma arose in the proximal humerus, and the remainder were primary cutaneous ALCL-type lymphomas, including one with a 6-year history of lymphomatoid papulosis, part of the spectrum of Ki-1+ cutaneous lymphomas (Paulli et al., 1995).

In the nodal cases 1–3 and 5, the infiltrate consisted of sheets of large blastic cells, sometimes with an intrasinusoidal growth pattern. Case 3 presented with simultaneous skin lesions, which consisted of atypical large cells and with small pleomorphic T-cells with clear cytoplasm. In the lymph node, only blast cells were observed. Case 4 was regarded as a lymphohistiocytic variant of ALCL as described by Pileri et al. (1990).

Case 6 consisted of biopsy specimens from gastric and duodenal mucosa in a patient with known enteropathy. Cytogenetic investigations were performed from ascitic fluid obtained by fine-needle aspirates. The diagnosis was enteropathy-associated primary T-cell lymphoma with ALCL-like morphology and involvement of the gastric mucosa, according to the REAL classification (Harris et al., 1994). In case 7, a lymph node biopsy showed a CD30+ ALCL-type lymphoma. Clinical information was obtained that a primary intestinal diffuse large cell lymphoma had been diagnosed 4 years earlier. Although immunophenotyping could not be performed on the material of the intestinal infiltrate, reevaluation of the slides showed that the lymph node infiltrate most probably was a metastasis of this primary intestinal lymphoma.

Immunophenotyping in all cases revealed strong positivity for CD30 and a T-cell phenotype in most of the cases; B-cell markers (CD19, CD20, CD22) or CD15 were not expressed. The exact phenotypes on frozen sections are listed in Table 2.

Cytogenetic Investigations

Metaphase cells were obtained from lymph node preparations of cases 1–4 and 7, a cell line obtained from peripheral blood of case 5, ascitic fluid of case 6, the bone tumor in case 8, and the skin infiltrates of patients 9, 10, and 11.

All primary nodal ALCL showed a clonal t(2;5)(p23;q35) in all tumor metaphases. Two cases were hyperdiploid and one case was near-tetraploid. In this case (Fig. 1), two rearranged chromosomes 2 and 5 each were present, indicating that tetraploidization took place after t(2;5) formation. Interestingly, in three lymphomas, an extra copy of chromosome 7 was seen; in case 1, six copies of this chromosome were present, so that it can be assumed that +7 was also an early event in this lymphoma. Case 4 displayed the t(2;5) as the sole cytogenetic abnormality in all metaphases analyzed.

Extranodal ALC lymphomas were near-diploid or near-tetraploid, with modal chromosome numbers ranging from 46 to 72–112. No translocation t(2;5)(p23;q35) was encountered, although case 6 showed a t(2;4) with a breakpoint at 2p23. In case 9,

TABLE 2. Immunophenotyping and Cytogenetic Data

Case	Tissue analyzed	Immunophenotype (positive)	Karyotype
1	Lymph node	CD30, CD2, CD3, CD4, CD7	90–94, XXYY, i(1)(q10), t(2;5)(p23;q35)x2, +7, +7, add(8)(q24), add(15)(q10)
2	Lymph node	CD30	47, XY, t(2;5)(p23;q35), +7
3	Lymph node	CD30, CD7	49–52, XY, +X, t(2;5)(p23;q35), ?add(2)(p?), add(3)(q27–29), del(6)(q13q23), +7, −9, der(12)t(9;12)(q12;q13), +13, +14, +14, +15
4	Lymph node	CD30, CD3, CD7, CD8	46, XX, t(2;5)(p23;q35)
5	Cell line	CD30, CD2, CD7, TCR βF1	49, XY, +1, der(2)t(2;5)(p23;q35)t(2;21)(q14;q11), der(5)t(2;5)(p23;q35), +8, +13, add(15)(q26), der(21)t(2;21)(q32;q11), i(22)(q10)
6	Ascitic fluid	CD30, CD3, CD7	45–47, X, −Y, t(2;4)(p23;q33), add(6)(p25), +12, der(18) t(1;18)(q12;q23)
7	Lymph node	CD30, CD4	45–46, X, t(X;6)(p11;p25), t(2;8)(p25;q21), t(8;18)(p11;q21), del(16)(q13) / 72–112, idem × 2
8	Bone tumor	CD30, CD3, CD4	44–46, XX, t(1;10)(p36;q23), t(4;15)(q31;q15), t(6;7)(p23;p15) / 87–92, idem × 2
9	Skin	CD30, CD2, CD4	50–59, XY, +X, +add(1)(p13)x2, +2, +2, +4, +del(6)(q21q23), +7, +9, dup(12)(q21q24), +der(12)dup(12)(q21q24), +14, +17, +21
10	Skin	CD30, CD 45 RO	49, Y, add(X)(?p11), −1, der(1)t(1;3)(q11;?p13), add(2)(q37)x2, del(2)(q13), +del(2)(q13), add(3)(?p13), add(4)(q31.1), add(5)(q33), del (6)(q21), del(7)(q22q32), add(8)(q24), +9, t(9;11)(q34;q23), add(10)(q26), del(11)(p11), −16, +21, der(22)t(1;22)(q13;p13), +der(?)t(?;1)(?;q21)
11	Skin	CD30, CD4	46, Y, add(X)(p10), add(1)(p36), t(2;?14)(p?25;q?24), del(3)(q?21), del(4)(q31), del(7)(p13), del(9)(q34), add(10)(q?26), der(12)add(12)(p10)add(12)(q22), del(14)(q24), i(17)(q10), −18, del(18)(q22), −20, −21, +mar1x2, +r

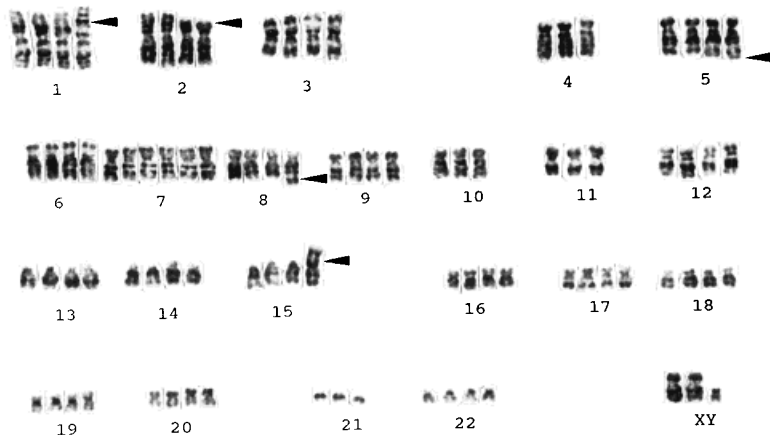

Figure 1. Representative karyotype of case 1 showing a hypotetraploid chromosome number with two rearranged chromosomes 2 and 5 and hexasomy 7. In addition, there are an isochromosome for 1q and an addition of genetic material at chromosome bands 8q24 and 15q10.

an extra chromosome 7 was observed, as was an interstitial deletion of 6q. In cases 8 (bone lymphoma) and 11 (skin tumor), rearrangements of the short arm of chromosome 1 with an identical breakpoint at 1p36 occurred (Fig. 2). Deletions in the long arm of chromosome 6 were observed in cases 3 (nodal), 9, and 10 (extranodal). Both in a primary nodal lymphoma (patient 1) and in a cutaneous ALCL (patient 10), a rearrangement of band q24 in chromosome 8 was noted. The complete karyotypes are listed in Table 2.

Molecular Analyses and p80 Expression

Results of RT-PCR analyses and anti-p80 immunostains are shown in Table 3. In all five nodal lymphomas, RT-PCR analysis showed the expression of a 175-bp fragment of the *NPM-ALK* fusion message. All six extranodal cases were negative in the first amplification, but a positive amplification product was seen after Southern blotting and in the nested RT-PCR assay in two of six extranodal ALCL, namely in one of the two samples investigated of the osseous lymphoma (case 8) and in one

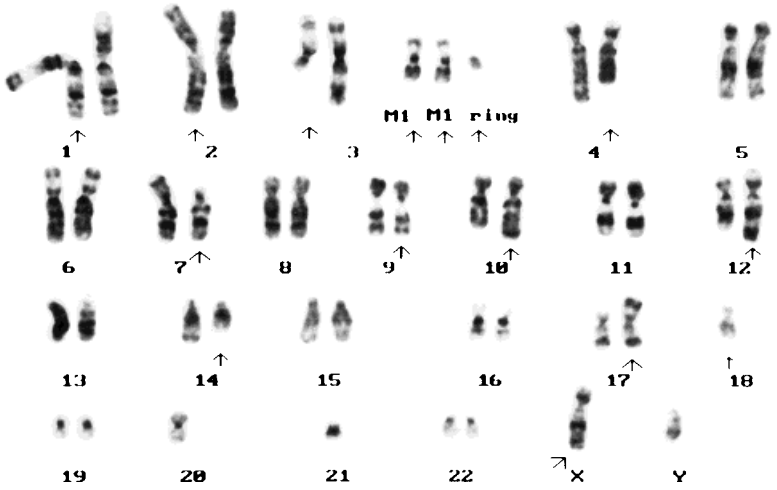

Figure 2. Representative karyotype of case 11. Note absence of a t(2;5)(p23;q35) and a structural rearrangement of chromosome 1 at p36.

TABLE 3. Comparison of Cytogenetic, Molecular Genetic, p80, and FISH Results for the Detection of t(2;5)(p23;q35) in 11 ALCL

			RT-PCR amplification product				
Case	Lymphoma origin	t(2;5)(p23;q35) Cytogenetics	First amplification	Southern transfer	Nested amplification	p80 Expression	FISH for 5q rearrangements
1	Nodal	Positive	−	+	+	+	n.d.[a]
2	Nodal	Positive	+	+	n.d.	+	n.d.
3	Nodal	Positive	+	+	n.d.	+	Positive (10.2%)
4	Nodal	Positive	+	+	+	+	n.d.
5	Nodal	Positive	+	n.d.	n.d.	+	n.d.
6	Intestinal	Negative	−	−	−	−	Negative
7	Intestinal	Negative	−	−	−	−	n.d.
8	Osseous	Negative					
		1	−	−	−	−	
		2	−	Weak +	+	−	Negative
9	Cutaneous	Negative	−	+	+	−	Positive (4%)
10	Cutaneous	Negative	−	−	−	−	n.d.
11	Cutaneous	Negative	−	−	−	−	n.d.

[a]n.d., not done.

primary cutaneous lymphoma (case 9). In four of six extranodal ALCL, no *NPM/ALK* fusion message was detectable by Southern blotting or by nested RT-PCR (Fig. 3).

The $p80^{NPM/ALK}$ stains of both paraffin and frozen sections revealed a strong positive reaction of all tumor cells in nodal lymphomas (cases 1–5). In contrast, all primary extranodal tumors were negative.

FISH Analyses

In cases 3 (nodal) and 9 (cutaneous), the signal constellation indicative of a 5q35 breakage was seen in 51/500 (10.2%) and 16/400 (4%) interphase nuclei, respectively (Table 3, Fig. 4). Therefore, these cases were regarded as positive for an NPM disruption. In contrast, cases 6 (2/600, 0.3%) and 8 (1/600, 0.15%) did not contain a significant percentage of interphase nuclei indicative of a rearrangement of 5q35 and, therefore, were interpreted to be negative.

DISCUSSION

This is the first study comparing results of chromosome banding with molecular and immunohistochemical analyses of t(2;5) expression in primary nodal and extranodal CD30+ ALCL. Our results of anti-$p80^{NPM/ALK}$ immunostaining matched those obtained from cytogenetic analysis concerning the t(2;5). All cases cytogenetically positive for the t(2;5) also demonstrated the presence of the *NPM/ALK* chimeric transcript in RT-PCR analyses. Two extranodal lymphomas negative for the t(2;5) by cytogenetics were shown to be positive by nested RT-PCR and Southern blotting, and one of these tumors (case 9) showed disruption of one *NPM* twin signal in a minority (4%) of cells in FISH analysis. The difference between classical cytoge-

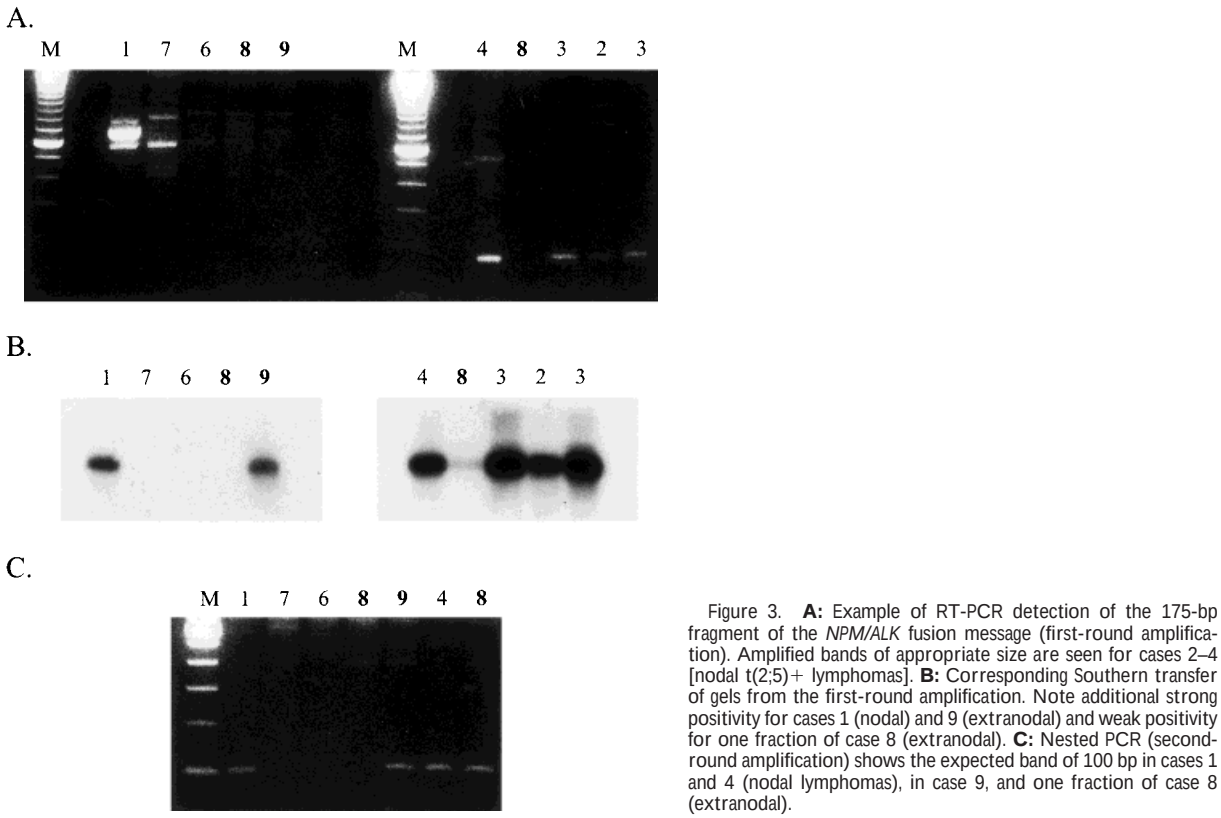

Figure 3. **A:** Example of RT-PCR detection of the 175-bp fragment of the *NPM/ALK* fusion message (first-round amplification). Amplified bands of appropriate size are seen for cases 2–4 [nodal t(2;5)+ lymphomas]. **B:** Corresponding Southern transfer of gels from the first-round amplification. Note additional strong positivity for cases 1 (nodal) and 9 (extranodal) and weak positivity for one fraction of case 8 (extranodal). **C:** Nested PCR (second-round amplification) shows the expected band of 100 bp in cases 1 and 4 (nodal lymphomas), in case 9, and one fraction of case 8 (extranodal).

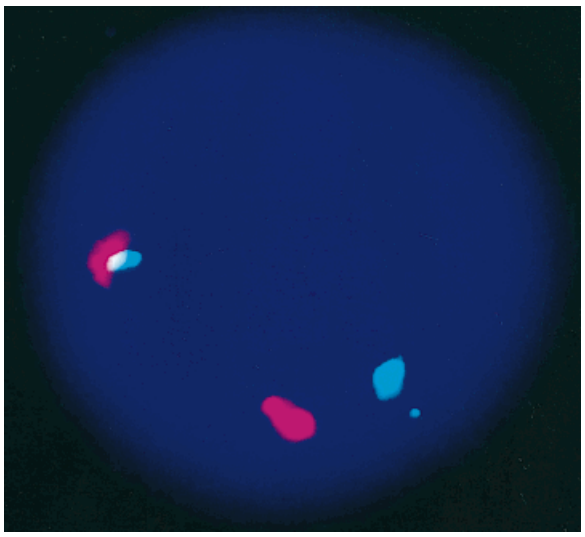

Figure 4. Interphase nucleus from the t(2;5)+ case 3 hybridized with the two *NPM* YACs. One regular red-green hybrid signal and two isolated red and green signals are indicative of a chromosomal breakage in 5q35.

netic and RT-PCR analyses in cases 8 and 9 may be due to the high sensitivity of the RT-PCR technique and the possibility of false-positive results. Alternatively, as is suggested by the data from case 9, only a minor fraction of tumor cells may carry the t(2;5)(p23;q35); this would mean, however, that in these cases the *NPM/ALK* fusion does not constitute a primary genetic aberration. Alternatively, the t(2;5) may have been lost during tumor progression in most tumor cells. Another explanation is suggested by the results from case 8, in which two fractions of the same tumor, excised separately on the same day, were analyzed. One fraction was positive for the chimeric protein in RT-PCR analysis, and the other was negative. Both specimens were negative in p80 staining. Cytogenetics were done from the fraction later proving to be RT-PCR positive: it failed to show the t(2;5). By FISH of 600 interphase nuclei, the percentage of cells displaying the signal constellation indicative for a 5q35 disruption was below the diagnostic threshold. These findings may point to the existence of untranslated (p80−) rearrangements not in a clonally amplified tumor cell but possibly in a nonneoplastic bystander cell. Trümper et al. (1996) demonstrated the t(2;5) in peripheral blood B lymphocytes of normal individuals, indicating that this translocation is not restricted to malignant lymphoid cells, a phenomenon that has also been described for the t(14;18) chromosome translocation (Limpens et al., 1995). These findings may also explain the results of Beylot-Barry et al. (1997) who found a low

frequency of ALK-1 protein expression (only 1 in 50 CD30+ cutaneous lymphoproliferative disorders) compared with more frequent (10 of 26) detection of *NPM/ALK* transcripts by nested PCR.

Lymphomas with the specific translocation t(2;5) seem to constitute a rather homogeneous entity characterized by nodal origin, early age of onset, and a morphologically and immunologically similar tumor cell (Mason et al., 1990; Shiota et al., 1995). In contrast, t(2;5)− ALCL may constitute one or more clinical and biological entities. De Bruin et al. (1993) pointed out that, although displaying identical morphologic features, nodal and cutaneous ALCL showed a marked heterogeneity regarding expression of surface markers and clinical outcome, with the prognosis being significantly worse in nodal ALCL. Indeed, the superior prognosis of primary CD30+ cutaneous ALC-type and non-ALC-type lymphomas has repeatedly been observed (Beljaards et al., 1989; Gianotti et al., 1991).

The present study constitutes the first report of cytogenetic data from primary intestinal T-cell and primary cutaneous ALC lymphomas. Interestingly, both intestinal lymphomas (cases 6 and 7) showed rearrangements of chromosomes 2 and 18, albeit with different breakpoints. Chromosome aberrations frequently characterizing nodal ALCL, such as trisomy 7, were not present. In contrast, rearrangements of the short arm of chromosome 6, which are more frequent in T-cell than in B-cell lymphomas (Mecucci et al., 1985), were documented in these cases and a primary bone ALCL (case 8). Another chromosomal alteration frequently encountered in T-cell lymphomas was detected in the bone tumor and two cutaneous lymphomas, namely rearrangements of the short arm of chromosome 1 (Fifth International Workshop on Chromosomes in Leukemia-Lymphoma, 1987). In cases 8 and 11, this rearrangement involved the breakpoint 1p36. Deletions in the long arm of chromosome 6 found in three of our patients (cases 2, 9, and 10) are frequent in lymphoid malignancies (Mitelman, 1994).

The most common secondary karyotypic alteration in our t(2;5)+ ALCL cases was a +7 occurring in three of five lymphomas. Trisomy 7 has been reported in other nodal ALCL and was also present in the cutaneous ALCL of our case 9. It had also been described in other high-grade T-cell lymphomas (Schlegelberger et al., 1994) and could also be documented in a case of lymphomatoid papulosis, a premalignant or low-grade CD30+ cutaneous lymphoma (Peters et al., 1995). Another chromosome region affected in both a nodal and an extranodal ALCL in our series (cases 1 and 10) was 8q24, the site of the *MYC* gene.

In summary, the t(2;5)(p23;q35) translocation and related expression of p80 is exclusively associated with primary nodal lymphomas. It is absent in extranodal ALCL-like malignancies, at least by classical cytogenetic and immunohistochemical methods. These observations support the view that these two groups of lymphoid malignancies, although characterized by a similar morphology, should be viewed as different disease entities.

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