

Large-cell anaplastic lymphoma-specific translocation (t[2;5] [p23;q35]) in Hodgkin's disease: indication of a common pathogenesis?

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Summary

Chromosomal aberrations are characteristic and specific events; the detection of chromosomal abnormalities often provides information on diagnosis and prognosis of disease.

Some patients with large-cell anaplastic lymphoma (Ki 1 lymphoma) have the translocation t(2;5) (p23; q35), involving a possible growth-regulating tyrosine kinase. We found this translocation in 11 patients with Hodgkin's disease of nodular sclerosis and mixed-cellularity types. This finding has implications for the understanding of the relation between large-cell anaplastic lymphoma and Hodgkin's disease, diseases with morphological and immunophenotypical similarities.

Study of this translocation may help understanding of the origins of cancer and cancer growth. It also allows a more precise definition of Hodgkin's disease and may be used as an indicator for clonality—which has long been sought.

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Introduction

Many cancers have been found to be characterised by specific chromosomal aberrations, which help in defining those with similar biological background. Detection of specific chromosomal aberrations can also be used for diagnosis or to assess prognosis.¹

Philadelphia chromosome (Ph1) is diagnostic of chronic myeloid leukaemia, and can be detected in more than 95% of cases. The molecular correlate of Ph1 is an aberrant conjoining of sequences from chromosome 22q1 to the *c-abl* protooncogene at 9q34. This hybrid gene encodes a chimeric protein with tyrosine-kinase activity.² Similar chromosomal aberrations have also been described in malignant lymphomas.

Large-cell anaplastic lymphoma (LCAL) has characteristic morphology, constant expression of the CD30 antigen,³ and is composed of large blastic, sometimes binucleated or multinucleated, pleomorphic cells with prominent, often elongated nucleoli and abundant cytoplasm, similar to Hodgkin and Sternberg-Reed cells. Some T-cell-derived LCALs have a specific translocation, t(2;5)(p23;q35)⁴ which may regulate cell growth, and be involved in tumorigenesis. This translocation has been shown to be a fusion product of the nuclear phosphoprotein nucleophosmin (npm) and the anaplastic lymphoma kinase gene (*alk*), resulting in a hybrid protein with tyrosine kinase activity.^{5,6} Other proteins with tyrosine kinase activity are the insulin receptor kinases, which have been shown to have transforming potential.⁷

Morphologically and immunohistochemically, the lymphoma most closely related to LCAL is Hodgkin's disease.^{8–10} In both entities there are variable numbers of Hodgkin and Sternberg-Reed cells, a pattern of fibrosis/sclerosis, foci of necrosis, and a variable mixture of histiocytes, epithelioid cells, neutrophils, eosinophils, lymphocytes, and plasma cells. Cases have been described that evolved from Hodgkin's disease into LCAL. Morphologically, the cellular phase of nodular sclerosis type II Hodgkin's¹¹ is sometimes difficult to differentiate from LCAL; there are no criteria for distinguishing LCAL from lymphocyte-depleted Hodgkin's disease. We and others have observed patients with Hodgkin's disease and LCAL who have the same pattern of T-cell receptors. Furthermore, immunophenotypically both tumours have similar expression of T-cell, B-cell, activation antigens,¹ and cytokines.^{12,13}

Do these diseases have a common origin in early steps of tumorigenesis and is their subsequent growth varied by other cellular factors or different immunological microenvironments? We investigated whether the t(2;5) translocation specific to LCAL could be detected in Hodgkin's disease.

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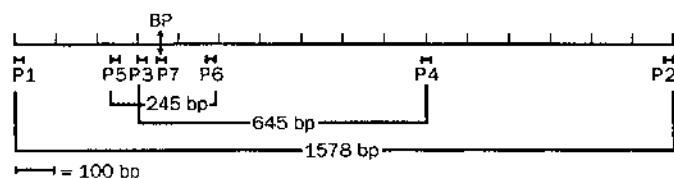
Materials and methods

Lymphoma and non-neoplastic lymphoid tissues were taken from patients under investigation and immunophenotyped, as were peripheral blood leucocytes and cultured cell lines, including a CD30 large-cell anaplastic cell line (L82) from a pleural effusion of a patient with LCAL carrying the specific t(2;5) translocation. As controls, we included two B-cell lymphomas (centroblastic-centrocytic lymphoma), two T-cell lymphomas (angioimmunoblastic lymphadenopathy), two lymph nodes with T-zone hyperplasia, and two human tonsils with follicular hyperplasia, as well as the T-cell lines Karpas299¹⁴ and HUT78, the B-cell lines Ramos and Raji, the myelomonocytic cell lines K562 and U937 (all cell lines except L82 were from DSM, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). Normal peripheral blood leucocytes were used either unstimulated or stimulated for 48 h with phorbol-myristate acetate (PMA) 10 ng/mL and calcium ionophore A23187 100 ng/mL (Sigma, Deisenhofen, Germany).

Extraction of cellular RNA and reverse-transcription polymerase chain reaction (RT-PCR) were done as previously described.¹⁵ RNA integrity was checked by agarose-gel electrophoresis and ethidium bromide staining. A β -actin fragment of 1800 bp was amplified to monitor first-strand synthesis and to control cDNA amplification.¹⁵ Bands for β -actin were scored: ++ (appearing after 20 cycles), + (appearing after 25 cycles), - (appearing after 30 cycles or not at all). 1/50 of the reverse-transcribed cDNA was used for PCR with 100 pmol 5' and 3' primers (P1/P2 or P3/P4, figure), and 1 U *Taq* polymerase (Biozym, Oldendorf, Germany) added to a final volume of 50 μ L. Visible bands were scored: ++ for strong positive bands, + for just detectable bands, - for smears or no detectable band. Each experiment was repeated at least two times, in different laboratories by two independent investigators from our staff with new sets of reagents to exclude contamination.

The amplified *npm/alk* cDNA fragments from cell line L82, the T-LCAL case 18 and Hodgkin's disease cases 4, 6, 10, and 13 were cloned into the pCRII vector with the TA cloning kit (Invitrogen, Leek, Netherlands). Sequence analysis for *npm/alk* cDNA was done on denatured double-stranded plasmid by the dideoxy chain termination method with Sequenase Kit (US Biochemical, Cleveland, Ohio, USA). Sequencing primers (P5/P6) are given in the figure, additionally the T7-primer (5'TAATACGACTCACTATAGGAGA 3') corresponding to the T7-promotor of pCRII was used.

The probes used for Southern blot analysis of PCR products were the 1.5 kb *Eco*RI fragment of *npm/alk* cDNA cloned from the cell line L82 into the pCRII vector, which was confirmed for 100% sequence identity with the published sequence⁹ and a breakpoint spanning oligonucleotide P7. 10 μ L of PCR products was subjected to electrophoresis and blotted onto a nylon membrane (Hybond N+, Amersham Corp, Arlington Heights, Illinois, USA). The probes were labelled with digoxigenin-dUTP by random priming reaction (for cDNA) or terminal deoxynucleotide transferase (for oligonucleotide) with DIG labelling and detection kits (Boehringer, Mannheim, Germany).



P1 5' ATG GAA GAT TCG ATG GAC ATG GAC 3' position 1-24 P2 5' CTA GGG ACT CGA ACA GAG ACC TCT 3' position 1578-1559 P3 5' TTG AAG TGT GGT TCA GGG CCA GTG C 3' position 304-328 P4 5' CAT CCC GAA GTC TCC AAT CTT GGC C 3' position 999-974 P5 5' TTG AAA ATG TCT GTA CAG CCA ACG 3' position 235-253 P6 5' GCC AGC AAA GCA GTA GTT GGG 3' position 480-460 P7 5' CAC TTA GTA GTG TAC CGC CGG 3' position 343-364

Figure: Scheme of *npm/alk* cDNA with location of breakpoint (BP), the breakpoint specific oligonucleotide (P7) and of primers used for RT-PCR (P1-P4) and sequencing (P5,P6)

The limit of detection for *npm/alk* was determined by diluting L82 cells down from 10 000 to 1 cell into 90 000 to 100 000 peripheral blood leucocytes. RNA extraction, RT-PCR analysis, and Southern blot hybridisation for confirmation of *npm/alk* were done as described above.

Additionally, one case of Hodgkin's disease (the most intensely positive case) was analysed by semiquantitative RT-PCR analysis. For this purpose 5 μ g of total cellular RNA of this case and from L82 cells were extracted and reverse transcribed. First-strand cDNAs were then adjusted to equal β -actin amplification, in which an approximately similar band intensity has to be obtained after 20, 22, and 25 cycles of amplification to record almost identical cDNA loadings. Equilibrated amounts of the cDNAs were then subjected to *npm/alk* amplification and compared after different numbers of cycles (20, 25, 30, 35). The results were again confirmed by Southern blot hybridisation.

Results

RT-PCR analysis for *npm/alk*

RT-PCR analysis showed the characteristic translocation *npm/alk* in eleven of thirteen patients with Hodgkin's disease, independent of the subtype, and in four of five patients with LCAL (T and 0 type), whereas specimens from other malignant lymphomas, lymph nodes with T-zone hyperplasia, hyperplastic tonsillar tissue, and normal peripheral blood leucocytes were negative. Hodgkin's disease and LCAL cases differed in the band intensities for *npm/alk*. RT-PCR results for primers P3/P4 were confirmed for specificity to *npm/alk* with Southern blot hybridisation. Hybridisation patterns were identical, whether the full-length cDNA clone was used as a probe or as the junction-specific oligonucleotide. Eleven of thirteen cases of Hodgkin's disease and five cases of LCAL showed the expected hybridisation signal.

Sequencing of RT-PCR-products of *npm/alk*

Four RT-PCR-positive cases of Hodgkin's disease and one RT-PCR-positive case of LCAL (cases 4, 6, 10, 13, and 18) had adequate cDNA available for large-scale amplification, cloning, and sequencing. Sequence analysis was done with T7-primers starting from the pCRII vector sequence, to confirm correct primer use. Sequence analysis of cases 4, 6, 10, 13, and 18 showed complete homology with the published *npm/alk* hybrid gene.

Sensitivity for detecting *npm/alk*

L82 cells were used to calculate the detection limit with RT-PCR. For this purpose L82 cells were serially diluted in peripheral blood leucocytes while the total number of cells was kept constant. RNA was extracted, photometrically quantified, and used for semiquantitative β -actin RT-PCR. After 20, 22, and 25 cycles of amplification, β -actin products were seen in ethidium-bromide-stained agarose gels. cDNAs were calibrated for equal β -actin loading and amplification rates. Subsequently RT-PCR was done for *npm/alk* for 35 cycles. There was a gradual decline in band intensities, but *npm/alk* could be demonstrated down to 1 cell. These results were confirmed by Southern hybridisation with digoxigenin-labelled breakpoint-specific oligonucleotide P7.

Semiquantitative RT-PCR analysis for *npm/alk*

DNA from Hodgkin's disease case 4 (with the most prominent band for *npm/alk* after 30 cycles, was compared with DNA from L82 cells by semiquantitative RT-PCR approach. First-strands were again equilibrated

Cases	P1/P2- RT-PCR 1578 bp	P3/P4- RT-PCR 695 bp	β -actin- RT-PCR
HDMC			
1	-	-	+
2	-	-	+
3	-	+	+
4*	++	+++	++
5	+	++	++
6	++	+++	++
7	++	++	+
HDMC			
8	++	++	++
9	+	+	+
10	++	+++	++
11	-	+	++
12	+	++	+
13	++	+++	++
LCAL			
14	nd	+	+
15	+	++	+
16	+	++	++
17	-	-	++
18	+	+++	++
AILD			
19	-	-	++
20	-	-	+
CBCC			
21	-	-	++
22	-	-	++
Controls			
Lymph-node 1	-	-	++
Lymph-node 2	-	-	++
Tonsil 1	-	-	++
Tonsil 1	-	-	++
L82-LCAL-T*	++	+++	++
Karpas299-LCAL-T	++	++	++
HUT 78-T	-	-	++
Ramos-B	-	-	++
RAJI-B	-	-	++
U937-M	-	-	++
K562-M	-	-	++
0 h PBLs	-	-	++
48 h PBLs	-	-	++

*Additionally confirmed by sequencing analysis. HDNS: Hodgkin's disease nodular sclerosis; *type II. HDMC: Hodgkin's disease mixed cellularity. LCAL: Large-cell anaplastic lymphoma. AILD=Angioimmunoblastic lymphadenopathy; CBCC=centroblastic-centrocytic lymphoma.

Table: *Npm/alk* RT-PCR analysis

by comparison of β -actin amplifications. Normalised cDNAs of L82 cells and the Hodgkin's disease case were amplified in parallel and visualised after various numbers of cycles. An amplified band appeared for L82 cells (10 ng RNA approx 1000 cells) after 25 cycles, whereas for the Hodgkin's disease case (10 ng whole cellular RNA) bands could be visualised after 30 cycles. In Southern blot, bands were confirmed to be true *npm/alk* products.

Discussion

The pathogenesis of Hodgkin's disease and LCAL is poorly understood; the diagnosis of both diseases is still based on complex morphological criteria, with Hodgkin and Sternberg-Reed cells embedded in a variable background of fibrous stroma and non-neoplastic cells.

In Hodgkin's disease, cytogenetic analysis has shown aneuploidy, hyperploidy, chromosomal triplications, and losses of, and recurring chromosomal alterations to, 6q, 8q, 11q, 14q, but no constant disease-specific chromosomal abnormality.¹⁶ In LCAL, however, some cases, especially those of T-cell type, carry the specific translocation t(2;5)(p23;q35), which has not yet been detected in any other lymphoma. So far, only one report

has described a case of Hodgkin's disease with a 5q35 abnormality.¹⁷

The scarcity of tumour cells is a major difficulty in representative chromosomal analyses in Hodgkin's disease, and the t(2;5) translocation may be missed by conventional chromosome analysis, as may other characteristic aberrations, for example t(14;18) in follicular lymphomas. The structure of t(2;5) has been resolved at the genetic level. A previously unidentified tyrosine kinase gene on chromosome 2p23 is fused to the nucleophosmin gene at 5q35, resulting in a highly transcribed, truncated, but active kinase gene. This kinase, termed *alk*, is normally silent in lymphoid cells and belongs to the insulin receptor kinase family of phosphotyrosine kinase genes, which regulate cellular growth and may trigger neoplastic transformation when mutated, translocated, and expressed aberrantly.⁷

Our study shows that eleven of thirteen cases of Hodgkin's disease and four of five cases of LCAL showed the LCAL-specific translocation t(2;5). Primer pairs P1,2 and P3,4 showed the same pattern for most cases. Two cases of Hodgkin's disease showed a product only for P3,4. This result may be due to degradation of RNA, leading to a reduction in amplifiable cDNAs. Alternatively, a similar, but different translocational event may be responsible for this difference, allowing only the small fragment to be amplified. Such discrepancies are frequently shown for other translocations, such as t(14;18)-*bcl2* oncogene, in which a major and a minor cluster breakpoint region exists. The two cases of Hodgkin's disease and the one case of LCAL that were negative for both sets of primers are likely to be true negative cases.

Our experiments show that starting with high quality RNA allows for the synthesis of long cDNAs and for early detection of β -actin gene transcription. Although band intensities declined gradually from 10 000 to 1 L82 cell, a signal could be detected even for one cell, suggesting for high sensitivity of *npm/alk* detection.

Hodgkin's disease is composed of a minority of tumour cells which may represent only a small percentage of the total cellular infiltrate. Only a few of the neoplastic cells may carry the t(2;5) translocation. To investigate this, we examined one case of Hodgkin's disease (4) by a semiquantitative approach. Amplification was delayed in the Hodgkin's disease case by about 5 cycles, which may be interpreted as a 32-fold lower amplification rate, suggesting a 32-fold smaller number of positive cells. This correlates well with the 3% to 5% Hodgkin and Sternberg-Reed cells in this case. Assuming that the Hodgkin and Sternberg-Reed cells express the same amount of *npm/alk* as L82 cells, it seems most likely that almost all of the neoplastic cells carry t(2;5). However, other cases showed weaker or very weak signals, which indicate lower numbers of cells with t(2;5).

These observations show that LCAL-specific translocation t(2;5) is found in an identical pattern in most cases of Hodgkin's disease and may be confined to a varying number of cells, probably the Hodgkin and Sternberg-Reed cells.

The t(2;5) translocation has been shown to involve a tyrosine kinase gene, which is most probably activated due to this translocation and may induce cellular proliferation, indicating that LCAL and Hodgkin's disease undergo at least one identical step during tumorigenesis.

This finding underlines the close biological relation between these two diseases, which is also shown by morphology, and genotypic and immunophenotypic analysis.

The method described for detection of this chromosomal translocation will allow better differentiation between borderline cases, most of which are T-cell lymphomas (eg, angioimmunoblastic lymphadenopathy type, lymphoepithelioid lymphoma, and pleomorphic T-cell lymphomas) and some B-cell lymphomas, such as rare cases of chronic lymphocytic leukaemia of B-cell type with a high content of Hodgkin and Sternberg-Reed cells. It will be of interest to study transitional cases developing from one entity to another, as the occurrence—or lack of—t(2;5) would allow us better to understand the biological relevance of this translocation.

It is almost impossible to detect rearrangements of the T-cell receptor or immunoglobulin genes in cases of Hodgkin's disease, because of the small number of tumour cells. The t(2;5) translocation may therefore be used as an indicator of clonality. It can also be used to monitor patients during disease-free intervals. The sensitivity demonstrated in this study allows the detection of one *npm1/alk*-positive cell in 100 000 negative cells.

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