# ALK-positive large B-cell lymphomas with cryptic *SEC31A-ALK* and *NPM1-ALK* fusions

Katrien Van Roosbroeck,[1,2] Jan Cools,[1,2] Daan Dierickx,[3] José Thomas,[4] Peter Vandenberghe,[1] Michel Stul,[1] Jan Delabie,[5] Chris De Wolf-Peeters,[5] Peter Marynen,[1,2] and Iwona Wlodarska[1]

[1]Center for Human Genetics, K.U. Leuven, Leuven, Belgium; [2]VIB Department of Molecular and Developmental Genetics, VIB, Leuven, Belgium; [3]Department of Hematology, K.U. Leuven, Leuven, Belgium; [4]Department of Oncology, K.U. Leuven, Leuven, Belgium; and [5]Department of Pathology, K.U. Leuven, Leuven, Belgium

*Citation: Van Roosbroeck K, Cools J, Dierickx D, Thomas J, Vandenberghe P, Stul M, Delabie J, De Wolf-Peeters C, Marynen P, and Wlodarska I. ALK-positive large B-cell lymphomas with cryptic* SEC31A-ALK *and* NPM1-ALK *fusions. Haematologica. 2010;95:509-513. doi: 10.3324/haematol.2009.014761*

## Supplementary Methods

### *Cytogenetics and fluorescent in situ hybridization (FISH)*

Conventional G-banding chromosomal analysis of lymph node biopsy samples followed routine protocols. The karyotype was described according to the International System for Human Cytogenetic Nomenclature (ISCN 2005).[6] Eighteen and five metaphase cells were analyzed in the respective cases.

FISH followed standard protocols. Experiments were performed on fixed cells from remaining cytogenetic harvests. Multicolor FISH (mFISH) was performed according to the manufacturer's protocols (MetaSystems, Altlussheim, Germany). Applied probes are listed in the *Online Supplementary Table S1*. Bacterial artificial chromosome (BAC) and fosmid clones were selected from the Ensembl (*www.ensembl.org*) and UCSC Genome Browsers (*www.genome.ucsc.edu*), respectively. Non-commercial probes were directly labeled with SpectrumOrange- and SpectrumGreen-dUTP (Abbott Molecular, Ottignies, Belgium) with random priming.

**Online Supplementary Table S1.** Results of FISH analyses of two ALK+ LBCL cases.

| Probe | Localization | Labeling | Reference | FISH pattern | Conclusion |
|---|---|---|---|---|---|
| **CASE 1** | | | | | |
| LSI ALK | 2p23 | 5' SG<br>3' SO | Abbott Molecular, Ottignies, Belgium | 2p F, 3qter R, add(20q) R | t(2;3)(p23;q?) with duplication of the terminal part of der(3) to chromosome 20 |
| CLTC | 17q23 | 5' SG<br>3' SO | Rudzki *et al.*[1] | 2 x 17F | *CLTC* gene not rearranged |
| LSI BCL6 | 3q27 | 5' SO<br>3' SG | Abbott Molecular, Ottignies, Belgium | 3q F, 2p F, add(13p) F | *BCL6* gene not rearranged, but translocated to 2p (BP centromeric to BCL6) |
| WI2-2194A15 (G248P86980A8) | 4q21/5' SEC31A | 5' SO (SEC31A) | UCSC Genome Browser (http://genome.ucsc.edu) | 3qter R, 4q F, 4q G, add(20q) R | Rearrangement of SEC31A with translocation of the 5' region to 3qter |
| WI2-2877K7 (G248P88440F4) | 4q21/3' SEC31A | 3' SG (SEC31A) | | | |
| WI2-2194A15 (G248P86980A8) | 4q21/5' SEC31A | 5' SO (SEC31A) | UCSC Genome Browser (http://genome.ucsc.edu) | 2p G, 3qter F, 4q R, add(20q) F | 5' end of *SEC31A* colocalizes with 3' end of *ALK* at 3q and add(20) |
| ALK (P1 clone 1111H1) | 2p23/3' ALK | 3' SG (ALK) | Mathew *et al.*[2] | | |
| WCP2 | Whole chromosome painting probe for chromosome 2 | TR | Abbott Molecular, Ottignies, Belgium Abbott Molecular, Ottignies, Belgium | 2R, 2R + 2pter G, 3G, 3G + 3qter R, 4q21 R, add(13p) G, add(20q) GR | t(2;3)(p23;q27) associated with duplication of the terminal part of der(3) to chromosome 20; |
| WCP3 | Whole chromosome painting probe for chromosome 3 | SG | | | Cryptic insertion of chromosome 2 material into the 4q21-region |
| **CASE 2** | | | | | |
| LSI ALK | 2p23 | 5' SG<br>3' SO | Abbott Molecular, Ottignies, Belgium | 2p F, 2p G, 5q R | Rearrangement of *ALK* with 3' end of ALK inserted at the terminal part of a normal-looking chromosome 5 |
| NPM1 | 5q35 | 5' SG<br>3' SO | Rudzki *et al.*[1] | 2 x 5q F | No rearrangement of *NPM1* detected by FISH |

*SG: Spectrum Green; SO: Spectrum Orange; TR: Texas Red; F: fusion signal; R: red signal; G: green signal.*

**Online Supplementary Table S2.** Primer sequences used in RT-PCR, 5' RACE-PCR and construct design.

| Name | Sequence |
|---|---|
| ALK-R2[3] | 5'-AGCACACTTCAGGCAGCGTCTTC-3' |
| 467 (1) | 5'-CCAGTGAGCAGAGTGACG-3' |
| ALK-R3[3] | 5'-ATTCCGGACACCTGGCCTTCATA-3' |
| 468 (1) | 5'-GAGGACTCGAGCTCAAGC-3' |
| ALK3' F[4] | 5'-GCAACATCAGCCTGAAGACA-3' |
| ALK3' R[4] | 5'-GCCTGTTGAGAGACCAGGAG-3' |
| ALK5' F[4] | 5'-CTCAGCGAGCTGTTCAGTT-3' |
| ALK5' R[4] | 5'-GGAGAAGGCATGTTTGTTG-3' |
| SEC31A-F1 | 5'- GCAATGTATCGACCTCAGCA -3' |
| SEC31A-F2 | 5'-CAGCCTGTTGCTCCTCCTAC-3' |
| NPM1-F1 (NPM1-ALK)[5] | 5'- TCCCTTGGGGGCTTTHAAATAACACC-3' |
| NPM1-F2 (NPM1-ALK)[5] | 5'- ACCAGTGGTCTTAAGGTTGA-3' |
| ALK-R1 (NPM1-ALK)[5] | 5'- CGAGGTGCGGAGCTTGCTCAGC-3' |
| ALK-R2 (NPM1-ALK)[5] | 5'- TTGTACTCAGGGCTCTGCA-3' |
| SEC31A-F-XhoI | 5'-ATCTCGAGCAGCATGGAGGACGGCAAGTTAAAGGAAGTAGATCGTACAG-3' |
| SEC31A-R-BglII | 5'-TTCAAGGCCAAAGCAATCTT-3' |
| SEC31A-F-BglII | 5'-TGAGGATGATTCTCGTGGAA-3' |
| SEC31A-R-NotI | 5'-AAGCGGCCGCTTCTGAGGACCTGTTCTTTGG-3' |
| ALK-F-NotI | 5'- ATAGCGGCCGCGGAAAAACATCACCCTCATT-3' |
| ALK-R-EcoRI | 5'-ATAGAATTCAGGGATCCCAAGGAAGAGAA-3' |

### *Molecular analysis*

Total RNA was extracted from 20µm sections of frozen tissue samples of both cases using TRIzol Reagent (Life Technologies, Merelbeke, Belgium). One microgram of this total RNA was reverse transcribed into cDNA using random hexamers and Superscript II (Life Technologies, Merelbeke, Belgium).

Reverse transcription (RT) PCR was performed on a cDNA template using conventional PCR with *Taq* DNA polymerase (Roche Diagnostics GmbH, Mannheim, Germany). The applied primers are listed in *Online Supplementary Table S2*. In order to show presence or absence of the 3' and 5' ends of ALK, primer pairs specific for the respective ends[4] were used. To amplify the *SEC31A-ALK* fusion, nested RT-PCR was carried out using forward primers in *SEC31A* and reverse primers in *ALK*.

A 5' RACE PCR experiment was performed using a previously described protocol and primers.[3] In the second round of nested PCR, however, reverse primer ALK-R3 was used (*Online Supplementary Table 2*). The final PCR products were cloned in pGEM-T-Easy (Promega, Madison, WI) and sequenced using an ABI3100 system (PE Applied Biosystems, Foster City, CA).

The IgH gene V-D-J functional variability and the Kappa deleting (Kde) rearrangements were examined by PCR and heteroduplex analysis.[7,8] The *TCR* gene VJ rearrangements of the TCRgamma locus were analyzed by PCR and denaturing gradient gel electrophoresis.[9] In case 2, an additional examination of the IG loci was performed with the Biomed-2 protocol.[10,11] Results of these molecular analyses of *TCR*, *IGH* and *IGK* can be found in Table 1.

### *Array-based Comparative Genomic Hybridization (aCGH)*

Array CGH was performed with Code Linked Slides (AP Biotech) containing the 3,527 BAC clones from the Wellcome Trust Sanger Institute 1 Mb Clone Set, extended with clones for all 90 protein tyrosine kinase genes, as previously described.[12]

### SEC31A-ALK *construct*

The *SEC31A* and *ALK* fragments were cloned from human tissue cDNA prepared from commercially available RNA (Clontech, Mountain View, CA, USA) with Superscript III reverse transcriptase (Life Technologies, Merelbeke, Belgium). The open reading frame of exon 1 to 24 of *SEC31A* was amplified from human thymus cDNA with primers SEC31A-F-XhoI and SEC31A-R-BglII, and with primers SEC31A-F-BglII and SEC31A-R-NotI (*Online Supplementary Table 2*). The *ALK* fragment was generated by PCR amplification from human fetal brain cDNA with primers ALK-F-NotI and ALK-R-EcoRI (*Online Supplementary Table S2*). The Phusion High Fidelity PCR kit (Finnzymes, Espoo, Finland) was used for all PCR reactions. Both generated *SEC31A* fragments and the ALK fragment were ligated into the retroviral pMSCV-puro vector (Clontech, Mountain View, CA, USA). The construct was verified by sequencing.

### *Cell culture and retroviral transduction*

HEK293T and Ba/F3 cells were cultured, transfected and transduced as previously described.[13] Transduced Ba/F3 cells were selected with puromycin (2.5 µg/mL). For growth curves, Ba/F3 cells were deprived of IL3 and seeded at $10^5$ cells per mL of medium. Viable cells were counted on five consecutive days with a Vi-CELL XR cell viability analyzer (Beckman Coulter, Fullerton, CA, USA). For dose-response curves, $1.5\times10^5$ cells (NPM1-ALK) and $3\times10^6$ cells (SEC31A-ALK) were seeded in 1 mL of medium and incubated in the presence of the ALK inhibitor TAE-684 (Axon Medchem, Groningen, The

Netherlands) for 48 h. Viable cell numbers were determined by the Celltiter AQueousOne Solution (Promega, Madison, WI, USA). For Western blotting, $4\times10^6$ cells were incubated with TAE-684 for 90 minutes and lysed in cold lysis buffer containing 1% Triton X-100 and phosphatase inhibitors.

### *Western blotting*

Standard Western blotting procedures were used to analyze total cell lysates with the following antibodies: anti-phospho-ALK (Tyr1604), anti-ALK (C26G7), anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), anti-phospho-AKT (Ser473), anti-AKT, anti-phospho-STAT3 (Tyr705) and anti-phospho-STAT5 (Tyr694) (Cell Signaling Technology, Danvers, MA, USA), anti-MAP Kinase (ERK1 + ERK2) and anti-STAT3 (ZYMED, Camarillo, CA, USA), anti-STAT5A (L-20) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-SEC31A (Sigma-Aldrich, St Louis, MO, USA).

## References


1. Rudzki Z, Rucinska M, Jurczak W, Skotnicki AB, Maramorosz-Kurianowicz M, Mruk A, et al. ALK-positive diffuse large B-cell lymphoma: two more cases and a brief literature review. Pol J Pathol. 2005;56(1):37-45.
2. Mathew P, Sanger WG, Weisenburger DD, Valentine M, Valentine V, Pickering D, et al. Detection of the t(2;5)(p23;q35) and NPM-ALK fusion in non-Hodgkin's lymphoma by two-color fluorescence in situ hybridization. Blood. 1997;89(5):1678-85.
3. Cools J, Wlodarska I, Somers R, Mentens N, Pedeutour F, Maes B, et al. Identification of novel fusion partners of ALK, the anaplastic lymphoma kinase, in anaplastic large-cell lymphoma and inflammatory myofibroblastic tumor. Genes Chromosomes Cancer. 2002; 34(4):354-62.
4. De Paepe P., Baens M, van Krieken H, Verhasselt B, Stul M, Simons A, et al. ALK activation by the CLTC-ALK fusion is a recurrent event in large B-cell lymphoma. Blood. 2003;102(7):2638-41.
5. Morris SW, Kirstein MN, Valentine MB, Dittmer KG, Shapiro DN, Saltman DL, et al. Fusion of a kinase gene, ALK, to a nucleolar protein gene, NPM, in non-Hodgkin's lymphoma. Science. 1994;263(5151):1281-4.
6. Shaffer LG, Tommerup N. ISCN 2005. An International System for Human Cytogenetics Nomenclature. Basel, Switzerland: S.Karger; 2005.
7. Aubin J, Davi F, Nguyen-Salomon F, Leboeuf D, Debert C, Taher M, et al. Description of a novel FR1 IgH PCR strategy and its comparison with three other strategies for the detection of clonality in B cell malignancies. Leukemia. 1995;9(3):471-9.
8. Beishuizen A, de Bruijn MA, Pongers-Willemse MJ, Verhoeven MA, van Wering ER, Hahlen K, et al. Heterogeneity in junctional regions of immunoglobulin kappa deleting element rearrangements in B cell leukemias: a new molecular target for detection of minimal residual disease. Leukemia. 1997;11(12):2200-7.
9. Theodorou I, Bigorgne C, Delfau MH, Lahet C, Cochet G, Vidaud M, et al. VJ rearrangements of the TCR gamma locus in peripheral T-cell lymphomas: analysis by polymerase chain reaction and denaturing gradient gel electrophoresis. J Pathol. 1996;178(3):303-10.
10. Evans PA, Pott C, Groenen PJ, Salles G, Davi F, Berger F, et al. Significantly improved PCR-based clonality testing in B-cell malignancies by use of multiple immunoglobulin gene targets. Report of the BIOMED-2 Concerted Action BHM4-CT98-3936. Leukemia. 2007;21(2):207-14.
11. van Dongen JJ, Langerak AW, Bruggemann M, Evans PA, Hummel M, Lavender FL, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia. 2003;17(12):2257-317.
12. Lahortiga I, De Keersmaecker K., Van Vlierberghe P, Graux C, Cauwelier B, Lambert F, et al. Duplication of the MYB oncogene in T cell acute lymphoblastic leukemia. Nat Genet. 2007;39(5):593-5.
13. Schwaller J, Frantsve J, Aster J, Williams IR, Tomasson MH, Ross TS, et al. Transformation of hematopoietic cell lines to growth-factor independence and induction of a fatal myelo- and lymphoproliferative disease in mice by retrovirally transduced TEL/JAK2 fusion genes. EMBO J. 1998;17(18):5321-33.