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

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ABSTRACT

We report 2 ALK-positive large B-cell lymphoma cases showing granular cytoplasmic and cytoplasmic/nuclear ALK immunostaining in which cryptic *ALK* rearrangements were identified by fluorescent *in situ* hybridization and molecular analysis. In the first case, the *ALK*-involving t(2;3)(p23;q27) masked the cryptic *SEC31A-ALK* fusion generated by an insertion of the 5' end of *SEC31A* (4q21) upstream of the 3' end of *ALK*. This rearrangement was associated with loss of the 5' end of *ALK* and duplication of *SEC31A-ALK* on der(20). In the second case with complex rearrangements of both chromosomes 2, a submicroscopic *NPM1-ALK* fusion created by insertion of the 3' end of *ALK* into the *NPM1* locus was evidenced. Further studies of *SEC31A-ALK* showed that this variant fusion transforms IL3-dependent Ba/F3 cells to growth factor independence, and that the ALK inhibitor TAE-684 reduces cell proliferation and kinase activity of SEC31A-ALK and its downstream effectors ERK1/2, AKT, STAT3 and STAT5.

Key words: fusion gene, oncogene, anaplastic lymphoma kinase, ALK-positive large B-cell lymphoma, cryptic translocation.

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Introduction

Anaplastic lymphoma kinase-positive large B-cell lymphoma (ALK$^+$ LBCL) is a rare neoplasm recognized as a separate entity by the latest WHO classification of hematologic malignancies.[1] The lymphoma displays a characteristic immunoblastic/plasmablastic morphology and a distinct immunophenotype (ALK$^+$, CD3$^-$, CD20$^-$, CD138$^+$). It spans all age groups (range 9 – 85 years with an average age of 37 years), has a male predominance (M:F ratio, 4:1) and frequently presents with extensive disease, an aggressive clinical course, poor response to therapy and short survival. So far, 84 cases of ALK+ LBCL have been reported.[2-5] The aberrant ALK expression hallmarking these cases is underlied by chromosomal translocations affecting the *ALK* gene (2p23). The t(2;17)(p23;q23) resulting in the CLTC-ALK fusion and a granular cytoplasmic ALK reactivity by immunohistochemistry (IHC)[6] was observed in at least 70% of informative ALK$^+$ LBCL cases. The t(2;5)(p23;q35) and/or NPM1-ALK fusion, flagged by a nuclear and cytoplasmic ALK immunostaining, was reported in 10% of ALK$^+$ LBCL cases.[3-4,7-10] It is noteworthy that *ALK*-involving translocations were originally identified in ALK$^+$ T-/null-cell anaplastic large cell lymphoma.[11] Interestingly, the same aberrations have been detected in inflammatory myofibroblastic tumors (IMT)[12] and non-small cell lung cancer.[13] More recently, oncogenic mutations of ALK kinase and ALK amplifications were reported in neuroblastoma tumors.[14] So far, at least 14 different partner genes targeted by *ALK* translocations have been identified in human tumors.[15] Molecular studies of the most common, *NPM1-ALK*, show that this fusion, and probably all ALK-involving fusions, leads to ligand-independent constitutive activation of ALK by its autophosphorylation, and promotes malignant cell transformation by activation of downstream signaling pathways.[16]

Although no ALK small-molecule inhibitors are currently available for clinical cancer therapy, the involvement of *ALK* in the pathogenesis of lymphoid and mesenchymal tumors has prompted developmental efforts in this area[17] with the recently identified inhibitor of ALK kinase activity, TAE-684, as a very promising agent.[18]

In the present study, we report 2 new ALK$^+$ LBCL cases characterized by cryptic *ALK* rearrangements, and functional studies of the *SEC31A-ALK* fusion detected in one of these tumors that, like *NPM-ALK*, was shown to be sensitive for TAE-684.

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The online version of this article has a Supplementary Appendix.

Design and Methods

Patients

Patients were selected from the lymphoma database of the Center for Human Genetics, K.U. Leuven. Morphology, immunophenotype and clinical data of both patients were reviewed. Local ethics approval for this study was obtained.

Cytogenetics and fluorescent in situ *hybridization (FISH)*

Cytogenetic and FISH analysis followed routine protocols. The applied FISH probes are listed in the *Online Supplementary Table S1*.

Molecular analysis

Detailed protocols of PCR, RT-PCR and Rapid Amplification of cDNA Ends (RACE PCR) are described in the Online Supplementary Methods. Briefly, PCR was performed on a DNA or cDNA template using Taq polymerase (Life Technologies). The primers are listed in the *Online Supplementary Table S2*. The 5' RACE PCR protocol has been published;[19] however, in the second round of the nested PCR, reverse primer ALK-R3 was used.

Functional analysis

Detailed protocols of the generation of the *SEC31A-ALK* construct, cell culture, retroviral transduction and Western blotting experiments are described in the *Online Supplementary Methods*.

Briefly, the *SEC31A* and *ALK* fragments were amplified from human tissue cDNA and cloned into the retroviral pMSCVpuro vector (Clontech). HEK293T cells and Ba/F3 cells were cultured in DMEM and RPMI1640, both supplemented with 10% fetal bovine serum. The Celltiter AQueousOne Solution (Promega) was used to obtain dose-response curves of SEC31A-ALK and NPM-ALK expressing Ba/F3 cells treated with TAE-684 inhibitor. Standard Western blotting was performed with anti-total and anti-phospho antibodies for ALK, SEC31A, ERK1/2, AKT, STAT3 and STAT5.

Results and Discussion

Relevant clinical, phenotypic and genetic data of both reported cases are shown in Table 1. Although case 2 was negative for CD20, CD79a and CD138 and showed a germline configuration of *IG* genes when analyzed by PCR, the expression of PAX5, MUM1 and IgA by neoplastic cells was indicative of the B-cell origin of this lymphoma. Cytogenetic analysis was performed in both cases. Case 1, showing a granular cytoplasmic expression pattern of ALK by IHC (Figure 1A), revealed a complex (diploid and tetraploid) karyotype without an overt 2p23 translocation (Table 1). To identify the genomic rearrangements underlying the aberrant ALK expression in this lymphoma, an extensive metaphase FISH analysis was performed (*Online Supplementary Table 1S*). Briefly, using LSI ALK, LSI BCL6 and mFISH, we identified a cryptic *ALK*-involving t(2;3)(p23;q27) associated with loss of the 5' end of *ALK* (green signal). The terminal part of the der(3) harboring the 3' end of *ALK* (red signal) was duplicated and translocated to chromosome 20 (Figure 1B). The partial loss of *ALK* was further evidenced by RT-PCR that showed expression of the 3' end of *ALK*, but not of its 5' end (Figure 2A). The cryptic involvement of *CLTC*/17q23, the most frequent *ALK* partner in ALK$^+$ LBCL,[3] was excluded by FISH with the appropriate break-apart probes. In order to identify the partner gene targeted by the t(2;3)(p23;q27), 5' RACE PCR was carried out. This analysis identified an in-frame fusion transcript in which exon 24 of *SEC31A* (4q21) was fused to exon 20 of *ALK* (Figure 2B). The *SEC31A-ALK* transcript was further confirmed by nested RT-PCR followed by sequencing with forward primers in the *SEC31A* gene combined with reverse primers in *ALK* (Figure 2C).

The molecular results were validated by metaphase FISH

Table 1. Relevant clinical and cytogenetic data of 2 ALK+ LBCL cases.

Case n.	Sex/ age	Stage	Treatment	Response/ Survival	ALK staining	Morphology	Immuno-phenotype	PCR analysis	Cytogenetics Sample/ Status	Karyotype	FISH identified aberration
1	M/27	IA	4 cycles of CHOP, IFR (30Gy)	CR/ 27 months	Cytoplasmic granular	Large cells with plasmatic differentiation	CD3- CD20- CD138$^+$ CD79a$^+$ PAX5+weak IgA+ (cyt) EMA$^+$	TCR*-g IGH-r IGK-r	LN/D	45-47,XY,+X[5],del(1)(q32),i(1)(q10)del(2)(q33)[1],del(6)(q22)[3], +7[2], der(13)t(3;13)(q25;p13)[7],-14[6],-15[2], add(16)(p13), add(20)(q13), +add(20)(q13)[3][cp8] 75-91,idem,add(2)(q25), der(16)t(1;16)(q21;q24),+1-3mar[cp4]	ish.t(2;3)(p23;q27), ins(4)(2;4)(?;q21), der(20)(20pter-> 20q13 ;;3q27;; 2p23->pter)
2	F/33	IVA	7 cycles of CHOP	CR/ 19 months	Nuclear and cytoplasmic	Large cells with plasmatic differentiation	CD3- CD20- CD138- CD79a- PAX5+ MUM1+ IgA+ (cyt/subm) CD30+ EMA+	TCR*-g IGH-g IGK-g IGL-g	LN/D	43-46,XX,+X,+X[1],der(1)t(1;17)(q21;q11),der(2)t(2;2)(p23;q21), der(2)?inv(2)(p13q21)del(2)(q21), der(7)t(1;7)(q21;q22),-15[2],-20[cp3]	

*M: male; F: female; CHOP: cyclophosphamide, adriamycin, vincristine, prednisone; IFR: involved field radiotherapy; CR: complete remission; cyt: cytoplasmic; subm: submembranous; *analyzed TCR alpha/delta, beta, gamma loci; g: germline; r: rearranged; LN: lymph node; D: diagnosis.*

with fosmid probes flanking the *SEC31A* gene that confirmed a cryptic rearrangement of *SEC31A* associated with an insertion of its 5' end in the vicinity of the 3' end of *ALK* at 3q27, and duplication of this region on add(20) (Figure 1B; *Online Supplementary Table S1*). The latter aberration was additionally proved by FISH with probes covering the 5' end of *SEC31A* (WI2-2194A15 in red) and the 3' end of *ALK* (P1 clone 1111H1 in green) (Figure 1B; *Online Supplementary Table S1*). Notably, mFISH followed by chromosome 2 painting identified a cryptic insertion of chromosome 2 material at 4q21, possibly at the locus of the rearranged *SEC31A* gene (Figure 1B; *Online Supplementary Table S1*). The more precise origin of these inserted sequences was not established. The genomic imbalances identified by cytogenetics and FISH (excluding the duplication of the 5' end of *SEC31A*) were confirmed by 1 Mb aCGH analysis (*data not shown*). Altogether, in case 1 we identified two copies of the *SEC31A-ALK* fusion and loss of the 5'end of *ALK*; these cryptic *ALK* rearrangements were masked by complex chromosomal rearrangements involving 2p23, 3q27, 4q21 and 20q13. Such rearrangements were probably required for the generation of a functional *SEC31A-ALK* fusion which, due to an opposite transcriptional orientation of *ALK* and *SEC31A*, cannot be generated by a simple reciprocal t(2;4)(p23;q21). Remarkably, similar complex aberrations were detected in a case of IMT in which the *SEC31A–ALK* fusion was originally identified.[20] Whether another case of ALK$^+$ LBCL with a cryptic insertion of the 3' end of ALK at 4q22-24 reported by Stachurski *et al.*,[21] targets *SEC31A* remains to be clarified. The duplication of the *ALK* fusion gene found in case 1 has been recurrently reported in ALK-positive lymphoma.[22-24] An increased level of ALK kinase possibly provides growth advantage for neoplastic cells, as shown in *in vitro* studies.[25] The significance of a focal deletion of the 5' end region of *ALK* recently reported in one ALK$^+$ LBCL case[8] remains unknown.

Case 2 was characterized by a nuclear and cytoplasmic ALK reactivity by IHC (Figure 1C) heralding the *NPM1-ALK* rearrangement. Cytogenetics, however, did not identify a t(2;5)(p23;q35) but showed complex aberrations involving both chromosomes 2 (Table 1). FISH with LSI ALK revealed a fused signal on one der(2), a green signal (5' end) on the second der(2) and a red signal (3' end) on the terminal part of a normal-looking chromosome 5 (Figure 1D; *Online Supplementary Table S1*). Further FISH with *NPM1* break-apart probe showed two fusion signals, each at 5q35 (Figure 1D; *Online Supplementary Table S1*). These results suggested a cryptic insertion of the 3' end of *ALK* into the *NPM1* locus at 5q35, as further proved by cDNA-based nested PCR. With forward primers in *NPM1* and reverse primers in *ALK*, an amplicon of approximately 150 bp was obtained and sequenced (Figure 2D). This analysis detected an in-frame fusion between exon 4 of *NPM1* and exon 20 of *ALK* (Figure 2B), which corresponds to the typical *NPM1-ALK* fusion commonly found in ALK$^+$ ALCL. In case 2, however, this rearrangement was generated by insertion of the 3' end of *ALK* into the *NPM1* locus.

The *SEC31A-ALK* fusion detected in case 1 has not previously been seen in lymphoma. To investigate its oncogenic potential, we designed a *SEC31A-ALK* construct and expressed it in the interleukin 3 (IL3)-dependent Ba/F3 cell line. These studies showed that SEC31A-ALK transformed the Ba/F3 cells to growth factor independent growth upon IL3 withdrawal (Figure 2E). Additionally, we tested the sensitivity of SEC31A-ALK to TAE-684, a selective inhibitor of ALK kinase activity. Ba/F3 cells expressing NPM1-ALK were used as a positive control since it has been shown that they respond to TAE-684.[18] Although both Ba/F3 cells expressing

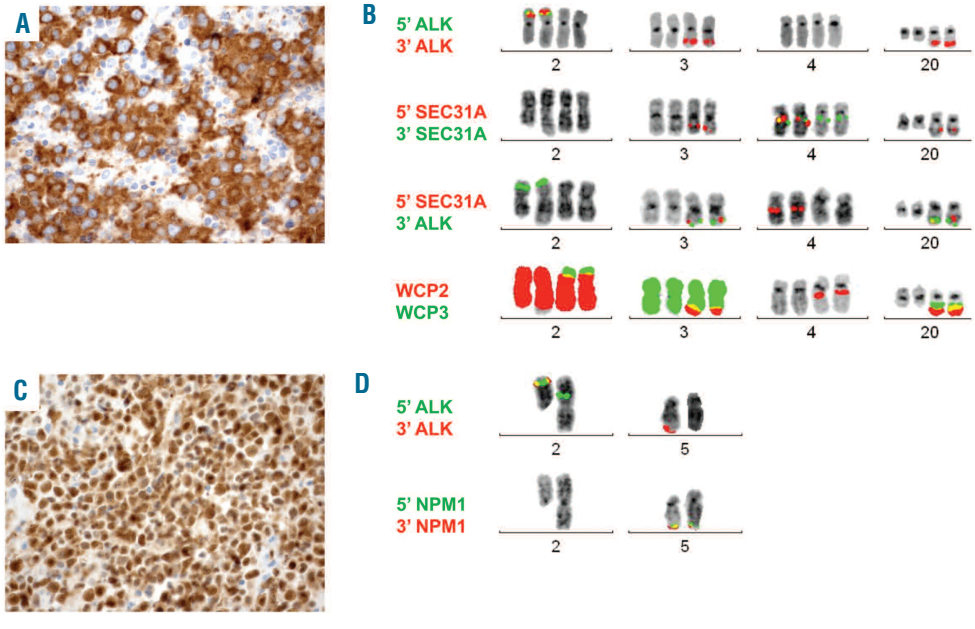

Figure 1. Immunohistochemical and FISH analysis. (A) ALK immunostaining in case 1 showing a granular cytoplasmic distribution pattern. (B) Results of FISH analysis of case 1 (partial karyotype). The applied probes included LSI ALK, probes flanking *SEC31A*, the 5' end probe of *SEC31A* combined with a probe covering 3' *ALK*, and Whole Chromosome Painting (WCP) probes for chromosomes 2 and 3, (*Online Supplementary Table S1*). Note the presence of the 3' end of *ALK* and the 5' end of *SEC31A* on both chromosomes 3 and 20, and the focal deletion of the 5' end of *ALK*. (C) ALK immunostaining in case 2 showing a nuclear and cytoplasmic distribution pattern. (D) Molecular cytogenetic analysis of case 2 with LSI ALK and probes flanking *NPM1* showed a cryptic insertion of the 3' end of *ALK* into the *NPM1* gene locus. FISH images were acquired with a 63x/1.40 oil-immersion objective in an Axioplan 2 fluorescence microscope equipped with an Axiophot 2 camera (Carl Zeiss Microscopy, Jena, Germany) and a MetaSystems Isis imaging system (MetaSystems, Altlussheim, Germany). Three to 12 abnormal metaphase cells and/or 200 interphase nuclei were evaluated in each FISH experiment.

NPM1-ALK and SEC31A-ALK responded to inhibitor treatment in a dose-dependent matter, with a 50% inhibitor concentration (IC_{50}) between 10 and 50 nM, NPM1-ALK showed a higher response with a lower inhibitor concentration than SEC31A-ALK (Figure 2F). When IL3 was added to these cells, proliferation became independent of the ALK pathway and was not disturbed by the ALK inhibitor TAE-684 (*data not shown*). Western blot analysis for SEC31A-ALK confirmed a decrease in ALK tyrosine phosphorylation with an increasing dose of TAE-684 (Figure 2G) while protein expression was unaffected. Downstream effectors ERK1/2, STAT3, and to a lesser extent AKT and STAT5, also showed decreased phosphorylation with increasing inhibitor concentrations while total protein expression remained unaffected (Figure 2G).

In summary, we showed that cryptic aberrations of *ALK* are recurrent in ALK$^+$ LBCL. These rearrangements include insertion of either the 5' region of the partner gene (*SEC31A*) in the vicinity of the 3' region of *ALK* (case 1), or insertion of the 3' region of *ALK* into the partner locus (*NPM1*) (case 2). So far, such cryptic *ALK* rearrangements have not been described in lymphoma. The detection of *SEC31A-ALK* in ALK$^+$ LBCL provides additional evidence of the same *ALK* rearrangements underlying ALK expressing tumors of lymphoid and mesenchymal origin. Functional analysis of this

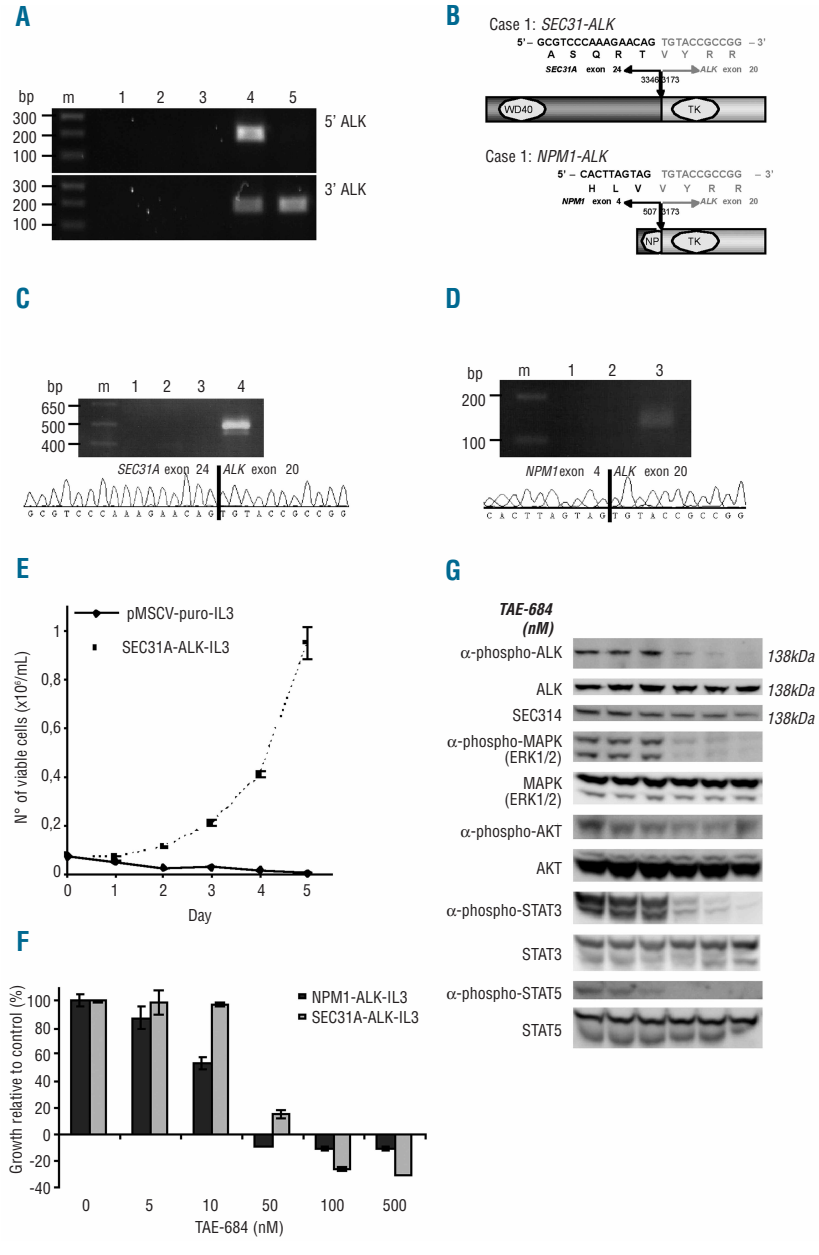

Figure 2. Molecular and functional analysis of the *SEC31A-ALK* and *NPM1-ALK* fusion. (A) Case 1: RT-PCR with primers specific for the 5' end (upper figure) and 3' end (lower figure) of *ALK* showed no expression on cDNA level of *ALK* in reactive lymph nodes, but presence of full length *ALK* (5' and 3' end) in normal brain cDNA. In case 1, only the 3' end of *ALK*, containing the tyrosine kinase domain, was expressed. Lane m, 1kb Plus; Lane 1, negative control (no cDNA); Lane 2 and 3, negative control (reactive lymph node cDNA); Lane 4, positive control (normal brain cDNA); Lane 5, cDNA from case 1. (B) Schematic representation of the identified fusion transcripts. The DNA and protein sequence at the fusion border (indicated by the arrowhead) are also shown. TK: tyrosine kinase, NP: nucleoplasmin. (C) Case 1: Nested RT-PCR with forward primers in *SEC31A* and reverse primers in *ALK* showed presence of the *SEC31A-ALK* fusion in case 1, but not in normal liver and reactive lymph node cDNA (upper figure). Lane m, 1kb Plus; Lane 1, negative control (no cDNA); Lane 2, negative control (normal liver cDNA); Lane 3, negative control (reactive lymph node cDNA); Lane 4, cDNA from case 1. An electropherogram with the *SEC31A-ALK* fusion sequence of the PCR product is also shown (lower figure). (D) Case 2: Nested RT-PCR with forward primers in *NPM1* and reverse primers in *ALK* showed presence of the *NPM1-ALK* fusion in case 2, but not in reactive lymph node cDNA (upper figure). Lane 1, negative control (no cDNA); Lane 2, negative control (reactive lymph node cDNA); Lane 3, cDNA from case 2. An electropherogram with the *NPM1-ALK* fusion sequence of the PCR product is also shown (lower figure). (E) IL3 deprivation of Ba/F3 cells transduced with *SEC31A-ALK* resulted in transformation to growth factor independent growth. When transduced with the empty pMSCVpuro vector, Ba/F3 cells were not able to survive without IL3 and died. The mean growth ± SEM of three separate measurements over five consecutive days is given. (F) SEC31A-ALK and NPM1-ALK expressing Ba/F3 cells were treated with the indicated concentrations of TAE-684 and cell survival was quantified after 48 h. Cell survival in the absence of TAE-684 (0 nM) was set at 100%. Mean ± SEM of four independent measurements is shown. (G) Western blot analysis showing the effect of TAE-684 treatment on Ba/F3 cells transduced with *SEC31A-ALK*. In addition to ALK phosphorylation, phosphorylation of ERK1/2, AKT, STAT3 and STAT5 decreased with increasing inhibitor concentrations. Expression of total SEC31A-ALK (138 kDa), ERK1/2, AKT, STAT3 and STAT5 remained unaffected.

fusion in Ba/F3 cells identified SEC31A-ALK as a constitutively activated tyrosine kinase. Inhibition experiments with the ALK-specific TAE-684 inhibitor demonstrated reduced phosphorylation of SEC31A-ALK and inhibition of downstream signaling. Downstream effectors ERK1/2, AKT, STAT3 and STAT5, previously found to be involved in NPM1-ALK signaling,[16] showed reduced phosphorylation with increasing inhibitor concentrations. *In vitro* proliferation of the Ba/F3 cells expressing NPM1-ALK and SEC31A-ALK was inhibited by TAE-684 with an IC_{50} between 10 and 50 nM, consistent with the previous findings by Galkin and colleagues.[18] These results suggest that TAE-684 is a very potent ALK inhibitor which can be used not only for inhibition of the typical NPM1-ALK protein, but also for inhibition of its variant fusions such as SEC31A-ALK.

The *SEC31A* gene is ubiquitously expressed in human cells; the SEC31A protein is localized to vesicular structures that scatter throughout the cell and is particularly concentrated at the perinuclear region. SEC31A is likely to be a part of coat protein complexes mediating transport from the ER to the Golgi machinery.[26] The biology of SEC31A may explain a granular cytoplasmic localization of the SEC31A-ALK protein in tumor cells. In contrast to other ALK-related fusions, a coiled-coil oligomerization domain of SEC31A located at the C-terminus of the protein is not involved in ligand-independent activation of the ALK tyrosine kinase. This function may be provided by WD40-like repeats located at the N-terminal end of SEC31A (Figure 2B) which interact with SEC13, but may also bind to other proteins.[26]

In conclusion, the cryptic *ALK* rearrangements identified in both presented ALK^+ LBCL cases underscore the need of comprehensive histopathological and genetic approaches in the diagnosis of ALK^+ neoplasms. Given that patients with ALK-expressing tumors may benefit in the future from targeted therapy, identification of such cases is clinically important.

Authorship and Disclosures

KVR designed and performed research, analyzed the data and wrote the paper; JC designed research and contributed to the paper; DD, JT, PV, JD and CDP provided patients' samples, clinical and pathological data, and revised the article for intellectual content; MS provided molecular data; PM revised the article for intellectual content; IW designed the study, provided cytogenetic data, analyzed the data, and contributed to the paper.

The authors reported no potential conflicts of interest.

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