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Research Article

Molecular Screening in Anaplastic Lymphoma Kinase–Positive Anaplastic Large Cell Lymphoma: Anaplastic Lymphoma Kinase Analysis, Next-Generation Sequencing Fusion Gene Detection, and T-Cell Receptor Immunoprofiling

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ABSTRACT

Anaplastic lymphoma kinase–positive anaplastic large cell lymphoma (ALK+ ALCL) originates from the T-lineage and is marked by rearrangements of the *ALK* gene. More than 10 fusion partners with the *ALK* gene are known, with the most common being the t(2;5)(p23;q35) translocation resulting in the *NPM1::ALK* fusion. In 10% to 20% of the ALK+ ALCL cases, the *ALK* gene fuses with various other partners. Modern molecular techniques, especially next-generation sequencing (NGS), have eased the identification of *ALK* gene fusion partners and have allowed in-depth characterization of the T-cell receptor (TCR) repertoire. We devised a real-time quantitative reverse-transcription polymerase chain reaction to measure the expression of the translocated portion of the *ALK* gene. Fusion partners for the *ALK* gene were analyzed using rapid amplification of 5′cDNA ends (RACE) method or NGS. TCR immunoprofiling was performed by amplicon NGS. We studied 96 ALK+ ALCL patients. *NPM1::ALK* fusion gene was observed in 71 patients, *ATIC::ALK* in 9, and *TPM3::ALK* in 3. *CLTC::ALK*, *MYH9::ALK*, and *RNF213::ALK* fusions were identified in 2 patients each. We also discovered the *TPM4::ALK* and *SATB1::ALK* fusion genes, plus the following 2 previously unidentified ALK+ ALCL fusions: *SQSTM1::ALK* and *CAPRIN1::ALK*. High expression of the translocated *ALK* gene segment was observed in all 93 analyzed samples. TCR testing was conducted on 23 patients with available DNA. In 18 (78%) patients, we discerned at least one (ranging from 1 to 4) clonal TCR rearrangement. In 59% of the patients, clonal TCR beta junctions corresponded with sequences previously observed in both healthy donors and under various pathological conditions. Reverse-transcriptase quantitative detection of *ALK* expression is a fast and reliable method for both diagnosing and monitoring treatment response in ALK+ ALCL patients, irrespective of the *ALK* gene translocation. NGS reveals new

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ALK translocation partners. Both malignant and reactive TCR repertoires in ALK+ ALCL patients are unique and do not consistently occur among different patients.

Introduction

Anaplastic lymphoma kinase–positive anaplastic large cell lymphoma (ALK+ ALCL) is a subtype of peripheral T-cell lymphoma according to the World Health Organization classification. It is characterized by the expression of ALK due to translocations involving the *ALK* gene located on chromosome 2p23. ALK+ ALCL constitutes approximately 3% of adult non–Hodgkin lymphomas; however, it represents 10% to 20% of the lymphomas diagnosed during childhood. Typically, patients are younger with a slight male predominance, and the median age at the time of diagnosis falls within the first 3 decades of life.[1,2]

Receptor tyrosine kinase ALK, located on 2p23, is physiologically expressed in fetal neural cells. In most cancer types (eg, lymphoma, lung cancer, inflammatory myofibroblastic tumors, Spitz tumors, and renal carcinoma), ALK is activated by chromosomal rearrangement.[3] The most prevalent rearrangement, accounting for approximately 80%, is t(2;5)(p23;q35). This leads to the fusion of the *ALK* gene with the *NPM1* (Nucleophosmin 1) gene.[4,5] In 15% to 20% of the ALK+ ALCL cases, the *ALK* gene fuses with various other partner genes. Specifically, t(1;2)(q25;p23) *TPM3::ALK* (Tropomyosin 3) is observed in 13% of the patients, and inv(2)(p23q35) *ATIC::ALK* (5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase) in 1%.[6,7] Additional translocations, each with a frequency below 1%, include the following: t(2;3)(p23;q12.2) *TFG::ALK* (TRK-fused gene), t(2;17)(p23;q23) *CLTC::ALK* (Clathrin heavy chain gene), t(X;2)(q11-12;p23) *MSN::ALK* (Moesin), t(2;19)(p23;p13.1) *TPM4::ALK* (Tropomyosin 4), t(2;22)(p23;q11.2) *MYH9::ALK* (Myosin heavy chain 9), and t(2;17)(p23;q25) *RNF213::ALK* (Ring finger protein 213).[1,6-9]

ALK is a member of the insulin receptor tyrosine kinase family. Typically, the translocation involves the fusion of the 5' end of the fusion partner gene, which includes its promoter and other regulatory regions, to the 3' end of the *ALK* gene, which encodes the intracellular tyrosine kinase domain. This leads to constitutively active chimeric protein. Chromosomal translocations and fusion proteins involving the *ALK* gene are also found in other hematologic malignancies (eg, diffuse large B-cell lymphoma) and solid tumors such as non–small cell lung cancer, inflammatory myofibroblastic tumor (IMT), breast cancer, colorectal cancer, esophageal squamous cell carcinoma, and renal cell carcinoma. ALK+ histiocytosis is a rare subtype of histiocytic neoplasm associated with ALK fusions (most frequent *KIF5B::ALK* fusion, rarely *CLTC::ALK*, *TPM3::ALK*, *TFG::ALK*, *EML4::ALK*, and *DCTN1::ALK* fusions).[10] Identification of *ALK* gene fusion partners is critical for diagnostics, prognosis, and subsequent targeted therapy using ALK inhibitors.[10-12] Moreover, detection of the fusion gene enables the assessment of minimal disseminated disease and minimal residual disease (MRD). The recent advancement of NGS technology has enabled complex molecular screening of diseases, including the detection of *ALK* gene translocation partners, other molecular alterations, and T-cell receptor (TCR) rearrangements repertoire (immunome). Analyses of clonal TCR beta (TCRB) and TCR gamma (TCRG) rearrangements in ALCL previously used qualitative methods.[13,14] NGS analysis of the TCR repertoire using amplicon sequencing allows for the detection of monoclonal TCR rearrangements not only in tumor cells but also in infiltrating T lymphocytes.

Materials and Methods

Patients

After obtaining approval from the ethical committee and informed consent, we collected and analyzed diagnostic tumor tissue samples from various sites (including lymph nodes, skin, soft tissues of the chest wall, tumors from the trachea, mediastinum, brain, duodenum, and other soft tissues). These samples were gathered from a cohort of 96 patients with ALK+ ALCL, who were consecutively diagnosed between 1992 and 2021 at the Department of Pathology and Molecular Medicine of the second Faculty of Medicine. The primary diagnosis of ALCL was based on a combined morphological and immunophenotype examination.

The median age of the patients was 16 years (range: 2 to 80 years), and the male/female ratio was 1.23:1. The age distribution of ALK+ ALCL patients, along with the number of patients, is depicted in Supplementary Figure S1. Complete data, including age, normalized copies of *ALK* gene expression, ALK protein localization, fusion genes, fluorescence in situ hybridization (FISH) results, histological variants, and outcomes for all individual patients, are shown in Supplementary Table S1.

Methods

Immunohistochemistry and Interphase Fluorescence In Situ Hybridization

Immunohistochemistry (IHC) was performed on thin histological tissue sections from formalin-fixed, paraffin-embedded tissues (FFPE) according to instructions provided by the standard manufacturer. We evaluated the expressions of the ALK protein, CD30, granzyme B, perforin, TIA1, and T/B-cell markers, such as CD2, CD3, CD4, CD7, CD8, CD20, PAX5, and CD79α. Fluorescence in situ hybridization on interphase nucleus (I-FISH) was performed to confirm the break on chromosomal region 2p23 harboring the *ALK* gene using the ALK Dual Color locus-specific probe (Zyto*Light* SPEC ALK Dual Color Break Apart Probe, ZytoVision).

Reverse-Transcriptase Quantitative Detection of the Expression of the Translocated Portion of the ALK Gene

Total RNA was extracted from 8- to 10 5-μm thick FFPE sections using the High Pure FFPE RNA Isolation Kit (Roche) and from 8 to 10 5-μm thick fresh frozen tissue sections using Trizol reagent (Life Technologies). The complementary DNA (cDNA) and RT-PCR

for the detection of the most frequent fusion gene, *NPM1::ALK*, were prepared as described elsewhere.[15] The second most common *ALK* fusion, *ATIC::ALK* was analyzed using reverse-transcriptase quantitative detection (RT-qPCR), based on the protocol published by Maes et al.[16] To quantify the mRNA of the translocated portion of the *ALK* gene (3′end), we used the LNA Probe 37 (TGCCCTGG), Universal probe library, (Roche) and primers ALK 37L (5′-tggagtttgtcaccagtgga-3′) and ALK37R (5′-ccagcactgagtcattatcc-3′). Beta 2 microglobulin (*B2M*) served as the control housekeeping gene to assess the quality and quantity of RNA/cDNA isolated from FFPE tissues, as described previously.[17] The cDNA sequence of the 3′*ALK* gene (the translocated portion) was cloned into a pCR2.1-TOPO vector and subsequently transformed into the TOP10 *Escherichia coli* strain, using the TOPO TA Cloning Kit (GIBCO BRL). Selected clones underwent screening for insert presence via PCR. After mass production, plasmids were extracted using the Miniprep method and quantified spectrophotometrically. The plasmid was then serially diluted in xeno DNA (Salmon sperm—Sigma) with a concentration of 0.2 g/L. Standard curves for the 3′*ALK* gene were prepared in the following final concentrations: 2×10^5, 2×10^4, 2×10^3, 2×10^2, and 2×10^1 copies/1 μL. RT-qPCR was performed according to the manufacturer's instructions (Roche) on the iCycler iQ Multicolor Real-Time PCR instrument (Biorad) using FastStart Tag Man Probe Master (Roche). Thermal cycling conditions were as follows: 2 minutes at 50 °C, 10 minutes at 95 °C, followed by 55 cycles of amplification for 20 seconds at 95 °C and 1 minute at 60 °C (fluorescence measurement). The RT-qPCR data were analyzed using adapted EuroMRD criteria,[18] achieving a sensitivity of 20 copies of the 3′*ALK* gene. Peripheral blood (PB) from 10 healthy donors, 10 samples from reactive nodes, 5 ALK-negative ALCL patients, and 6 Burkitt lymphoma patients served as negative controls, each showing no ALK expression.

Detection of ALK Gene Translocation Partners

Lymphomas that were *NPM1::ALK* PCR-negative were analyzed using the ALK-specific rapid amplification of 5′cDNA ends (RACE) method (ThermoFisher Scientific). In total, 14 patients were studied using this 5′ RACE technique. More recently, we used an NGS approach to identify fusions, point mutations, and expression levels across 125 genes associated with lymphoma (using the FusionPlex Lymphoma Kit) or in 17 genes related to lung malignancy. The latter is a more concise NGS panel specifically designed to detect translocation partners of the *ALK* gene (FusionPlex Lung kit, Archer/Invitae). We examined 12 patients using the FusionPlex kits. These kits can identify fusions without needing previous knowledge of fusion partners or breakpoints. For library preparation using the FusionPlex Lymphoma or Lung Panel, we used 125 ng of the total RNA. The completed libraries were sequenced on the MiSeq instrument using the 2 × 300 v3 kit (all from Illumina). The NGS data were interpreted using both Archer Analysis software (Invitae) and Arriba software.[19] Molecular findings were then correlated with IHC localization of the ALK protein, I-FISH results, and the expression of the 3′end of *ALK* mRNA as determined by qPCR.

Next-Generation Sequencing for T-Cell Receptor Rearrangement Profiling

DNA from diagnostic specimens was extracted from 8 to 10 5-μm thick sections of FFPE or fresh frozen tissue using the QIAamp FFPE Tissue Kit (Qiagen) or Trizol reagent (Life Technologies), respectively. NGS libraries for TCRB and TCRG rearrangements were prepared according to the protocols established by the EuroClonality-NGS Working Group[20] and sequenced on the MiSeq instrument with the 2 × 250 v2 kit (Illumina). Data interpretation was done using the ARResT/Interrogate pipeline.[21,22] Sequences representing at least 5% of the total TCR rearrangements for their respective type (TCRB or TCRG) were classified as clonal, considering background clone frequencies, following the EuroClonality-NGS recommendations for B-Cell Clonality Evaluation.[23] Sequences with abundance greater than 5% but less than 10 times higher frequency than the subsequent background sequences were categorized as "clonal weak." Selected TCRB sequences were cross-referenced with previously reported datasets using the TCRdb database.[24]

Results

Fusion Genes and Anaplastic Lymphoma Kinase Expression

We examined a total of 55 children (aged 2-18 years) and 41 adult patients (aged 19-80 years) diagnosed with ALK+ ALCL. The male-to-female ratio among the patients was 1.23:1. Chromosomal breakpoints affecting the ALK locus (2p23) were detected by I-FISH in all examined tumors (91 patients). However, material was either unavailable or not evaluable in 5 patients. The *NPM1::ALK* fusion gene was identified in 71 of the 96 patients (74%). Through the NGS panel and/or 5′RACE method, the *ATIC::ALK* fusion gene was identified in 9 patients (9.4%), *TPM3::ALK* in 3 patients, whereas 2 patients each presented with the *CLTC::ALK*, *MYH9::ALK*, or *RNF213::ALK* fusions. Other fusion genes, including *TPM4::ALK*, *SQSTM1::ALK*, *SATB1::ALK*, and *CAPRIN1::ALK*, were identified individually in separate patients. Notably, the *SQSTM1::ALK* and *CAPRIN1::ALK* fusions have not been previously described, and only a single case of the *SATB1::ALK* fusion has been reported in the literature for ALK+ ALCL patients.[25]

The *SQSTM1::ALK* fusion was detected in a 75-year-old man exhibiting a 2p23 break and cytoplasmic positivity for the ALK protein as confirmed by IHC. The diagnosis was derived from a stomach tumor biopsy. The fusion occurred between exon 5 of the *SQSTM1* gene encoding Sequestome 1 and exon 20 of the *ALK* gene (as illustrated in Fig. 1). The *ALK* gene expression level was 75×10^4 copies. Unfortunately, the patient passed away shortly after diagnosis.

The *CAPRIN1::ALK* fusion gene was identified in a relapsed sample taken from a lymph node cluster 10 years after the initial diagnosis of ALK+ ALCL. This male patient was 15 years old at the time of his initial diagnosis. Regrettably, the diagnostic biopsy sample was not retrievable. Alongside the novel *CAPRIN1::ALK* fusion gene (a fusion between exon 18 of the *CAPRIN1* gene encoding cell cycle–associated protein 1 and exon 20 of the *ALK* gene, illustrated in Fig. 1), we identified a 2p23 break and observed an *ALK* gene expression level of 14×10^6 copies. The ALK protein was exclusively localized in the cytoplasm, as revealed by IHC. Currently, the patient remains in his second remission. Both these novel fusions resulted in a break in the *ALK* gene in the same region (breakpoint chr2:29223528, GRCh38/hg38).

The *SATB1::ALK* fusion was identified in a 27-year-old woman. Unfortunately, no sample was available to examine the 2p23 break. This fusion happened between exon 5 of the *SATB1* gene encoding SATB homeobox 1 and exon 20 of the *ALK* gene (Fig. 1). The breakpoint in the *ALK* gene was located in exon 20 at breakpoint chr2:29223455, GRCh38/hg38. The diagnosis was based on a

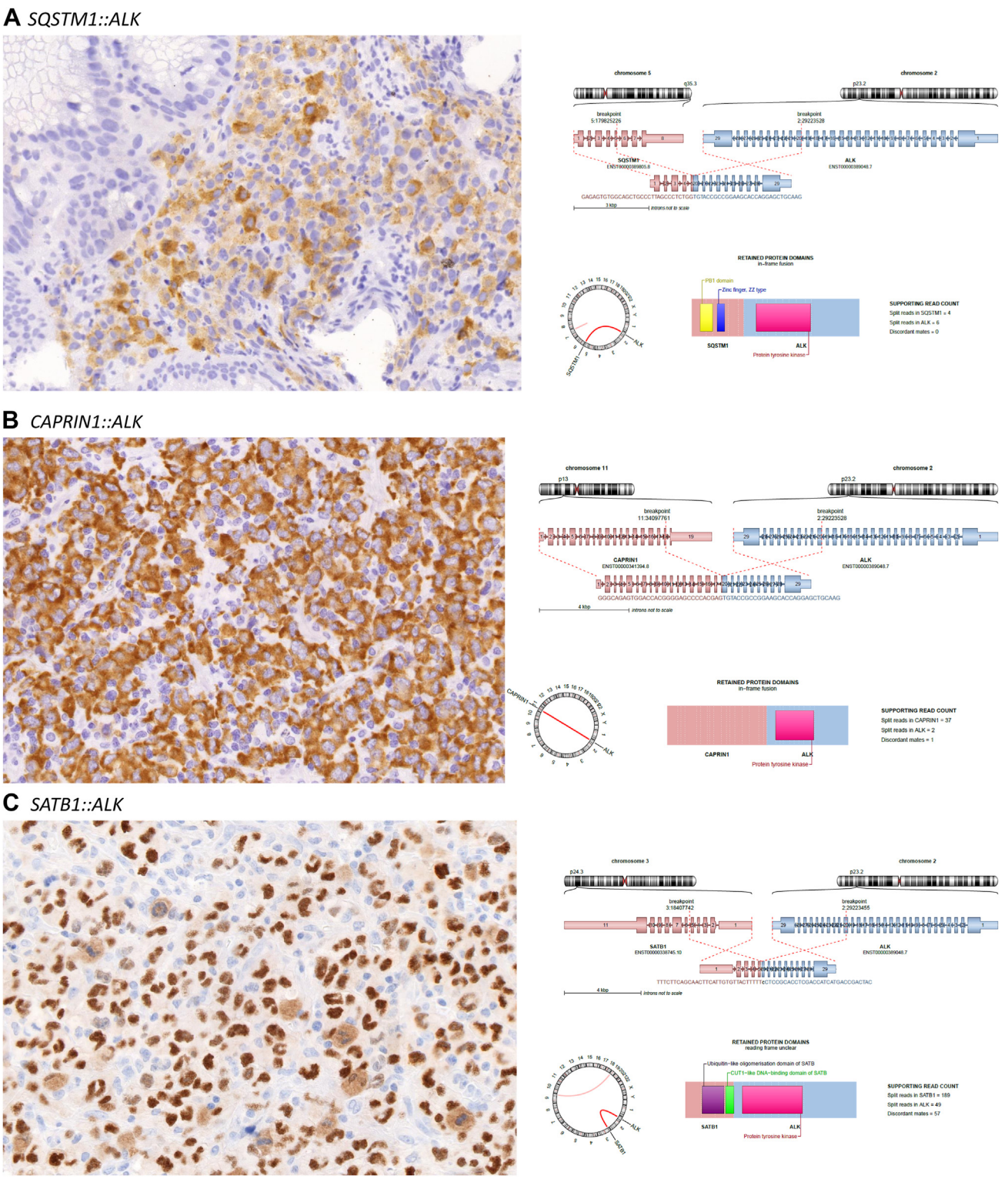

Figure 1.
Newly discovered fusions. (A) Cytoplasmic positivity for ALK protein from a stomach tumor biopsy; ×400; *SQSTM1::ALK* fusion, (B) lymph node with cytoplasm ALK positivity; ×400; *CAPRIN1::ALK* fusion, and (C) lymph node with cytoplasmic and nucleus positivity; ×400; *SATB1::ALK* fusion. Detection and visualization of fusions is by using FusionPlex Lymphoma/Lung Panel (Archer/Invitae) and Arriba SW.

lymph node biopsy that exhibited both cytoplasmic and nuclear positivity for the ALK protein. The *ALK* gene expression level was documented at 10×10^6 copies. This patient currently lives in complete remission.

Only 4 biopsy samples (4%) indicated nucleic acid degradation, rendering them unsuitable for expression level evaluation. In all the remaining samples studied ($n = 92$), there was an overexpression of the 3' end *ALK* mRNA, with a median value of 10×10^4 normalized

Table 1
Molecular screening of ALK+ ALCL (numbers of patients, fusion gene, immunohistochemical localization of ALK protein, examination of the *ALK* gene break on chromosome 2 chromosomal region 2p23 by I-FISH, and median copy number of the translocated part of the *ALK* gene by qPCR)

No. of patients	Fusion gene	Localization of ALK/IHC	Break 2p23	ALK expression (median—copies)
71	*NPM1::ALK*	64× nucleus and cytoplasm 5× cytoplasm 2× ND	66× break 5× nonevaluable/ unavailable	72413 8× positive, not quantifiable
9	*ATIC::ALK*	9× cytoplasm	9× break	345927 1× positive, not quantifiable
3	*TPM3::ALK*	3× cytoplasm	3× break	85922
2	*CLTC::ALK*	2× granular in cytoplasm	2× break	825000
2	*MYH9::ALK*	2× cytoplasm	2× break	9087
2	*RNF213(ALO17)::ALK*	1× cytoplasm 1× nucleus and cytoplasm	2× break	7812500 1× nonevaluable
1	*TPM4::ALK*	Cytoplasm	Break detected	710204
1	*SQSTM1::ALK*	Cytoplasm	Break detected	750000
1	*CAPRIN1::ALK*	Cytoplasm	Break detected	1480000
1	*SATB1::ALK*	Nucleus and cytoplasm	Unavailable	10187969
3	Nonevaluable	3× cytoplasm	3× break	3× nonevaluable

ALK, *ALK* receptor tyrosine kinase gene; *NPM1*, nucleophosmin 1 gene; *ATIC*, 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase gene; *TPM3*, tropomyosin 3 gene; *CLTC*, clathrin heavy chain; MYH9, myosin heavy chain 9 gene; *RNF213*, ring finger protein 213 gene; *TPM4*, tropomyosin 4 gene; *SQSTM1*, sequestosome 1 gene; *CAPRIN1*, cell cycle–associated protein 1 gene; *SATB1*, *SATB* homeobox 1 gene.

copies (ranging from 343 to 19 × 10^6 copies). The correlation of molecular findings in the 96 ALK+ ALCL patients is shown in Table 1 and Supplementary Table S1. In 9 patients, *ALK* gene expression was detected, but the levels of the control gene were below the quantitative range of the assay, making the quantification less reliable. We labeled these levels as "positive, not quantifiable" and excluded them from further analyses. We observed no difference in ALK expression levels between the morphological groups (common type vs small cell/mixed variant). Similarly, we did not observe any differences based on the type of the most frequent fusion genes (*NPM1::ALK* vs *ATIC::ALK*). In the *NPM1::ALK*-positive subgroup, we did not observe differences in the level of ALK expression based on clinical and biological factors, including ALK localization (nucleus vs nucleus and cytoplasm), tumor site (lymph node vs other), or between patients who relapsed and those who remained in complete remission (data not shown).

Figure 2 shows the clinical utility of *ALK* expression detection in MRD monitoring in 2 patients. In both cases, *ALK* transcript levels in bone marrow (BM) and PB correlated with the clinical course of the disease and were used to make clinical decisions.

T-Cell Receptor Rearrangement Profiling

We studied TCRB and TCRG rearrangements of 23 ALK+ ALCL patients for whom diagnostic FFPE or frozen samples were available. In 18 (78%) patients, we identified at least one clonal TCR rearrangement (ranging from 1 to 4), and at least one of these rearrangements was always productive. Based on the recent recommendation of EuroClonality-NGS, 7 sequences (from 4 patients) whose frequency, compared with the following background sequences, was less than 10× higher were classified as "clonal weak."[23] Clonality in these cases was evidenced by a rearrangement at a different locus or the nonproductive nature of both rearrangements in 3 patients. In 1 patient (No. 24) the result remains ambiguous. In 16 (70%) patients, we observed simultaneous rearrangements of both TCRB and TCRG. Except for one, all cases with TCRG rearrangements exhibited 2 major TCRG rearrangements. Conversely, 72% of the cases (13 of 17) with a TCRB rearrangement only had one major rearrangement, although 2 cases did display 2 TCRB rearrangements with corresponding frequency each. One particular patient (No. 32) possessed a major TCRB clone with a frequency (80%) corresponding to the percentage of ALCL cells in the sample, characterized by an unproductive junction. This was accompanied by another clone with a productive rearrangement, having a frequency of 5%. This might signify either a lymphoma subclone or a large tumor-infiltrating T-lymphocyte clone. We attempted to determine the antigen specificity of TCR sequences using the TCRdb database, referencing previously published cohorts. In 10 patients (59%), the clonal TCRB junction sequence (underlined in Table 2) perfectly matched a previously reported sequence. In most scenarios, these sequences appeared in multiple datasets, encompassing healthy donors, patient blood samples, or virus-specific T cells. We could not identify any recurring motif in the remaining rearrangements originating from nonmalignant T lymphocytes.

Discussion

ALK+ ALCL is an uncommon type of non–Hodgkin lymphomas that originates from the T-cell lineage and has its highest incidence between 10 and 14 years of age. We present a cohort of ALK+ ALCL patients diagnosed by our Department of Pathology. This cohort includes both primary diagnosis and second opinion cases, thanks to the collaborative efforts of the Lymphoma Group at Motol University Hospital, which partners with institutions both within the Czech Republic and Slovakia. Furthermore, the Department of Hematology and Oncology at the second Faculty of Medicine, Motol University Hospital, is the reference center for all pediatric lymphomas in the Czech Republic. As such, our cohort almost completely encompasses the consecutive group of pediatric patients and also includes the majority of adult patients diagnosed within that period. Our patient cohort had a peak incidence between 10 and 14 years, with a higher representation of boys, consistent with the findings in the World Health Organization report.[2]

The most prevalent fusion partner of *ALK* in ALK+ ALCL is *NPM1* on chromosome 5q35.[5,9] This fusion is observed in 75% to 83% of the childhood ALK+ ALCL cases.[26] In our cohort, we identified the *NPM1::ALK* fusion in 74% of the patients aged between 2 and 80 years (median 14 years). When analyzing only the childhood ALK+ ALCL subset (2-18 years, 55 patients), we detected the *NPM1::ALK* fusion gene in 85%. When the *ALK* gene fuses with the *NPM1* gene, the resultant fusion protein (ALK protein) is detected both in the nucleus and the cytoplasm.[8,27]

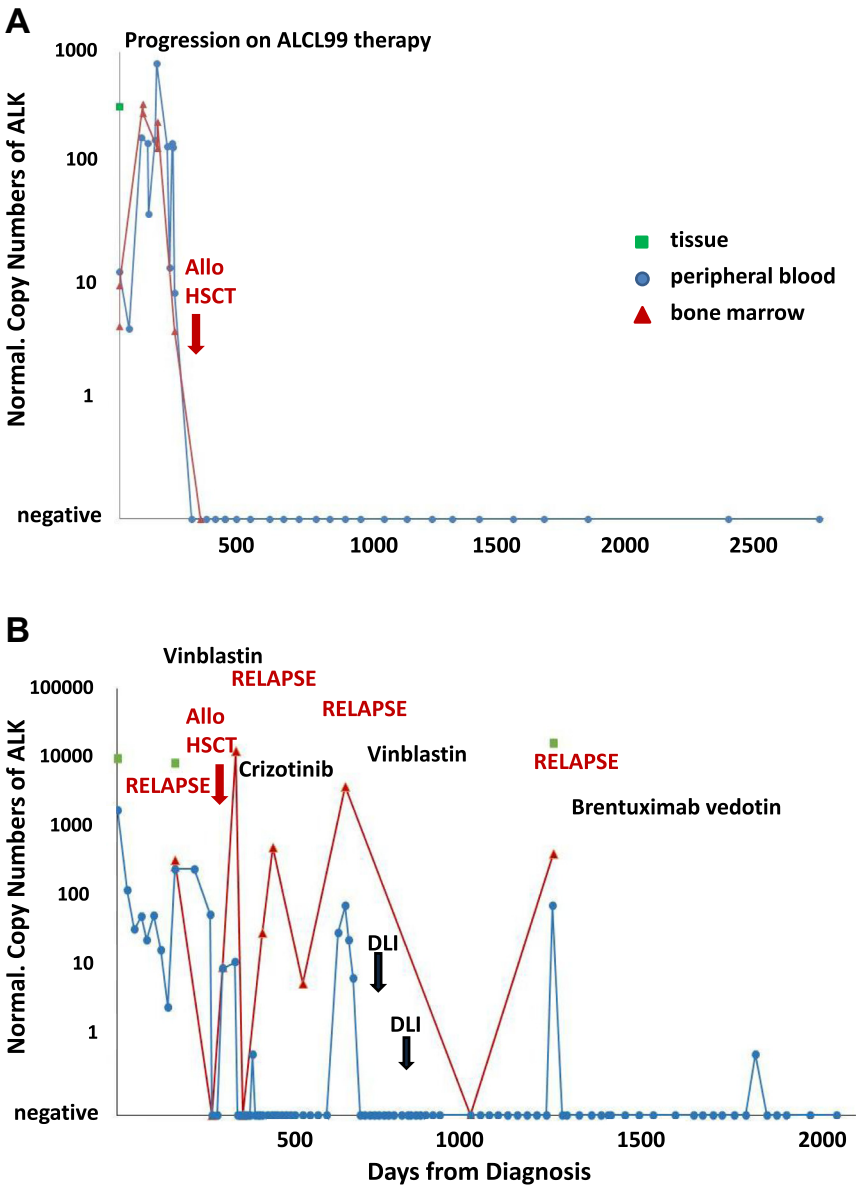

Figure 2.
Examples of minimal residual disease (MRD) monitoring in patients with ALK+ ALCL. (A) A 6-year-old girl who experienced disease progression during ALCL99 therapy and subsequently underwent allogeneic hematopoietic stem cell transplantation (HSCT). Following HSCT, she has remained in complete remission with MRD negativity observed in both peripheral blood (PB) and bone marrow (BM). (B) A 3-year-old patient boy who underwent HSCT after experiencing a relapse of the disease. Subsequently, BM relapse was detected based on morphology and flow cytometry (FC) analysis (BM 1% by FC and 1.6% atypical cells in morphological examination). Crizotinib treatment was initiated, resulting in MRD negativity in both PB and BM by day 18 of treatment. However, on discontinuation of crizotinib, a subsequent increase in transcript levels correlated with 0.2% infiltration by FC in BM. This prompted the initiation of vinblastine therapy, accompanied by 2 donor lymphocyte (DLI) infusions, ultimately leading to MRD negativity in both PB and BM. Seven months after the removal of vinblastine, another relapse was detected in a cervical lymph node, coinciding with an increase in MRD. Vinblastine therapy was reintroduced, and brentuximab vedotin therapy was initiated (consisting of 16 doses). Since then, the patient has remained in complete remission for 40 months with ALK transcript negativity.

The second most frequently detected fusion in our ALK+ ALCL patient cohort was the fusion with the *ATIC* gene on chromosome 2, resulting from the recurrent cryptic chromosomal inversion, inv(2)(p23q35). The *ATIC::ALK* fusion gene has been identified not only in ALK+ ALCL patients but also in those with IMT and non–small cell lung cancer.[7,28,29] ALK protein, in agreement with existing literature, was identified exclusively in the cytoplasm of all *ATIC::ALK* cases.[7,30] All samples exhibited a 2p23 break, and the median expression of the translocated portion of the *ALK* gene was 3 × 105 copies (Table 1). Other fusions in our cohort (involving *TPM3*, *TPM4*, *CLTC*, *MYH9*, *RNF213*, *SQSTM1*, *CAPRIN1*, and *SATB1* genes) were less common. We observed high expression levels of the translocated portion of the *ALK* gene and a chromosome 2 break (chromosomal region 2p23) in all cases examined. *ALK* expression, therefore, serves as a suitable diagnostic marker and a metric for MRD monitoring in all ALK+ ALCL patients, regardless of the fusion partner.

Of the fusion partners of the *ALK* gene described above, *SQSTM1* and *CAPRIN1* have not yet been reported in ALK+ ALCL, whereas the *SATB1* fusion partner has only been observed in a

Table 2
Characteristics of clonal rearrangements of TCRB and TCRG (rearranged segments, numbers of deleted/inserted nucleotides, and amino acid sequence of the junction)

Patient	ALCL cells %	Clonotype TCRG	Frequency (%)	Clonotype TCRB	Frequency (%)
22	20	V3 -0/2/-0 J1=J2 CATWDS*IIIRN	32	V19 -6/7/-4 J1-3 CASSGGGNTIYF	25
		V4 -0/15/-3 J1=J2 CATWDGPMGGPLL*ET	13	V24-1 -4/16/-2 J1-5 CATSDPQGAK#NQPQHF	21
24	NA	Not detected		V28 -3/16/-1 J1-5 CASSFPRTGSSNQPQHF	8[w]
				V7-9 -3/9/-9 J1-5 CASSFTAWPQHF	10[w]
32	90	V10 -2/12/-4 JP1 CAAWDTPQGHWLVQ	43	V19 -0/27/-5 J1-5 CASSIDGR*GETGW##QPQHF	80
		V11 -0/8/-3 J1=J2 CACWIRHPIRYYKK	36	V24-1 -3/19/-4 J2-3 CATSDPTGLVDADTQYF	5
38	25	V2 -0/2/-3 JP2 CATWDGL**LDQ	18[w]	V9 -4/16/-5 J1-5 CASSPGGGGYQPQHF	8[w]
		V3 -4/6/-2 JP2 CATWVLE**LDQ	15[w]		
45	35	V10 -0/0/-3 JP1 CAAWDYHWLVQ	40	V5-6 -4/5/-5 J1-4 CASSG#NEKLFF	62
		V5 -5/1/-0 JP1 CATWVYHWLVQ	34	V6-5 -0/14/-2 J2-7 CASSYSGSGRAYEQYF	20
46	80	V9 -2/2/-9 J2 CALWED#KKLF	42	V6-1 -11/19/-5 J2-2 CADPDRHR#TGELFF	76
		V4 -4/3/-3 J1=J2 CATWDVYYKK	37		
49	30	V8 -0/7/-5 J1=J2 CATWDRGVDYKK	31	V28 -7/18/-5 J2-1 CASTMLGRHHNEQFF	82
		V10 -4/4/-0 JP1 CAAWGVYHWLVQ	55		
50	60	V10 -0/4/-2 J1=J2 CAAWD*WIIIRN	17	not detected	
		V8 -4/5/-8 J2 CATWESDKKLF	15		
53	25	V10 -17/1/-9 JP1 SWFK	31	V6-5 -6/16/-1 J2-7 CASRRGSSLSYEQYF	8[w]
		V10 -0/5/-5 JP1 CAAWDYKDWLVQ	11		
54	80	V10 -4/6/-11 J2 CAAWGREKLF	41	V20-1 -1/14/-2 J2-5 CSARVAPPLQETQYF	38
		V2 -0/7/-5 J1=J2 CATWDGPGDYKK	16		
58	15	V10 -1/3/-2 JP1 CAAWDLDHWLVQ	37	V20-1 -5/20/-2 J2-3 CSAFPLVVFDTDTQYF	66
		V5 -3/3/-1 J1=J2 CATWDQNYYKK	20		
59	NA	V4 -5/6/-6 J1=J2 CATWGGVIRN	46	V7-9 -0/13/-4 J2-1 CASSLAQGFPYNEQFF	66
		V10 -7/9/-3 J1=J2 CAAYKLFIIRN	37		
60	60	V10 -3/11/-8 JP1 CAAWDKSARLVQ	69	V28 -4/9/-3 J1-4 CASSSGSANEKLFF	90
62	40	V2 -0/11/-3 J1=J2 CATWDGLLPTYYKK	25	V7-9 -1/20/-4 J2-7 CASSLGPDRRYSYEQYF	61
		V8 -0/1/-2 J1=J2 CATWDRDYYKK	25		
63	70	V9 -0/7/-8 J2 CALWEVHVNKKLF	49	V11-2 -4/18/-2 J2-1 CASSSGLAGVPYNEQFF	89
		V8 -6/6/-4 J1=J2 CATWPRYYKK	43		
64	85	V10 -3/15/-8 J2 CAAWE*VSI##KKLF	73	V6-1 -6/7/-8 J1-4 CASRDGEKLFF	90
		V4 -0/0/-1 J1=J2 CATWDGNYYKK	19		
69	80	V2 -0/2/-0 J1=J2 CATWDGPNYYKK	55	V5-1 -6/16/-0 J2-1 CASTSGTGFSYNEQFF	74
		V2 -0/10/-7 J2 CATWDGPR*#YKKLF	11[w]		
76	30	V8 -8/11/-0 J1=J2 CATLVGEELL*ET	5	V12-3=V12-4 -2/10/-2 J1-5 CASSLWGWGNQPQHF	43
		V8 -2/12/-2 J1=J2 CATWDIVRWDYYKK	5		
20	NA	Not detected		Not detected	
31	85	Not detected		Not detected	
47	60	Not detected		Not detected	
52	30	Not detected		Not detected	
75	80	Not detected		Not detected	

Frequency was calculated as a percentage of clonotype reads out of total reads with identified junction (TCRG or TCRB). Underlined sequences were detected with an exact match in previous studies using the TCRdb database. The percentage of tumor cells in the samples was estimated by hematoxylin and eosin staining together with ALK and CD30 immunohistochemistry.
[w]Sequences evaluated as "clonal weak."
NA, not available; TCRB, T-cell receptor beta; TCRG, T-cell receptor gamma.

single case. The *SATB1* gene is situated on chromosome 3 (chromosomal region 3p24.3) and plays a crucial role in the self-renewal and lymphopoiesis of adult hematopoietic stem cells.[31] The SATB1 protein is predominantly expressed during the CD4+CD8+ cell stage and regulates the transcription with both activator and repressive roles.[32] Only one ALK+ ALCL case with *SATB1::ALK* fusion has been documented. Drieux et al[25] described a fusion transcript where exon 20 of the *ALK* gene was fused to 3 intronic bp of *SATB1* gene, followed by the exon 5 of SATB1.[25] In our patient, we detected a fusion between intron 5 of the *SATB1* gene (breakpoint chr3:18407742, GRCh38/hg38) and part of exon 20 of the *ALK* gene (breakpoint chr2:29223455, GRCh38/hg38).

In this study, 2 novel fusion partners, *SQSTM1* and *CAPRIN1*, have been identified in ALK+ ALCL. The *SQSTM1::ALK* fusion has been described in isolated cases, such as ALK+ large B-cell lymphoma, epithelioid cell histiocytoma, IMT of the head and neck, and lung carcinoma. However, it has not been described in ALK+ ALCL to date.[33-35] The *SQSTM1* gene encodes a multifunctional protein that binds to ubiquitin, regulates the activation of the nuclear factor kappa-B signaling pathway, and plays a role in oxidative stress response, cell signaling, and autophagy.[36] The fusion of exon 5 of the *SQSTM1* gene with exon 20 of the *ALK* gene was first described by Takeuchi et al[37] in ALK+ large B-cell lymphoma. This discovery correlated with the presence of t(2;5)(p23;q35.3). The same fusion has been described in epithelioid cell histiocytoma and lung cancer.[33,38] Fusions of the *SQSTM1* gene with genes other than *ALK* have also been reported. Specifically, fusions of *SQSTM1*/*NTRK2* (in Spitz tumor) and *SQSTM1*/*NTRK3* (in thyroid cancer) have been documented. These fusions also have potential therapeutic implications.[39,40]

The *CAPRIN1* gene is located on chromosomal region 11p13 and is highly expressed in hematopoietic cells. Caprin-1 selectively

binds to MYC and cyclin D2 mRNAs, which facilitates cell progression from the G1 phase to the S phase, enhances cell viability, and promotes cell growth.[41] Only one fusion involving the *CAPRIN1* gene has been previously described. Donati et al[42] detected a fusion of the *CAPRIN1* gene with the *ROS1* gene in a 34-year-old female patient with a Spitz tumor. In our study, we identified a previously undescribed *CAPRIN1::ALK* gene fusion that involves a fusion between exon 18 of the *CAPRIN1* gene and exon 20 of the *ALK* gene. The breakpoint in the *ALK* gene corresponds to those seen in other fusions, with the exception of the fusion with the *SATB1* gene.

In our study, we implemented reverse-transcriptase quantitative detection (RT-qPCR) to measure the expression of the translocated portion of the *ALK* gene for diagnosing all ALK+ ALCL patients (irrespective of the *ALK* gene fusion partner) and detecting MRD. We constructed a plasmid standard calibration curve with sensitivity capable of detecting 20 copies of *ALK* mRNA. This methodology enables swift diagnosis from various samples, including FFPE tissue, frozen tissue, BM, PB cells, and liquor cells, facilitating an assessment of disease spread. All patients with evaluable RNA (97%) exhibited high *ALK* expression, and the control group showed no expression. Lamant et al[43] conducted gene expression profiling for individual morphological subtypes of ALK+ ALCL. Based on the gene expression data, they were able to distinguish 2 groups of ALK+ ALCL, correlating with the morphological subtype. Our cohort included 92 patients with the common type, 2 patients with the small-cell variant, and 2 with the mixed variant, as opposed to the 23 common types and 9 with morphological (small cell or mixed) variants reported in their study. We observed no difference in ALK expression levels between the groups, a result that could be attributed to the small number of cases in the subgroups and the absence of analysis for other genes. Pomari et al[44] noted a bimodal distribution of *NPM1::ALK* expression in *NPM1::ALK*-positive cases. Our results also showed a bimodal distribution of ALK expression, but the high and low expression groups were not as distinctly separated (Supplementary Fig. S2). Like them, we did not observe differences in the level of ALK expression based on clinical and biological factors. However, they reported that 9 of the 12 patients who experienced relapse belonged to the ALK-high group. We did not observe a significant difference in ALK expression between patients who relapsed and those who remained in complete remission. This difference could be attributed to the fact that the study by Pomari et al[44] exclusively analyzed pediatric patients.

ALK expression is a promising target for monitoring MRD in ALK+ ALCL patients.[45,46] We demonstrated this in the case of 2 patients in whom MRD levels correlated with the clinical course and served as the basis for timely treatment administration. From the current limited data, it appears that the level of ALK transcript in BM better predicted relapse than levels in PB. However, a more extensive study will be needed for a definitive conclusion regarding the prognostic significance of measuring ALK levels.

We used NGS-based profiling of TCRG and TCRB to evaluate the clonality and T-cell repertoire of ALCL samples. Clonal TCRG VJ rearrangements were observed in 17 of the 23 patients (74%), and clonal complete TCRB VDJ rearrangements were identified in 17 of the 23 patients (74%). In 5 samples (22%), we found no clonal rearrangements. Malcolm et al[14] identified no TCRB, TCRG, or TCRD rearrangements in 14% of the 57 ALK+ ALCL patients using PCR with Genescan analysis. Their study detected a similar proportion of TCRG rearrangements (75%) but a slightly lower number of TCRB rearrangements (58%). Both mentioned studies used multiplex primer assays developed by the BIOMED-2 group.[47] The advantage of the NGS-based approach in our study lies in the direct reading of the V-(D)-J junction sequence and a superior distinction between biallelic or oligoclonal sequence patterns. Notably, in 10 of the 23 patients (43%), the clonal sequences of TCRB had previously been reported in multiple T-cell data sets, including normal blood, blood of patients with tumors, and virus-specific T cells.[24] This indicates that some ALK+ ALCL lymphomas may arise from polyreactive T lymphocytes with broad antigen specificity. The public nature of these TCRB rearrangements should be acknowledged when considering antigen receptor–based MRD detection, even if they are likely to present at low frequencies. Both clonal and reactive TCR repertoires were unique for each patient. We identified no shared clonotypes between patients that could potentially be reactive against neoantigens and serve as disease markers. This fact also makes it difficult to distinguish the sequences of a potential large T-cell clone from the clonal rearrangement of a lymphoma. Here, the knowledge of the sequence and its possible nonproductivity could help. However, most of the sequences in our cohort were productive for both TCRB and TCRG. Moreover, an unproductive sequence may indicate a rearrangement of a second allele of the same clone. The frequency of TCRG rearrangements was lower than the frequency of TCRB rearrangements in some patients, suggesting that infiltrating γδT cell clones might be involved. TCRG is rearranged in the vast majority of T lymphocytes and often on both alleles.[48] The method used in our study is based on the creation of sequencing libraries by multiplex PCR. The lower frequency of TCRG rearrangements compared with TCRB can rather be caused by a different background and abundance of these rearrangements in the sample; therefore, the relative quantification of these rearrangements can differ, although they belong to the same clone. However, a definitive exclusion that these are not large reactive clones would only be possible using modern methods such as spatial transcriptomics.

In conclusion, we developed a qPCR-based tool for rapid ALK+ ALCL diagnostics grounded on *ALK* expression and demonstrated that *ALK* expression is consistently present, regardless of the fused gene. The detection of TCR rearrangements by NGS shows interesting data from a research perspective and at least the same diagnostic benefit as the classical BIOMED-2 method. However, neither method can distinguish a potential large clone of infiltrating T cells. Both ALK expression and TCR rearrangements (present in 78% of the patients with TCR rearrangement) can serve as markers for monitoring MRD. ALK expression seems more suitable due to its presence in all patients, along with its ease of feasibility and interpretation. The detection of the 2p23 break by FISH, ALK expression through IHC (possibly supplemented by RT-qPCR), and the *NPM1::ALK* fusion via RT-PCR are currently sufficient for routine diagnostics, although TCR clonality offers additional value primarily from a research perspective. NGS offers insights into ALK partner genes, which can be instrumental in understanding their clinical relevance in the future.

Author Contributions

M.K., E.F., and R.K. designed and coordinated the study, analyzed data, and interpreted the results. M.M. performed I-FISH analysis. Z.P. and R.K. performed the histopathological analyses. E.K., A.K., Z.K., and M.D. provided clinical information and coordinated sample collection of the patients. M.S. and A.S. performed the TCR analysis, M.K. and E.F. wrote the paper, and all authors reviewed and contributed significantly to the manuscript.

Data Availability

The raw data are available on request (eva.fronkova@lfmotol.cuni.cz).

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Declaration of Competing Interest

The authors declare no competing financial interests.

Ethics Approval and Consent to Participate

The patients/their parents were informed about the aim of the study and gave their written consent. The study was performed in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of the Second Faculty of Medicine, Charles University, Prague, Czech Republic.

Supplementary Material

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