Complex Genomic Rearrangement of *ALK* Loci Associated with Integrated Human Epstein-Barr Virus in a Post-Transplant Myogenic Liver Tumor

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Epstein-Barr virus (EBV) is a ubiquitous viral agent, well known to be associated with lymphoid, epithelial, and smooth-muscle malignancies in immunocompromised individuals. This report describes a 10-year-old patient with an EBV-related liver tumor occurring after kidney transplantation. The neoplasm presented a phenotypic spectrum, ranging from a smooth-muscle tumor to an inflammatory pseudotumor (IPT). The neoplastic cells failed to disclose CD21, CD35, or *ALK* expression, the latter confirmed by reverse-transcription polymerase chain reaction. Cytogenetic analysis revealed a single clonal cell population showing 46,XY,del(2)(p23),der(3)t(2;3)(p23;q29),der(21)t(Y;21)(q12;p13) karyotype. By metaphase FISH analysis, the neoplastic cells demonstrated the presence of two molecularly different but related aberrant clones, one with the loss of one *ALK* allele and the second with translocation of the 3' end of *ALK* kinase domain on the der(3) chromosome. Using FISH with an EBV-specific and 3' end *ALK* DNA probes, a co-localization of the viral DNA and the *ALK* sequences was found on the der(3) chromosome. Metaphases with loss of rearranged *ALK* did not show integrated virus; instead, viral particles together with an associated 3' end *ALK* domain formed an ex-chromosomal, episomal-like type configuration. The interphase study, using dual-color 5'/3' end *ALK* FISH assay, revealed 30% of nuclei with only one fused signal, confirming the total loss of one *ALK* allele in the subset of tumor cells. A combined immunofluorescence and FISH study indicated this separate clonal variant to correspond to desmin-positive smooth-muscle cells. In contrast, desmin-negative myofibroblasts showed the presence of both normal and rear-

ranged *ALK* alleles. Our results indicate that *ALK* locus may be a target of EBV integration, a hitherto unreported finding. Although the sustained clonal expansion in EBV-related smooth-muscle tumors/IPTs may depend on functions provided by the EBV oncogenic proteins, the tumor phenotype may be further modified by the secondary genomic rearrangements imposed by the virus during and/or after the integration event. In this respect, the observed phenotypic heterogeneity most likely reflects divergence during neoplastic progression, with the subsequent expansion of morphologically and molecularly distinct but cytogenetically related clones. (*Am J Pathol* 2003, 163:913–922)

Smooth-muscle tumors are increasingly recognized in children with various immunodeficiencies occurring after organ transplantation.^{1–5} Since clonal Epstein-Barr virus (EBV) is consistently demonstrated in these tumors but not in sporadic smooth-muscle tumors, the virus probably plays a pivotal role in tumorigenesis. EBV, a common and the causative agent of infectious mononucleosis, is known to be associated also with other malignancies including lymphomas, nasopharyngeal carcinomas, human immunodeficiency virus-associated and posttransplant lymphoproliferative disorders, and inflammatory pseudotumors (IPTs)/IPT-like follicular dendritic cell tumors.^{6–9} EBV is a double-stranded DNA-enveloped virus with a variety of gene products.¹⁰ EBV-associated smooth-muscle tumors exhibit EBV type III latency, characterized by expression of immortalizing Epstein-Barr nuclear antigen (EBNA)-2 in addition to the oncogenic, latent membrane protein (LMP)-1.¹¹ The latent proteins are the main targets of cytotoxic T cells, and their expres-

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sion is better tolerated in cases of profound immunodeficiency.

Anaplastic lymphoma kinase (ALK) is a member of the insulin receptor family of receptor tyrosine kinases and its expression is normally restricted to the nervous system.¹² *ALK* gene fusion was first identified in anaplastic large-cell lymphoma (ALCL), in which most commonly a t(2;5) chromosomal translocation generates an *NPM-ALK* gene fusion and constitutively activated chimeric protein.¹³ Activation of *ALK* by variant *ALK* fusions have also been described in a subset of inflammatory myofibroblastic tumors (IMTs) occurring in children and young adults.^{14–17} These fusions are associated with striking ALK expression in the myofibroblastic spindle cells in a significant (up to 40%) subset of tumors.¹⁸ Genetic changes that underlie ALK-negative IPTs remain unclear. It is feasible that ALK-negative IPTs might represent a spectrum of entities with overlapping histological features but different underlying transforming mechanisms.^{15,18} Some investigators have proposed a role for viruses in the pathogenesis of IPTs, including EBV and human herpesvirus-8.^{6,19–21}

This report describes a 10-year-old patient who developed a post-transplant EBV-related liver tumor with histologically combined smooth-muscle and IPT characteristics. Tumor cells revealed a spectrum of serial genomic rearrangements, involving the *ALK* gene and integrated EBV sequences, with the subsequent expansion of morphologically and molecularly distinct but cytogenetically related clones. Our findings indicate that the *ALK* loci may be a site of EBV virus integration. Virus integration could facilitate secondary genome rearrangements, leading consequently to the development of tumors with morphological features resembling IPTs and to related smooth-muscle tumors.

Case Report

A 10-year-old boy presented in March 2002 with a mass in the right liver lobe. He had a history of a single renal transplantation in February 2000 due to chronic renal insufficiency and bilateral nephrectomy, subsequent to two successive hemolytic uremic syndromes in 1996 and 1997. An immunosuppressive agent, FK 506, was administered in subtoxic serum levels. Annual follow-up echography and nuclear magnetic resonance scan of the abdomen revealed a heterogeneous and T1-hypodense well-circumscribed nodular solid lesion, 5.3 cm in diameter, in the right lobe of the liver. The transplant kidney and spleen were normal. Laboratory tests revealed serum conversion for EBV, Hepatitis A virus and Polyomavirus but not for Cytomegalovirus, Hepatitis B and C viruses, or Toxoplasma. The mass was removed by a right lobotomy of the liver. In May 2002, the boy was admitted to the hospital following a supracondylar fracture of the humerus. Physical examination and imaging techniques could not reveal new or residual tumor activity.

Figure 1. Map of *ALK* gene on 2p23 with localization of the FISH probes used in the study.

Materials and Methods

Histopathology and Immunohistochemistry

After sampling for frozen material, tumor samples were fixed in 6% buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin (H&E) for routine histological examination. Indirect immunohistochemistry was performed, either on paraffin or frozen sections, using monoclonal desmin (dilution 1:20; ICN-Cappel, Gent, Belgium), α -smooth-muscle actin (α -SMA, dilution 1:40; DAKO, Glostrup, Denmark), cytokeratin (dilution 1:50; Biodesign, Saco, ME), CD21 (dilution 1:10; DAKO, Merelbek, Belgium), CD35 (dilution 1:10; DAKO), myogenin (dilution 1:30; Novocastra, Gent, Belgium) and ALK-1 (dilution 1:10; DAKO) antibodies.

Electron Microscopy

Small fragments of the biopsy were immediately fixed in 2.5% glutaraldehyde, 0.1 mol/L phosphate buffer. After postfixation in 1% osmiumtetroxide 0.1 mol/L phosphate buffer, the samples were dehydrated in graded series of alcohol and embedded in epoxy resin. Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and examined using a Zeiss EM10 electron microscope.

Cytogenetic and FISH Analysis

Chromosome metaphases were obtained after short-term culture of a primary tumor sample, using standard procedures. G-banded chromosomes were evaluated and classified according to the International System of Human Cytogenetic Nomenclature.²²

FISH was performed on either metaphase chromosomes or interphase nuclei using probes homologous to 3' or 5' regions of *ALK* gene, as shown in Figure 1. The *ALK* locus was evaluated with the LSI *ALK* Dual-Color Rearrangement Probe (Vysis, Inc., Downer's Grove, IL), composed of a mixture of two differentially labeled DNAs that map either 3' telomeric (SpectrumOrange) or 5' centromeric (SpectrumGreen) to *ALK*. For subsequent study, the digoxigenin-labeled *ALK* P1 (PAC RP11–111H1) (specific for 3' end of *ALK* catalytic domain) or PAC RP11–223F18 (specific for 5' end of *ALK*) clones were used.²³ Additional FISH experiments used the *EcoRI*-B fragment of EBV DNA probe (kindly provided by Dr. John Arrand from the Paterson Institute for Cancer Research, Manchester, UK) and a probe specific for the subtelomeric sequences of chromosome 3q. FISH was performed according to the protocol previously described.²⁴ The FISH data were obtained on a Leica DMRB (Leica, Wetzlar, Germany) fluorescence microscope equipped

with a cooled black and white charged couple device camera (Photometrics, Tuscon, AZ), run by Quips Smart-Capture FISH Imaging Software (Vysis, Bergisch-Gladbach, Germany).

For simultaneous ascertainment of cytogenetic and immunophenotypic features in individual cells, a sequential immunocytological/FISH analysis was performed on cytological touch preparations of the frozen tumor specimen, using primary monoclonal anti-desmin antibody (dilution 1:20; ICN-Cappel), according to procedure previously described.²⁵ Thirty randomly chosen immunonegative or positive cells were photographed using an Axioplan 2-fluorescence microscope. Subsequently, FISH was performed on the same slide with the LSI *ALK* probe described above, and the same cells were rephotographed.

ALK RT-PCR Analysis

Total RNA was extracted from the frozen tissue of the present case and from an IMT with *ALK-AT1C* fusion (positive control) using Trizol (Invitrogen, Carlsbad, CA), according to manufacturer's protocol. The RNA was reverse-transcribed using MMLV reverse transcriptase. A positive control reverse transcription-polymerase chain reaction (RT-PCR) for the quality of the cDNA synthesis, using the primers 5'-CCCATTTCGGCTCCCATGATT and 5'-GGTCTCGGTGTGTGACTTGGA, was performed to amplify the *CIZ* gene. To analyze *ALK* transcripts, two primer sets were developed. The first, *ALK3f* (GCAACATCAGCCTGAAGACA) and *ALK3r* (GCCTGTTGAGAGACAGGAG), amplifies a 192-bp fragment derived from the 3' end of the *ALK* transcript. The second set, *ALK5f* (CTCAGCGAGCTGTTCAGTTG) and *ALK5r* (GGAGAAGGCATGTTTGTGG), amplifies a 200-bp fragment derived from the 5' end of the *ALK* transcript.

Results

Histopathologic Findings

Generally, the tumor was relatively well delineated from the surrounding parenchyma, although nodular extensions were occasionally seen (Figure 2b). A striking phenotypic variability was present in the lesion (Figure 2a). Part of the tumor consisted of a typical smooth-muscle tumor, showing fascicles of eosinophilic cells with cigar-like shaped nuclei and strong desmin and α -SMA expression (Figure 2, c and e). Some mononuclear inflammatory cells were present between the smooth-muscle cells. Other parts of the tumor revealed an inflammatory, loosely textured background, in which α -SMA-positive and desmin-negative myofibroblasts were present (Figure 2, d and f). The inflammatory component consisted mainly of lymphocytes and plasma cells (Figure 2d). This part of the tumor showed characteristics of an IPT. Both phenotypes were easily discriminated in most parts of the tumor, albeit that both components were intermingled in some areas (Figure 2). Quantitatively, the IPT component outnumbered the smooth-muscle component and the lat-

ter was mainly present at the edge of the lesion. Mitotic figures were occasionally present, mainly in the smooth-muscle component. Necrosis was focally seen. Immunostaining on frozen tumor sections using the *ALK*-1 antibody showed no reactivity in the IPT component or in the smooth-muscle compartment. Frozen sections as well as paraffin sections were repeatedly negative on CD21 and CD35 staining. No expression of cytokeratin or myogenin was found.

Electron Microscopic Findings

The tumor consisted of scattered spindle-shaped cells lying in an inconspicuous stroma with some collagen fibers and amorphous matrix components. Two types of spindle-shaped tumor cells could be distinguished. The most frequent type in our sections presented characteristics of myofibroblastic cells. The cells had a large nucleocytoplasmic ratio with an oval nucleus and prominent nucleolus.

The cytoplasm contained some rough endoplasmatic reticulum (RER) cisternae, numerous free ribosomes and polysomes, a small fat droplet and sometimes bundles of peripherally located actin microfilaments (Figure 3). The other cell type was more oblong and presented with a more voluminous cytoplasm almost completely filled with bundles of actin microfilaments and densifications. Plasmalemmal attachment plaques became obvious on the peripheral cell membrane as well as caveolae (Figure 3). These cells presented characteristics of smooth-muscle cells.

Some tumor cells revealed in their cytoplasm the presence of enveloped viral particles or virions. Identical viral particles could be seen in necrotic cell fragments.

Cytogenetic Analysis

A total of 13 metaphase spreads were analyzed. Six metaphases revealed an abnormal karyotype: 46,XY,del(2)(p2;3),der(3)t(2,3)(p23;q29),der(21)t(Y;21)(q12;p13) (Figure 4). Seven cells confirmed a constitutionally normal karyotype.

FISH Findings

Dual-color FISH analysis using LSI *ALK* assay showed two different patterns of hybridization in cells with abnormal karyotype. Sixteen out of 25 analyzed metaphases revealed only one fused (orange/green) signal on normal chromosome 2p23 (Figure 5A), while the remaining nine spreads, besides the normal (fused) signal on chromosome 2, disclosed the presence of the 3' end *ALK* signal (orange) on derivative chromosome 3 (Figure 5B). This result indicated the presence of two major clones, both showing a common loss of sequences 5' end to *ALK* (green). However, in the first clone the region 3' end to *ALK* (orange) was lost, leading to the loss of the entire *ALK* allele, while in the second clone it was translocated on 3q29. The existence of two clones was supported also by the results of interphase FISH, using the same dual-

Figure 2. Overview of the histopathological and immunohistochemical findings. In many areas, the transition between the smooth-muscle component (**left**) and the IPT component (**right**) is easily seen (H&E stain, $\times 25$) (**a**). The tumor is relatively well delineated from the surrounding parenchyma (arrows) (H&E stain, $\times 25$) (**b**). Higher magnification of the smooth-muscle component shows bundles of eosinophilic spindle cells with cigar-shaped nuclei (H&E stain, $\times 160$) (**c**). Higher power of the IPT component discloses vessels, lymphocytes, plasma cells (**arrowheads**), and spindle cells in a myxoid background (H&E stain, $\times 160$) (**d**). The smooth-muscle component is strongly desmin-positive (indirect immunoperoxidase stain, $\times 400$) (**e**). The spindle cells of the IPT component only express α -SMA (indirect immunoperoxidase stain, $\times 400$) (**f**).

color LSI ALK probe. The touch preparation of the tumor specimen showed normal, adjacent (orange/green) signals (two fusions) in 15% of cells. The remaining 85% were abnormal, with 30% of nuclei showing only one fused (orange/green) signal, 39% of nuclei having one

fused signal and one orange signal, and 16% of cells with variable number of fused and orange signals (Figure 5C).

The translocation of the ALK catalytic domain in a subset of metaphases was further confirmed by FISH analysis with the ALK P1 probe (specific for ALK 3' end),

Figure 3. Electron micrograph showing tumor cells with smooth muscle (asterisks) and fibroblast-like (wide arrow) characteristic ($\times 9,200$). Inset: detail of EBV virus particles in a cytoplasmic cell fragment (arrow) ($\times 72,500$).

which revealed seven out of 11 metaphases with only one signal on the normal chromosome 2p23, while the remaining spreads showed signals both on the normal 2p23 and der(3). By contrast, hybridization of abnormal metaphases with the PAC 223F18 probe (specific for *ALK* 5' end) showed only one signal, at the normal 2p23, in all analyzed cells. Altogether, rearrangement of *ALK* associated with loss of the 5' end of the gene was shown. FISH results are summarized in Table 1 and Table 2.

Hybridization with the probe specific for subtelomeric 3q sequences disclosed signals only on the terminal end of normal chromosome 3. This result confirmed the presence of an unbalanced der(3)t(2;3) translocation (data not shown).

Co-hybridization of digoxigenin-labeled ALKP1 together with biotin-labeled EBV DNA probes with the interphase nuclei of tumor cells revealed one red and one fused green/red (yellow) signal in 43% (Figure 5D), one red and one green signal in 35% and two red signals in 17% of cells. While the abnormal chromosome spreads were evaluated, a single red signal at normal chromo-

some 2p23 and overlapping red/green hybridization signal at the breakpoint of a der(3) was present (Figure 5E). This result indicated the integration of EBV DNA in the translocated 3' end *ALK* region in the molecularly distinctive clone. In contrast, the metaphases that failed to show translocated 3' end *ALK* did not show chromosomal integration of the virus DNA. In five of 14 of these metaphases, however, the overlapping green/red signal was found outside the counterstained chromosomal chromatin borders, pointing the presence of co-localized EBV and *ALK* sequences in an autonomous (non-integrated) episomal form (Figure 5F). Normal metaphases showed only red signals from *ALK* P1 probe on two normal homologous chromosomes 2p23 and no green signals from EBV probe.

Combined Immunofluorescence and FISH Study

The immunofluorescence and subsequent FISH study was performed to find out if the molecular heterogeneity was in any way associated with the phenotypic diversity of tumor cells. Using anti-desmin antibodies, positive cytoplasmatic immunoreactivity was found in 27% of cells. Immunopositive cells were randomly dispersed among negative ones. Twelve out of 14 desmin immunopositive cells that were evaluated demonstrated loss of one *ALK* allele using LSI *ALK* probe (Figure 5, G and H); two cells showed two fused, red and green, signals. Eleven out of 16 desmin immunonegative cells revealed the presence of one fused plus one single red signal. The remaining five cells showed the varying number of fused and/or single red signals.

ALK RT-PCR Analysis

To confirm *ALK* expression status, we performed RT-PCR analysis of total RNA prepared from the tumor specimen and from control IMT with *ATIC-ALK* chimeric fusion. As shown in Figure 6, the tumor cells of the present case did not show *ALK* specific transcripts using primers for either the 5' or the 3' end of the *ALK* transcript. A control experiment demonstrated RT-PCR detection of cDNAs for an unrelated gene, *CIZ*, in the same sample. In contrast, when cDNA derived was used from an *ATIC-ALK* positive control case, a PCR product was obtained with the primers amplifying 3' *ALK* but not with 5' *ALK* primers. These findings confirm the lack of detectable *ALK* expression in the tumor under the study.

Discussion

Recent studies demonstrate that in addition to inflammatory myofibroblastic tumors, *ALK* rearrangements and *ALK* expression are found in other neoplasms, especially neuroblastomas, rhabdomyosarcomas, and malignant peripheral nerve sheet tumors, supporting the concept of *ALK* involvement in a broader group of malignancies.²⁶ In this report we present an abnormalities of the *ALK* gene in

Figure 4. G-banded karyogram of abnormal tumor cells showing 46,XY,del(2)(p23),der(3)t(2;3)(p23;q29),der(21)t(Y;21)(q12;p13) karyotype. Arrows point to the breakpoints.

Table 1. Results of FISH Analysis

Probe (localization)/specificity (fluorochrome*)	FISH results [†] (% of interphase nuclei)	Conclusion
LSI <i>ALK</i> dual-color (2p23)/ 3'telomeric to <i>ALK</i> (O) 5'centromeric to <i>ALK</i> (G)	Metaphase FISH [‡] : Chr 2 GO [16] [§] Chr 2 GO, der(3) O [9] Interphase FISH [200]: 2 × GO (15%) 1 × GO (30%) 1 × GO, 1 × O (39%) Other (16%)	Clone 1: loss of one <i>ALK</i> allele Clone 2: loss of sequences centromeric to <i>ALK</i> 5'end and translocation of sequences telomeric to <i>ALK</i> 3'end on 3q29 Normal cells: 15% cells with two normal chr 2 Clone 1: loss of one <i>ALK</i> allele (30% of cells) Clone 2: loss of the sequences centromeric to <i>ALK</i> 5'end (39% of cells)
ALKP1 RP11-111H1 (2p23)/ 3'end <i>ALK</i> (O)	Metaphase FISH: Chr 2 O [7] Chr 2 O, der(3) O [4]	Confirms loss of 3'end <i>ALK</i> sequences in clone 1 Confirms translocation of 3'end <i>ALK</i> to 3q29 in clone 2
RP11-223F18 (2p23)/5'end <i>ALK</i> (G)	Metaphase FISH: Chr 2 G [10]	Confirms loss of <i>ALK</i> 5'end in all abnormal cells
ALKP1 (2p23)/3'end <i>ALK</i> (O) EBV/EcoRI-B (G)	Metaphase FISH: Chr 2 O [9] Chr 2 O, der(3) GO [5] Chr 2 O, episomal GO [5] Interphase FISH [100]: 1 × G, 1 × O (35%) 1 × GO, 1 × O (43%) 1 × GO (5%) 2 × O (17%)	Cells of clone 1: without EBV in metaphase Cells of clone 2: EBV DNA associated with 3'end <i>ALK</i> on der(3) or in episomal form Cells of clone 1: with EBV not associated with 3'end <i>ALK</i> Cells of clone 2: with EBV sequences associated with 3'end <i>ALK</i> Normal cells: two chr 2 and without EBV

*O, orange; G, green.

[†]Only abnormal metaphases were selected.

[‡]GO, fused green/orange; G, green; O, orange signals.

[§]Number in square brackets is number of analyzed cells.

a case of hepatic posttransplant smooth-muscle tumor. The unusual and hitherto unreported finding in this tumor is its phenotypic spectrum. One phenotype corresponds to the typical EBV-related smooth-muscle tumor in immunosuppressed patients, based on the histology, immunohistochemistry (desmin), and ultrastructural features. The other phenotype shows features of an inflammatory pseudotumor: a desmin-negative, α -SMA-positive spindle-cell population, admixed with mononuclear inflammatory cells, including plasma cells and vessels, in a myxoid background. IPTs have been described in immunosuppressed patients and IPTs from the liver, spleen, and epididymis have been associated with EBV.^{27–30} A major proportion of EBV-related liver and spleen IPTs corre-

spond to EBV-associated follicular dendritic reticulum cell proliferations, but this is not the case in the tumor we describe, since specific follicular dendritic reticulum cell markers (CD21 and CD35) were negative. In addition, no ultrastructural features of follicular dendritic reticulum cells could be found. In the differential diagnosis of the IPT component, an inflammatory leiomyosarcoma could be considered. However, the latter entity generally does not show the loosely textured stroma as seen in our case and tend to be more cellular. In addition, one would expect desmin to be expressed in an inflammatory leiomyosarcoma. Moreover, these tumors often show foam cells, or even psammoma bodies, which are completely lacking. Finally, inflammatory leiomyosarcoma

Figure 5. Molecular cytogenetic analysis of abnormal tumor cells carrying del(2)(p23) and der(3)t(2;3)(p23;q29) chromosomes. **A:** Metaphase FISH using LSI *ALK* (Vysis) probe: dual-color signals are present only on normal chromosome 2p23 (**arrow**), indicating clone 1 with the loss of *ALK* allele (**arrowhead**). **B:** Metaphase FISH using LSI *ALK* probe: dual-color signal is present on normal chromosome 2p23 (**arrow**) and the red signal is present on der(3) chromosome, indicating clone 2 with the rearranged *ALK* gene (**arrowhead**). **C:** Dual-color hybridization to interphase nuclei from tumor specimen using LSI *ALK* probe: two separate clones, one with loss (**arrow**) and the second with rearranged *ALK* allele (arrowheads) are evident. **D:** Co-hybridization of digoxigenin-labeled ALKP1 and biotin-labeled EBV probes: a single red and overlapping red/green (**arrows**) signals in interphase nuclei indicate the integration of EBV at the site of *ALK* in subset of cells; metaphase cell reveals only red signal on normal chromosome 2. **E:** Co-hybridization of digoxigenin-labeled ALKP1 and biotin-labeled EBV probes: metaphase cell showing single red signal on normal chromosome 2p23 (**arrowhead**) and fused green/red signal on der(3) chromosome (**arrow**). **F:** Co-hybridization of digoxigenin-labeled ALKP1 and biotin-labeled EBV probes: metaphase cell showing single red signal on normal chromosome 2p23 (**arrow**) and fused red/green signal outside chromosome, indicating the presence of EBV together with 3' end *ALK* sequences in the episomal form (**arrowhead**). **G:** Identification of desmin-positive cells by immunostaining (**arrow**). Nuclei are counterstained with DAPI. **H:** Double-color interphase FISH analysis of cells corresponding to **E**, using double-color LSI *ALK* probe. Loss of rearranged *ALK* allele was revealed only in the nuclei that corresponded to desmin-positive cells (**arrow**).

Table 2. The Hybridization Signals in Interphase Nuclei by FISH Using LSI *ALK* Dual-Color (Vysis) Probe in Desmin-Positive and Desmin-Negative Cells

Cells phenotype by immunostaining	Number of cells with signals/total number of cells (%)			
	Two adjacent 5'/3' (green/orange) signal*	One adjacent 5'/3' (green/orange) signal†	One adjacent 5'/3' (green/orange) + one 3' (orange) signal‡	Other
Desmin-positive	2/30 (7%)	12/30 (40%)	0	0
Desmin-negative	1/30 (3%)	0	11/30 (37%)	4/30 (13%)

*Normal, diploid cells.

†Clone 1, with the loss of one *ALK* allele.

‡Clone 2, with the loss of 5'*ALK* end only.

shows a near haploid karyotype, which is not the case in the tumor we present.³¹

Cytogenetic analysis demonstrated an unbalanced translocation involving bands 2p23 (*ALK* locus region) and 3q29. FISH analysis confirmed that the 2p23 rearrangement was within the *ALK* gene in a significant proportion of cells. Furthermore, among visually the same abnormal metaphase cells two molecularly distinctive clones were recognized: one with loss and the second with retained 3' *ALK* catalytic domain on derivative chromosome 3. The former resulted probably from the latter by a secondary rearrangement during aberrant cell expansion and tumor formation. Using an EBV-specific probe, FISH revealed integration of viral EBV DNA sequences at the 3q29 translocation breakpoint site and EBV co-localization with the translocated 3' end *ALK* kinase domain in the latter. The clone that revealed loss of translocated *ALK* contained viral sequences together with 3' end *ALK* in the nuclear episomal form. It is unknown whether the chromosomal integration of EBV sequences occurred before or after the translocation. However, the EBV integration site itself was shown to be a chromosomal region prone to enhanced fragility due to an altered chromatin structure.³² The loss of the integrated EBV viral genome due to chromosomal breaks proximal to the integration site was documented in BL cells and NPC cells in culture.^{33–34} This observation led to the hypothesis that under certain conditions EBV might act by a so-called “hit and run” mechanism during oncogenic transformation. Accordingly, due to increased chromosomal instability and a strong positive selection pressure toward cells retaining only episomal EBV, tran-

sient EBV integration into the host genome might be followed by loss of the virus together with the adjacent host cell chromosome fragments. Similarly, the loss of integrated EBV with the adjacent translocated *ALK* sequences in a subset of cells was observed in our tumor, suggesting the hit-and-run mechanisms of EBV-driven transformation that occurred *in vivo*.

In this context, the biological implications of EBV infection associated with tumor formation are of special interest. An integration of the viral particles into the human genome in EBV-associated disease has been well documented.³⁴ The integration of EBV within the vicinity of the *ALK* receptor tyrosine kinase gene was never reported. As is known from other RNA and DNA tumor viruses, integration of viral DNA might modify the expression of viral as well as of cellular genes and might also cause chromosomal instability. The known mechanism of *ALK* activation in ALCLs and IMTs is formation of chimeric fusions that result in constitutive, ligand-independent receptor oligomerization. In the present case *ALK* kinase domain was also involved in the translocation. However, in contrast to the subset of IMTs with known variant chromosomal translocations involving the *ALK* gene, activation of *ALK* could not be demonstrated in myofibroblastic cells of the tumor, as shown by immunostaining and as confirmed by RT-PCR analysis. Thus, the IPT component of the present case belongs to the category of *ALK*-negative IPTs, although the possibility of transient *ALK* expression during the EBV integration near or within *ALK* gene or during the subsequent *ALK* secondary rearrangements cannot be excluded. Alternatively, the clonal expansion of cells harboring the *ALK* translocation could gain the required proliferative advantage through EBV association, and not by direct *ALK* activation. This hypothesis is supported by the recent finding that a small proportion of normal lymphoid cells in non-neoplastic lymph nodes are positive for chimeric *ALK* transcripts, suggesting that *ALK* gene rearrangement by itself might be insufficient to induce tumor formation.³⁵ Furthermore, it is known that EBV-associated smooth-muscle tumors contain oncogenic latent membrane proteins that alone may implicate neoplastic cellular transformation.³⁶ The transforming ability of LMP-1 is well recognized in lymphoid malignancies and LMP-1 might play a role in the pathogenesis of *ALK*-negative ALCLs.^{11,38}

The very intriguing relation between distinct cellular genotypes, with respect to *ALK* changes and desmin-positive/-negative phenotypes that was observed within

Figure 6. RT-PCR analysis of *ALK* cDNA. RT-PCR was performed with primers designed to amplify respectively the 3'-end (lanes 1 and 2) or the 5'-end (lanes 3 and 4) of *ALK*. Lanes 1 and 3 show the absence of any product using cDNA of the tumor. Lanes 2 and 4: PCR was performed with cDNA from an ATIC-*ALK* positive control case. Lane 5 is a positive control for the cDNA synthesis showing amplification of the *CIZ* gene, starting from cDNA of the tumor.

tumoral cells of the present case, requires further consideration. Both metaphase and interphase FISH confirmed the spectrum of serial genomic changes involving the *ALK* gene and integrated EBV sequences. Immunofluorescence anti-desmin staining combined with subsequent FISH analysis demonstrated the presence and the loss of translocated 3' end of *ALK* tyrosine kinase domain in desmin-negative and -positive cells, respectively. The desmin-negative cells most likely correspond to IPT-related myofibroblasts while the desmin-positive cells represent the smooth-muscle tumor component. Although myofibroblasts can also express desmin, on the histological sections the desmin-positive cells were only seen in the histologically typical smooth-muscle component of the tumor. The combined cytogenetic, immunofluorescence and morphological findings indicate that, although phenotypically different, both components of the tumor shared a common single-cell origin. We believe that this case represents a single immunosuppression-related EBV-driven lesion, which shows a phenotypic spectrum, ranging from a smooth-muscle tumor to an IPT. Thus, the observed phenotypic heterogeneity most likely reflects divergence during neoplastic progression, with the subsequent expansion of morphologically and molecularly distinct but cytogenetically related clones. The reason for this diversity remains obscure. One may hypothesize, however, that secondary genomic rearrangements induced by virus integration and subsequent relocation could impose somatic changes of cellular genes encoding functions responsible for tumor's gene expression profile.

Notably, the EBV-ALK interaction in EBV-associated smooth-muscle tumors or IPTs has never been investigated. Whether these tumors might be induced by direct ALK-EBV fusion warrants further studies.

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