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Attenuated isolated 3' signal: A highly challenging therapy relevant ALK FISH pattern in NSCLC

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ABSTRACT

Objectives: Targeted therapies in the management of patients with lung cancer provide significantly better outcome compared to chemotherapy. Detection of the anaplastic lymphoma kinase (ALK) gene rearrangement has great predictive value for treatment with small molecule tyrosine kinase inhibitor (crizotinib and alectinib commonly). Fluorescent *in situ* hybridisation (FISH) assay is a basic diagnostic test designed for detecting ALK gene rearrangements. Although being considered as gold standard method by IASLC's guideline, it is often regarded as difficult and error prone. Our aim was to examine a unique atypical ALK FISH pattern, revealed during a systematic large-scale monitoring, which carries the great risk of misinterpretation, hence may result in loss of patients eligible for targeted therapy.

Materials and Methods: Tissue and cytology samples from nearly one thousand patients with advanced stage non-small cell lung cancer (NSCLC, n = 996) were routinely examined by ALK FISH and immunohistochemistry (Ventana ALK-D5F3-CDx assay). Anchored Multiplex PCR based Next Generation Sequencing (AMP-NGS) was used to detect fusion gene transcripts in ambiguous cases.

Results: Fifty-nine (5,9%) of the cases were positive with ALK FISH test. Three cases showed atypical pattern with a significantly reduced sized red (3') signal and complete loss of green signals. Digital signal measurement confirmed this finding, showing consistent attenuation of 3' signals throughout the tumours. In all three cases AMP-NGS and ALK IHC verified the presence of a fusion gene and expressed oncoprotein, respectively.

Conclusion: Approximately 5% of the 59 ALK positive cases exhibited atypical attenuated isolated 3' signal pattern. The immunohistochemistry and AMP-NGS examinations helped to clarify the presence of oncoprotein and the fusion gene, respectively. Our results emphasize the importance of extensive exploration of the genetic background of any unexpected FISH finding to avoid false diagnosis. This enables clinicians to indicate the adequate therapy with higher efficiency for patients suffering from NSCLC.

1. Introduction

Lung cancer is the leading cause of cancer death in the developed world. Targeted therapy in non-small cell lung cancer (NSCLC)

reshaped the landscape of thoracic oncology. Among numerous identified driver mutations in NSCLC, several genetic abnormalities - resulting in activated and/or overexpressed oncoproteins (including EGFR, ALK, ROS1, BRAF) - are already druggable [1]. A paracentric

Abbreviations: NSCLC, Non-Small Cell Lung Cancer; NSCLC-NOS, Non-Small Cell Lung Cancer-Not Otherwise Specified; EGFR, Epidermal Growth Factor Receptor; ALK, Anaplastic Lymphoma Kinase; ROS1, c-ros oncogene 1; EML4, Echinoderm Microtubule Associated Protein Like 4; inv(2)(p21;p23), chromosomal inversion in the short arm of chromosome 2, between bands p21 and p23; IASLC, International Association for the Study of Lung Cancer; FISH, Fluorescence *in situ* hybridization; DC BA, Dual Colour Break Apart probe; NGS, Next Generation Sequencing; IHC, immunohistochemistry; DAPI, 4', 6-diamidino-2-phenylindole; RT-PCR, Reverse Transcription Polymerase Chain Reaction; AMP, Anchored multiplex PCR; TKD, tyrosine kinase domain; TKI, tyrosine kinase inhibitor

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chromosomal inversion (inv(2)(p21;p23)) affecting the genes echinoderm microtubule-associated protein like 4 (EML4) and anaplastic lymphoma kinase (ALK), both located on the short arm of chromosome 2, is one such abnormality that is detectable with different methods. ALK may be involved in other gene rearrangements affecting other partners on different chromosomes with a similar oncogenic effect [2,3]. In the International Association for the Study of Lung Cancer (IASLC) atlas for testing ALK in lung cancer (IASLC 2013) [4], the fluorescence in situ hybridization (FISH) assay using a dual colour, break apart probe (ALK DC BA FISH, Vysis, Abbott Molecular) has been the gold standard for testing the ALK gene associated rearrangements [5]. Immunohistochemistry (IHC), reverse transcriptase PCR (RT-PCR) and next generation sequencing (NGS) have also become accepted as diagnostic methods for ALK rearrangement testing by now [6]. The IASLC guideline describes two different positive signal patterns for ALK DC BA FISH analysis. Beside the expected split signal pattern (separation of signals) in case of inv(2), the 5' deletion pattern (also termed 'isolated red signals') is also recognized as a frequent positive FISH result in NSCLC. In addition to these scenarios, the guideline mentions further "atypical signal profiles": (i) isolated green signals (considered as negative result, but some studies actually describe such cases harbouring NGS and IHC proved rearrangement) [7,8], (ii) red-doublet or triplet signals and (iii) isolated red signals in the absence of normal ALK allele, but these are rare findings [9]. Recently, a novel atypical signal pattern was described by Li et al. [10] as "attenuated" isolated red signal. In this case, a considerable fraction of fluorescence intensity of the isolated 3' signals appear to be lost resulting in a consistently smaller signal compared to both green and red signals of the intact allele indicating a rearrangement of the chromosome region covered by the FISH probe.

During our recent large-scale attempt to explore the frequency of different kinds of atypical patterns we also encountered the aforementioned attenuated isolated 3' signals. As nothing is known of the background of this pattern, the primary aim of our study was to examine this phenomenon in detail using state of the art molecular genetics and image analysis.

2. Methods

2.1. Samples

ALK-FISH samples from 996 cases of the FISH laboratories in two pathology departments (2nd Department of Pathology, Semmelweis University and Department of Pathology, Medical School and Clinical Center, University of Pécs) were re-examined to identify those with atypical FISH signals. Endobronchial excisional biopsies, transbronchial needle aspiration (TBNA), bronchial brush cytological samples and surgical specimens were included into the analysis. All cases with the diagnosis of pulmonary adenocarcinoma or non-small cell lung cancer, not otherwise specified (NSCLC-NOS) have been involved in this study. Cytology samples included cell blocks and smears (73 and 74 respectively). Slides made from cell blocks (in case of cytology samples) and FFPE tissue slides were used for both IHC and FISH examinations. IHC was not performed on smears. FISH was used as the basic screening method; IHC was applied as confirmatory method in a subset of the cases. At Semmelweis University the IHC was done for confirmation in specific cases, e.g. due to atypical FISH signal pattern, biopsy samples with low tumour cell content or clinicopathological features (like adenocarcinoma with signet ring cells or young age, below 40 years) characteristic for ALK-rearrangement in FISH-negative case. IHC at University of Pécs was done for all biopsy samples except for cytological smears. Altogether, IHC was performed in 338 cases out of 996.

2.2. Fluorescence in situ hybridisation (FISH) and image processing/quantification

ALK FISH analysis was performed with both conventional dual colour break apart probe (Vysis, Abbott Molecular, Illinois, USA; ALK 5' – green, ALK 3' – red) and a three-colour combined break apart and gene fusion probe (ZytoLight® SPEC ALK/EML4 TriCheck™, Zytovision GmbH, Bremerhaven, Germany; ALK 5' – green, ALK 3' – red, EML4 – aqua). Nuclei were stained by 4',6-diamidino-2-phenylindole (DAPI).

The evaluation scheme provided by IASLC Atlas⁴ was applied in the assessment of ALK rearrangement. A case was considered as positive, if $\geq 15\%$ cells showed split signal and/or isolated red signal among 100 tumour cells (or $\geq 50\%$ cells among 50 tumour cells).

FISH was evaluated on composite images created by merging multiple tissue planes (image stacks) separately for each colour channel (blue/green/red/aqua). Stacking was carried out using Zeiss (University of Pécs) or Leica (Semmelweis University) motorized automated fluorescent microscopes and MetaSystems™ or Leica CW4000 FISH evaluation software (applying $10 \times 0.5 \mu\text{m}$ and $11 \times 0.4 \mu\text{m}$ z-stacks), respectively. Involvement of EML4 was detected by red-aqua signal fusion when applying TRiCheck prob-set. In cases with visible smaller isolated red FISH signals, fluorescence intensity was quantitatively evaluated with ImageJ, an image processing and analysis platform (ImageJ 1.47) [11]. Red channel of 8-bit multichannel TIF images was imported into ImageJ. In 50 tumour cells, peak and total intensity were separately measured for each attenuated and normal red signal as well. Peak signal intensity was defined by the maximum grey scale value of signal intensity curve generated by a 10 pixel-long measurement tool superimposed across the largest diameter of the given signal. Total fluorescence intensity was defined as area under the intensity curve (AUC). If a tumour cell contained more than one of either the isolated or normal red signal, the highest intensity was used for the peak and the average of the AUCs for total intensity comparisons.

2.3. Immunohistochemistry

Monoclonal antibody against ALK was used to detect ALK protein overexpression. Ventana™ ALK (D5F3) CDx Assay (Roche Diagnostics™) "ready to use" antibody with OptiView DAB IHC Detection kit™ and Optiview Amplification™ kit was applied on Ventana Benchmark Ultra system.

2.4. Anchored multiplex PCR-Based targeted next-generation sequencing

Examination of fusion variants in ALK gene rearrangements was carried out by Anchored Multiplex PCR (AMP)-Based Targeted Next-Generation Sequencing technique (ArcherDX™, Boulder, USA). In certain cases, total nucleic acid was isolated from smear, while in others it was extracted from formalin fixed paraffin embedded tissue block. AMP NGS was performed as described previously by Morreau et al. [12].

2.5. Statistical analysis

Both peak and total intensities of isolated 3' and normal red ALK signals were compared between the three different cases by Kruskal-Wallis test. Differences between the qualitatively determined attenuated red and normal signal groups were examined by using exact two-sided Wilcoxon signed rank test.

3. Results

3.1. ALK positivity rate

Out of 996 NSCLC cases analysed with ALK DC BA FISH, 59 (5.9 %) showed positive signals in at least 15 % of the tumour cells. IHC was performed on 22 out of 59 FISH positive cases (38 %) and it gave

Table 1

Clinical details and results of ALK analysis in case of three patients with atypical FISH pattern. (FNA: fine needle aspiration, ADC: adenocarcinoma).

	Gender/ age	Stage	Sample type	Localization	Histological type	ALK IHC	DC BA FISH	Tri-check FISH	NGS
Case 1	Male 59 y/o	IV.	Surgical biopsy	Bone (spine)	ADC	+	+	+	EML4-ALK Variant 3 (Ex6; Ex20)
Case 2	Female 42 y/o	IV.	FNA	Liver	ADC	+	+	+	EML4-ALK Variant 1 (Ex13; Ex20)
Case 3	Female 83 y/o	IV.	FNA	Cervical lymph node	ADC	+	+	+	EML4-ALK Variant 5 (Ex18; Ex20)

concordant results in all cases. One of the fifty-nine positive cases (1.6 %) was diagnosed based on previously Giemsa stained smear, while 53 were detected in tissue specimens (89.9 %) and 5 in cell block preparations (8.4 %).

3.2. FISH signal patterns of the positive cases including atypical signal pattern

Split signal was observed in 61 % of the cases (36 out of 59), while 34 % (20 out of 59) showed 5' deletion pattern. Three additional patients (1 of 36 positive cases at Semmelweis University and 2 of 23 positives at University of Pécs) with NSCLC harbouring ALK rearrangement showed atypical FISH signals (Table 1). All three atypical patterns represented attenuated isolated 3' (red) signals (with consistently absent 5' signal) (Fig. 1). Tricheck ALK/EML4 FISH examination revealed the presence of EML/ALK fusion in all three cases. Duplication of EML4 labelling aqua signals and colocalization of attenuated red signal with one of the two aqua signals proved the fusion between the ALK tyrosine kinase domain (TKD) and a portion of EML4 gene. The attenuated diameter/intensity of the red signal was observed with both types of FISH assays (Fig. 1) and were present in vast majority of tumour cells (68 %, 84 % and 68 % in case one, two and three, respectively). Non-neoplastic stroma cells, serving as internal negative control, in tissue sample of patient one showed red signals with normal appearance. The tumour cells harboured 1.29, 1.81 and 1.62 isolated

attenuated red signals on average in case 1, 2, 3 respectively, while intact alleles (indicated by fusion signals) were present 1.92, 1.92 and 1.62 on average in the same cases, respectively.

3.3. Signal intensity measurement

Median and mean of peak signal intensities of the normal red signals were 75.3 % and 72.9 %, respectively, while the same parameters of attenuated red signals were 38.6 % and 39.7 %, respectively, on average throughout the three cases. Median and mean total signal intensity of normal signals were found to be 275.9 % and 331.9 %, respectively, while these were 102.5 % and 186.8 % for the attenuated red signals, respectively. Overall, the peak and total signal intensity of normal red signals on average throughout the three cases were significantly higher in comparison with the isolated 3' signals ($p < 0.001$) (Table 2). Not surprisingly, the same difference between normal and attenuated signals was experienced within the individual cases regarding peak and total intensity. Both values for attenuated red signals were significantly lower in the individual cases (in comparison with normal signals, mean values were 50 % and 34 % in case 1, 55 % and 55 % in case 2, 58 % and 67 % in case 3, respectively; $p < 0.001$ for each pairwise comparison) (Table 3).

The peak and total intensities of attenuated red signals showed great cell to cell variety in all cases. Notably, although total signal intensity of normal red signal varied among the cases, the peak intensity exhibited

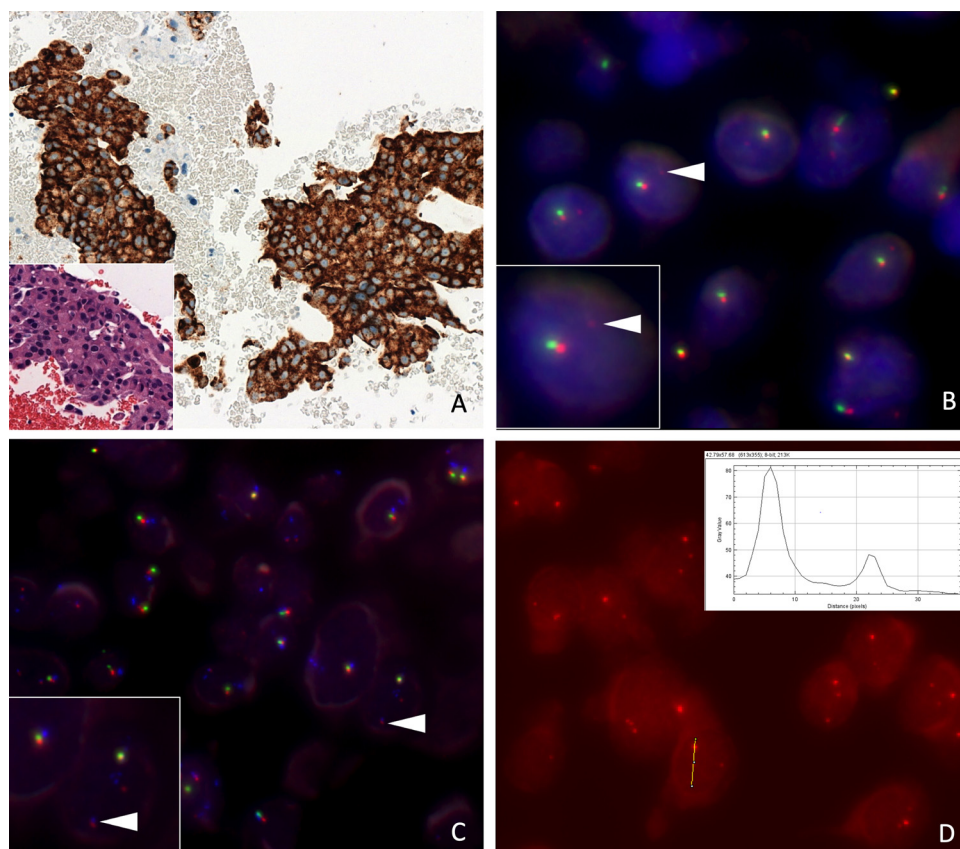


Fig. 1. (A) ALK D5F3 IHC on cell block made from aspiration cytology sample of Case 2 (insert shows HE staining). (B) ALK DC BA FISH reveals the reduced red signal beside the normal fusion signal of intact allele. (C) TriCheck tricolour FISH shows colocalization of reduced red signal and one segment of duplicated aqua signal confirming EML4/ALK rearrangement. (D) Measurement of signal intensity in red channel (3' end of probe in ALK FISH) with ImageJ shows significantly diminished signal intensity in case of allele involved by the genetic event.

Table 2

Comparison of total and peak fluorescence intensities of 3' signals of normal and rearranged ALK alleles (with isolated attenuated 3' signal) of the tumour cells.

	total intensity normal	peak intensity normal	total intensity attenuated 3'	peak intensity attenuated 3'
N	150	150	150	150
Median	275.8615	75.2882	102.4505	38.6482
Mean	331.8834	72.8889	186.8369	39.6688
Chi-Square	79.840	1.120	86.560	6.880
df	2	2	2	2
Significance	p < 0.001	p = 0.571	p < 0.001	p = 0.032
Kruskal-Wallis H	79.740	3.329	74.253	9.161
df	2	2	2	2
Significance	p < 0.001	p = 0.189	p < 0.001	p = 0.010

no significant difference (p = 0.189) (Table 2).

3.4. Immunohistochemistry and next-generation sequencing

All three cases with atypical FISH signal pattern showed strong cytoplasmic positivity after immunohistochemical staining when using the FDA approved D5F3 clone and Ventana platform. (Fig. 1) The staining was homogenous throughout the tumour in the tissue sample and on the whole tumour cell population in the cytology samples. Anchored Multiplex PCR–Based Targeted NGS revealed three different, previously described EML4/ALK fusion variants (variant 3, 1 and 5' in patient one, two and three respectively) (Table 1).

3.5. Patient outcomes

Patient 1 received ALK tyrosine kinase inhibitor as first line therapy, and the CT scan showed stable disease (according to RECIST criteria) three months after initiation of the treatment. Patient 2 refused any further medical intervention including the therapy after the diagnosis and was lost from follow-up. The first line ALK TKI (crizotinib) therapy has also been started in patient 3, but four weeks later she died as a result of a severe lower airway infection.

4. Discussion

Diagnostic samples of nearly one thousand patients from two

Table 3

Comparison of the total and peak fluorescence intensities of 3' signals of normal and rearranged ALK alleles, separately in the three individual cases. *TIN1-2-3: total intensity of normal (non-rearranged) red signals in case 1-2-3; PIN1-2-3: peak intensity of normal (non-rearranged) red signals in case 1-2-3; TI3'1-2-3: total intensity of 3' isolated (rearranged) red signals in case 1-2-3; PI3'1-2-3: peak intensity of 3' isolated (rearranged) red signals in case 1-2-3;

	Parameter	N	Mean	Std. Dev.	Minimum	Maximum
Case 1	total intensity normal	50	209.8685	50.6697	125.7980	322.0920
	peak intensity normal	50	72.6660	10.9813	51.6973	85.0000
	total intensity isolated 3'	50	70.2417	21.8042	17.0150	119.8450
	peak intensity isolated 3'	50	36.6037	7.38549	14.0740	52.7028
Case 2	total intensity normal	50	295.8343	135.6812	114.3980	560.6000
	peak intensity normal	50	71.0642	10.8490	42.2198	85.0000
	total intensity isolated 3'	50	163.3481	140.5302	33.6380	451.8330
	peak intensity isolated 3'	50	39.2459	11.9129	14.8487	65.0000
Case 3	total intensity normal	50	489.9474	84.9541	311.9440	764.9330
	peak intensity normal	50	74.9366	9.43852	48.4549	85.0000
	total intensity isolated 3'	50	326.9208	90.7581	158.9670	594.6330
	peak intensity isolated 3'	50	43.1568	10.9095	23.7764	84.2417
Test Statistics*	TIN1-TI3'1	PIN1-PI3'1	TIN2-TI3'2	PIN2-PI3'2	TIN2-TI3'2	PIN3-PI3'3
	Z	-6,154	-6,154	-6,154	-6,135	-6,144
	Significance	p < 0.001	p < 0.001	p < 0.001	p < 0.001	p < 0.001

centres were examined with ALK FISH and 59 proved to be positive. Fifty-six of those were featured with typical positive patterns, while 3 cases showed atypical FISH pattern difficult to recognize. This 5% ratio of atypical signals is in line with a previous report [13]. Meticulous FISH image analysis confirmed the significant reduction in size and intensity of these 'attenuated' signals. Immunohistochemistry revealed overexpression of the ALK oncoprotein and the NGS confirmed the ALK gene rearrangement in these cases.

The 'attenuated' 3' signal pattern is not yet recognized as positive finding in IASLC atlas (guideline)[4]. Beside the indication of apparent EML4/ALK fusion by IHC and TriCheck (tricolour) FISH analysis, Anchored Multiplex PCR (AMP)–Based Targeted Next-Generation Sequencing examination confirmed well known fusion variants in the background which qualifies this pattern as a positive finding in the everyday routine diagnostic work. Recognition of this atypical pattern would improve diagnostic accuracy and result in appropriate targeted therapy in an increased number of patients.

The true background of the unambiguous signal attenuation is to be clarified; however, we hypothesize a small chromosomal deletion (or more complex rearrangement) downstream of ALK, affecting the region where the 'red' FISH probe normally hybridizes (Fig. 2). It is also important to note that the total intensity of the attenuated red signals was not only lower than that of the normal, but the attenuated / normal total intensity ratio also significantly varied among the individual cases. This raises the possibility that the predicted ALK downstream deletion in the background is variable in length (see Table 3. in supplement).

Similar atypical signals were demonstrated in only one previous publication. Li et al. [10] recorded a case with 'attenuated' red signals (with novel fusion partner gene called CEBPZ), along with two additional cases with isolated green signals, as uncommon FISH patterns among the 228 ALK IHC positive NSCLC cases. They also found 8 cases with normal FISH pattern (colocalized signals) and detectable fusion gene when NGS was applied at the same time. However, all our cases showed EML4-ALK rearrangements indicating that the attenuated signal is not necessarily associated with novel fusion partners.

Takeuchi et al. [7] found two ALK IHC positive cases with isolated green signal FISH pattern. Although this pattern has previously been considered as negative, RT-PCR detected the fusion gene in both cases and the patients responded to ALK TKI therapy. To unlock this contradiction, Takeuchi and his colleagues hypothesized that a large deletion, involving the DNA segment labelled by red probe, may lead to complete loss of the red signal; hence the isolated green pattern. This

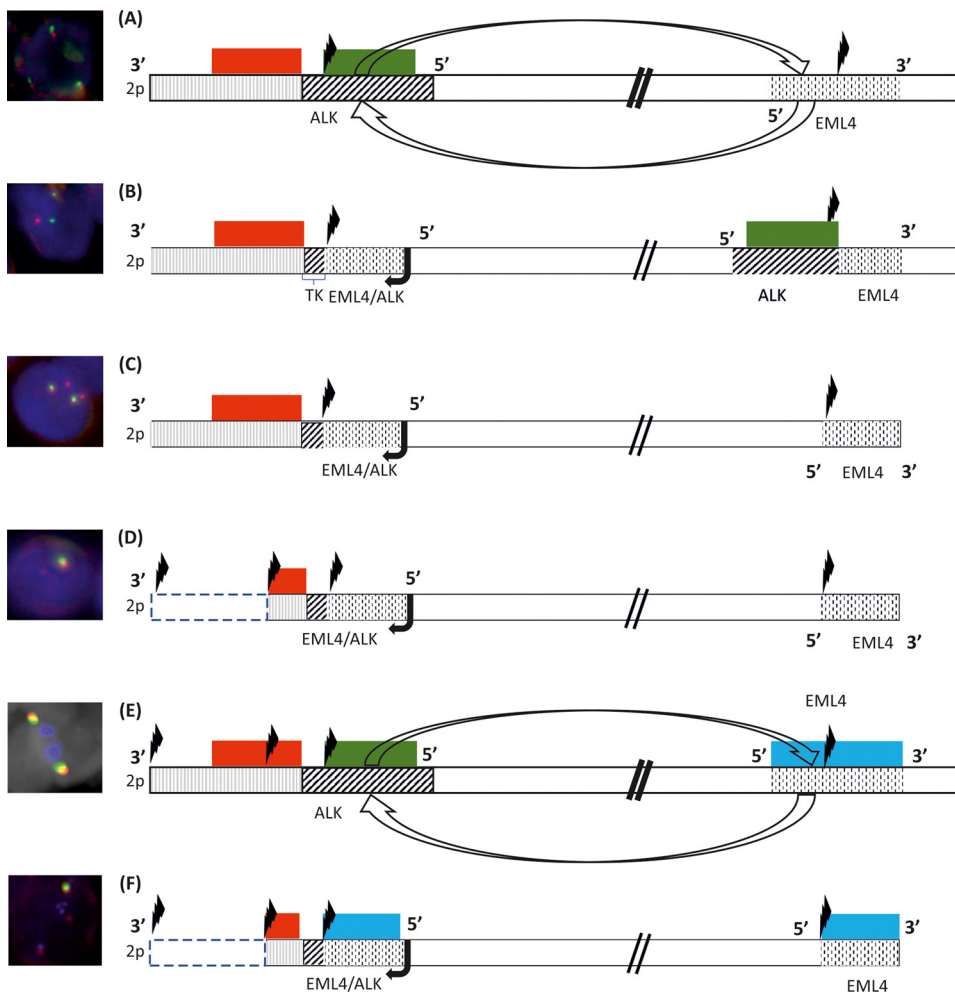


Fig. 2. (A) Fusion signals in a tumour cell with intact ALK gene. The schematic drawing demonstrates position of red and green probe after hybridization in absence of gene rearrangement (flash sign: breakpoint; arrows: paracentric inversion; dash lines: EML4 gene; diagonal lines: ALK gene; TK: gene region responsible for TK domain). (B) Separation of signals (split signal) in a tumour cell harbouring EML4/ALK inversion. (C) Isolated red signal observable after complex genetic event: during inversion between ALK and EML4 the 5' part ALK simultaneously undergoes deletion. (D) Attenuated isolated red signal can be a consequence of combined inversion, 5' deletion, and ALK 3' downstream deletion. (E) 'Tricheck' tricolour FISH probe labels not only the ALK gene but the EML4 gene as well, with a third colour (aqua). (F) Confirmation of EML4/ALK rearrangement in case of attenuated isolated red signal pattern. The small red signal is colocalized with one fragment of the duplicated aqua signal.

work mentions yet another atypical pattern (in which rearrangement was also confirmed by RT-PCR), termed "wild type pattern". This is featured with complete loss of one fusion signal, hypothesized to be the result of a simultaneous up- and downstream deletion of the DNA segments straddling the part of the ALK gene responsible for coding the tyrosine kinase domain. This loss of genetic material is speculated to result in either complete absence of DNA segment to be labelled or only minute retained FISH probe fragments which are too small to detect with fluorescent light microscope.

The loss of the region in 5' upstream direction (labelled green in Figure 2) of the ALK TKD is a well-known phenomenon. However, the loss of a region in 3' direction (labelled red in Figure 2) has rarely been described before [14]. Takeuchi termed this condition as "ALK downstream deletion". Considering also our hypothesis on the background of the definitive signal attenuation described in this paper, the two phenomena shall be distinguished as complete/incomplete ALK downstream deletion in the future.

In addition to the attenuated red signal pattern, we also observed 5 FISH positive cases with 5' deletion pattern harbouring more than one isolated red signals. We do not assume that these are atypical patterns since the red signals did not colocalize with fused ones as in the rare phenomenon of 'red-doublet pattern' described earlier by Peled et al. [9]. Double or triple isolated red signals can be the result of simple copy number gain of the rearranged allele. In rare cases, the unquestionable positive ALK IHC can be accompanied by equivocal FISH result. Fusion pattern - considered as negative FISH result - in the otherwise IHC and NGS positive tumour cells could be the result of random colocalization [15], or non-canonical type of EML4/ALK rearrangement (e.g.: cryptic insertion) [7,9].

AMP-based NGS has been already used by other authors [16] to reveal the presence or absence of gene fusion in the background of unusual signals during FISH testing of ALK and ROS1. The method allows detection of ALK gene rearrangements even without prior knowledge of the partner gene [17,18]. As both new and known fusion variants can be detected with AMP, ambiguously positive FISH cases can be sorted out confidently in this way.

Probably the most practical and cost-effective approach for ALK examination is to start with immunohistochemistry. The positive or equivocal cases can be confirmed by a second method like FISH or NGS. FISH is the most prevalent, but AMP-based NGS is an appropriate secondary method in laboratories where it is available for daily routine purposes. While ALK/EML4 Tricheck FISH can be a helpful test in most of the cases, it is unable to detect fusion gene partners other than EML4. In case of atypical signal patterns samples should be tested by NGS to clarify the presence of gene rearrangements and the fusion partners.

Significance of atypical ALK FISH signals appears to be non-negligible, as more and more observations point out that known or previously unknown fusions may lie behind these unexpected signal patterns. This means that more patients are potentially eligible for ALK TKI therapy as previously was concerned.

5. Conclusion

We observed attenuated red (3') FISH signals in 5% of the NSCLC cases carrying ALK rearrangement. This poses a significant risk of overlooking these true positive cases. In all these cases NGS, TriCheck FISH and ALK IHC methods verified the presence of EML4/ALK fusion and oncoprotein expression, respectively. Our findings underline the

importance of careful interpretation of the ALK FISH assay. Diverse diagnostic modalities are highly recommended in ambiguous cases to insure the proper treatment for patients eligible for TKI therapy [19–23]. Based on our findings we strongly recommend the inclusion of the highly challenging atypical pattern of 3' signal attenuation into the official guidelines.

CRedit authorship contribution statement

Gábor Smuk: Conceptualization, Investigation, Visualization, Writing - original draft. **Gábor Pajor:** Conceptualization, Investigation, Visualization, Methodology, Writing - original draft. **Károly Szuhai:** Investigation, Writing - original draft. **Hans Morreau:** Investigation, Resources. **Ildikó Kocsmár:** Formal analysis, Investigation. **Éva Kocsmár:** Formal analysis, Investigation. **László Pajor:** Writing - review & editing. **Béla Kajtár:** Writing - review & editing. **Veronika Sárosi:** Writing - review & editing. **Gábor Lotz:** Formal analysis, Investigation, Visualization, Validation, Writing - original draft. **Tamás Tornóczky:** Supervision, Resources, Project administration, Writing - original draft.

Declaration of Competing Interest

The authors declare that there is no business relationship resulting in conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.lungcan.2020.03.007>.

References

- [1] G. Los, Q. Li, B. Mroczkowski, et al., Cytoreductive antitumor activity of PF-2341066, a novel inhibitor of anaplastic lymphoma kinase and c-Met, in experimental models of anaplastic large-cell lymphoma, *Mol. Cancer Therap.* 6 (2007) 3314–3322, <https://doi.org/10.1158/1535-7163.mct-07-0365>.
- [2] M. Soda, Y.L. Choi, M. Enomoto, et al., Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer, *Nature* 448 (2007) 561–566, <https://doi.org/10.1038/nature05945>.
- [3] S. Perner, P.L. Wagner, F. Demichelis, et al., EML4-ALK fusion lung cancer: a rare acquired event, *Neoplasia* 10 (2008) 298–302, <https://doi.org/10.1593/neo.07878>.
- [4] IASLC Atlas of ALK-ROS1 Testing in Lung Cancer, https://issuu.com/iaslc/docs/alk-ros1_atlas_low-res.
- [5] D.R. Camidge, S.A. Kono, A. Flacco, et al., Optimizing the detection of lung cancer patients harboring anaplastic lymphoma kinase (ALK) gene rearrangements potentially suitable for ALK inhibitor treatment, *Clin. Cancer Res.* 16 (2010) 5581–5590, <https://doi.org/10.1158/1078-0432.CCR-10-0851>.
- [6] X. Niu, J.C. Chuang, G.J. Berry, et al., Anaplastic Lymphoma Kinase Testing: IHC vs. FISH vs. NGS, *Curr. Treat. Option. On.* 18 (2017) 71, <https://doi.org/10.1007/s11864-017-0513-x>.
- [7] K. Takeuchi, Y. Togashi, Y. Kamihara, et al., Prospective and clinical validation of ALK immunohistochemistry: results from the phase I/II study of alectinib for ALK-positive lung cancer (AF-001JP study), *Ann. Oncol.* 27 (2016) 185–192, <https://doi.org/10.1093/annonc/mdv501>.
- [8] S. Ren, F. Hirsch, M. Varella-Garcia, et al., Atypical negative ALK break-apart FISH harboring a crizotinib-responsive ALK rearrangement in non-small-cell lung cancer, *J. Thorac. Oncol.* 9 (2014) e21–3, <https://doi.org/10.1097/JTO.000000000000013>.
- [9] N. Peled, G. Palmer, F. Hirsch, et al., Next-generation sequencing identifies and immunohistochemistry confirms a novel crizotinib-sensitive ALK rearrangement in a patient with metastatic non-small-cell lung cancer, *J. Thorac. Oncol.* 7 (2012) e14–e16, <https://doi.org/10.1097/JTO.0b013e3182614ab5>.
- [10] W. Li, J. Zhang, L. Guo, et al., Combinational analysis of FISH and immunohistochemistry reveals rare genomic events in ALK fusion patterns in NSCLC that responds to crizotinib treatment, *J. Thorac. Oncol.* 12 (2017) 94–101, <https://doi.org/10.1016/j.jtho.2016.08.145>.
- [11] C.A. Schneider, W.S. Rasband, K.W. Eliceiri, NIH Image to ImageJ: 25 years of image analysis, *Nat. Methods* 9 (2012) 671–675, <https://doi.org/10.1038/NMETH.2089>.
- [12] K. van der Tuin, M. Ventayol Garcia, W.E. Corver, et al., Targetable gene fusions identified in radioactive iodine refractory advanced thyroid carcinoma, *Eur. J. Endocrinol.* 4 (2019) 235–241, <https://doi.org/10.1530/EJE-18-0653>.
- [13] D.R. Camidge, M. Skokan, P. Kiatsimkul, et al., Native and rearranged ALK copy number and rearranged cell count in non-small cell lung cancer: implications for ALK inhibitor therapy, *Cancer* 119 (2013) 3968–3975, <https://doi.org/10.1002/cncr.28311>.
- [14] A. Yoshida, K. Tsuta, H. Nitta, et al., Bright-Field Dual-Color Chromogenic In Situ Hybridization for Diagnosing Echinoderm Microtubule-Associated Protein-Like 4-Anaplastic Lymphoma Kinase-Positive Lung Adenocarcinomas, *J. Thorac. Oncol.* 6 (10) (2011) 1677–1686, <https://doi.org/10.1097/JTO.0b013e3182286d25>.
- [15] G. Smuk, T. Tornóczky, L. Pajor, et al., Immense random colocalization, revealed by automated high content image cytometry, seriously questions FISH as gold standard for detecting EML4-ALK fusion, *Cytometry A*. 93 (2018) 653–661, <https://doi.org/10.1002/cyto.a.23489>.
- [16] Z. Zheng, M. Liebers, B. Zhelyazkova, et al., Anchored multiplex PCR for targeted next-generation sequencing, *Nat. Med.* 20 (2014) 1479–1483, <https://doi.org/10.1038/nm.3729>.
- [17] K. Chang, A. Tay, C. Kuick, et al., ALK-positive histiocytosis: an expanded clinicopathologic spectrum and frequent presence of KIF5B-ALK fusion, *Mod. Pathol.* 32 (2019) 598–608, <https://doi.org/10.1038/s41379-018-0168-6>.
- [18] S.W. Lam, A.M. Cleton-Jansen, A.H.G. Cleven, Molecular analysis of gene fusions in bone and soft tissue tumors by anchored multiplex PCR-Based targeted next-generation sequencing, *J. Mol. Diagn.* 20 (2018) 653–663, <https://doi.org/10.1016/j.jmoldx.2018.05.007>.
- [19] A. Scattone, A. Catino, L. Schirosi, et al., Discordance between FISH, IHC, and NGS analysis of ALK status in advanced non-small cell lung Cancer (NSCLC): a brief report of 7 cases, *Transl. Oncol.* 12 (2019) 389–395, <https://doi.org/10.1016/j.tranon.2018.11.006>.
- [20] M.I. Ilie, C. Bence, V. Hofman, et al., Discrepancies between FISH and immunohistochemistry for assessment of the ALK status are associated with ALK 'borderline'-positive rearrangements or a high copy number: a potential major issue for anti-ALK therapeutic strategies, *Ann. Oncol.* 26 (2015) 238–244, <https://doi.org/10.1093/annonc/mdu484>.
- [21] S. Dacic, L.C. Villaruz, S. Abberbock, et al., M. N. ALK FISH patterns and the detection of ALK fusions by next generation sequencing in lung adenocarcinoma, *Oncotarget* 7 (2016) 82943–82952, <https://doi.org/10.18632/oncotarget.12705>.
- [22] M. Pekar-Zlotin, F.R. Hirsch, L. Soussan-Gutman, et al., Fluorescence in situ hybridization, immunohistochemistry, and next-generation sequencing for detection of EML4-ALK rearrangement in lung cancer, *Oncologist* 20 (2015) 316–322, <https://doi.org/10.1634/theoncologist.2014-0389>.
- [23] S. Savic, J. Diebold, A. Zimmermann, et al., Screening for ALK in non-small cell lung carcinomas: 5A4 and D5F3 antibodies perform equally well, but combined use with FISH is recommended, *Lung Cancer* 89 (2015) 104–109, <https://doi.org/10.1016/j.lungcan.2015.05.012>.