Immunohistochemistry with 3 different clones in anaplastic lymphoma kinase fluorescence in situ hybridization positive non-small-cell lung cancer with thymidylate synthase expression analysis: a multicentre, retrospective, Italian study

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Summary

Introduction. ALK rearrangement is the only druggable oncogenic driver detectable by immunohistochemistry (IHC) not requiring further confirmation of positivity in accessing first-line specific inhibitors. ALK-positive patients experience clinical benefit from pemetrexed-based chemotherapy possibly due to lower thymidylate synthase (TS) levels. This study assesses agreement with three different ALK IHC clones in 37 FISH-positive NSCLC. TS expression by real time (RT)-PCR was compared with ALK FISH-negative cases.

Materials and methods. 37 ALK FISH-positive NSCLC cases diagnosed between 2010 and 2015 in 7 Italian centres were investigated with ICH using three different anti-ALK antibodies (ALK1, 5A4 and D5F3). Staining for ALK1 and 5A4 was graded as 0+, 1+, 2+, and 3+, while the scoring for D5F3 was recorded as negative or positive. Proportion agreement analysis was done using Cohen’s unweighted kappa (k). TS and β-actin expression levels were analysed by quantitative RT-PCR. Comparison between TS expression in ALK FISH-positive specimens and a control cohort of ALK FISH-negative ones was performed with the Mann-Whitney and Kruskal-Wallis tests.

Results. Considering 2+ and 3+ as positive, the proportion of IHC agreement was 0.1691 (95% CI 0-0.4595) for ALK1/5A4, 0.1691 (95% CI 0-0.4595) for ALK1/D5F3, and 1 for D5F3/5A4. Considering 3+ as positive, it was 0.1543 (95% CI 0-0.4665) for ALK1/5A4, 0.0212 (95% CI 0-0.1736) for ALK1/D5F3, and 0.2269 (95% CI 0-0.5462) for 5A4/D5F3. Median TS expression was 6.07 (1.28-14.94) and ALK-positive cases had a significant lower TS expression than ALK-negative tumours (p = 0.002).

Conclusions. IHC proved to be a reliable tool for the diagnosis of ALK-rearranged NSCLC. D5F3 and 5A4 clones have the highest percentage of agreement. TS levels are significantly lower in FISH-positive patients.

Key words: non-small-cell lung cancer, ALK, FISH, immunohistochemistry, thymidylate synthase
Introduction

Lung cancer is the leading cause of cancer-related death worldwide and national data confirm this finding as it is responsible for 27% of cancer-related deaths in men, while it is at the third place in women. Non-small cell lung cancer (NSCLC) accounts for about 85% of all primary lung cancer, with two major histotypes, namely adenocarcinoma (ADC) and squamous cell carcinoma (SqCC). The discovery of druggable genetic alterations in subsets of patients with NSCLC (particularly in adenocarcinoma) paved the way to the introduction of molecular targeted drugs in thoracic oncology, mainly tyrosine kinase inhibitors (TKIs). Among others, ALK rearrangements are found in approximately 2-5% of advanced NSCLC patients. ALK is a receptor tyrosine kinase of the insulin growth factor superfamily, initially identified in anaplastic large cell lymphoma patients. In lung cancer, the most frequent alteration is represented by a translocation of ALK with echinoderm microtubule-associated protein-like 4 (EML4) gene, resulting in an oncogenic fusion protein where ALK is constitutively active. Moreover, other ALK partners have been described to date, such as nucleophosmin (NPM) and tropomysin (TPM). Crizotinib, a small molecule originally developed as a MET inhibitor and also active against ALK and ROS1 kinases, was approved in 2011 by the U.S. Food and Drug Administration (FDA) for the treatment of ALK-rearranged advanced lung tumours. Its approval also in treatment-naive ALK-positive patients was based on the evidence of its activity in heavily pre-treated ALK-positive NSCLC patients showing a significant increase in response rate (RR), as well as in progression free survival (PFS), as compared to first-line platinum-based chemotherapy. The identification of ALK rearrangements was initially based on the break-apart fluorescence in situ hybridization (FISH) test (Abbott Molecular, Abbott Park, IL) adopted as companion test in crizotinib registration studies. This assay uses two fluorescent probes to flank the highly conserved break point within ALK. A positive result, which means a value necessary for drug prescription, requires that at least 15% of 50 evaluated cells harbour split signals. The FISH test is characterized by a good sensitivity and specificity, but still remains operator-dependent, relatively expensive, time-consuming and it requires technical expertise, potentially limiting its wide adoption in pathology laboratories. A recent Italian observational study assessing real life molecular tests in 1787 advanced NSCLC patients, showed that ALK FISH evaluation was made in only 920 of 1345 ADC, possibly reflecting such technical issues. Among alternative methods, reverse transcription- polymerase chain reaction (RT-PCR) can detect ALK rearrangements with great specificity and sensibility. However, this method is time-costly and can suffer from the poor quality of RNA obtained from formalin-fixed paraffin-embedded tissues and the necessity of PCR multiplexing, because of the wide variation of fusion types. More recently, next-generation sequencing (NGS) assays have been shown to be highly sensitive and able to identify even novel ALK variants and co-existing mutations. Immunohistochemistry (IHC) analysis of ALK fusion protein expression is a formidable screening tool in terms of turnaround time, costs and tumour tissue preservation. Recently, Mok et al. have demonstrated the excellent performance of IHC using Ventana D5F3 clone in ALK FISH uninformative patients suggesting its use alone as a standard testing method for ALK fusion. In 2015, the FDA approved the D5F3 clone (Ventana/Roche, Tucson, AZ) as alternative companion diagnostic test for Crizotinib treatment after the demonstration of 94% overall agreement when compared with FISH break-apart test. D5F3-Optiview System (Ventana) was validated as part of the European Harmonization Study where a binary assessment (i.e., positive or negative) was applied. This assay was highly sensitive (90%) and specific (95%) as well as accurate (93%) relative to the FISH results. Another IHC assay, based on the 5A4 clone by Novocasta, was validated by the European Thoracic Platform (ETOP) and showed a sensitivity of 81.3% with a 99% specificity considering scores 2+ and 3+ as positive. The molecular testing guidelines for the selection of lung cancer patients for treatment with tyrosine kinase inhibitors (TKI), suggests either IHC or FISH for ALK rearrangements detection, without making specific recommendations on which specific IHC clone should be used. Novel ALK inhibitors have been developed using different assays, including IHC. Finally, chemotherapy represents a valid option for the treatment of ALK-positive patients when developing resistant disease after TKIs, particularly when using a pemetrexed-based regimen. Pemetrexed is a potent antifolate agent currently registered for the treatment of advanced non-squamous NSCLC and malignant mesothelioma. High expression of thymidylate synthase (TS), an enzyme involved in DNA synthesis, confers resistance to pemetrexed, while low levels are associated with cell sensitivity. Shaw et al. first evaluated thymidylate synthase (TS) expression in ALK-rearranged NSCLCs reporting lower levels when compared to ALK-negative specimens. This Italian multicentre retrospective study is designed to compare 3 different ALK IHC clones (D5F3, 5A4,
ALK1) in FISH positive NSCLC specimens, in order to verify their agreement. Moreover, we evaluated TS expression by real time-PCR in ALK rearranged cases compared to a FISH ALK-negative control cohort, to further characterise this molecularly distinct subgroup of patients. A description of the association between molecular data and patient’s outcome in terms of response to therapies (both ALK inhibitors and pemetrexed chemotherapy) was also performed.

Materials and methods

All the tumours included in this study were reviewed by expert pathologists at each institution and the histologic diagnosis was performed according to the 2021 World Health Organization (WHO) criteria. This study was approved by the ethics committee of each oncologic centre involved. Patients gave written informed consent before inclusion in the study. Each investigator sent the anonymised data to the San Luigi Hospital that had full access to the dataset as coordinator centre. FISH analysis was performed with a commercially available assay (Vysis LSI ALK dual colour, break-apart rearrangement probe, Abbott Molecular, Abbott Park, IL) according to the manufacturer’s recommendations. FISH test was done locally in accredited molecular pathology laboratories. At least 50 tumour cells in each sample were analysed and scored according to international guidelines.

IHC staining was performed on 4-μm sections obtained from formalin-fixed and paraffin-embedded tissue blocks and then mounted on charged slides. After deparaffinization and rehydration, antigen retrieval was performed with Cell Condition Solution-1 (CC1) for 64 minutes at 95°C. ALK IHC assay was performed using 3 different clones: Novocastra mouse monoclonal antibody p80 ALK (clone 5A4, Leica Biosystems, Newcastle Upon Tyne, United Kingdom); Companion Diagnostic Kit Ventana anti-ALK rabbit monoclonal primary antibody (clone D5F3, Cell Signaling Technology); mouse monoclonal anti-human CD248 (clone ALK1, Dako/Agilent, Carpentry, CA). All assays were performed using an automated immunostainer (ULTRA, Ventana Medical System, Tucson, AZ). The expression of all samples was scored by experienced pathologists (LR, PG, GR). Expression level with ALK1 and 5A4 was quoted using an IHC scoring considering a 4-tried intensity score (0, negative; 1+, weak; 2+, moderate; 3+ strong), while a dichotomous negative/positive system was adopted with the CDx D5F3 Ventana kit. In all batches, a negative (lack of the primary antibody) and positive (ganglions and nerves of the appendiceal tip and/or a sample from an ALK FISH-positive resected pulmonary adenocarcinoma) control was employed to evaluate the appropriateness of the IHC analysis. When using the 4-tried scoring system, cases showing no staining were considered as negative, cases with 2+/3+ staining were considered as positive/rearranged and cases with 1+ intensity expression were considered as indeterminate.

Quantitative RT-PCR for TS and β-actin were performed in the present case cohort and in a FISH ALK-negative control group (173 ALK-negative and EGFR-negative advanced ADC including 127 men and 46 women) as previously described. Ten-μm thick sections were used for RNA extraction. The sections were serial to a 4-μm-thick section from the same formalin-fixed paraffin embedded tumour block used for H&E staining to select appropriate neoplastic areas. RNA isolation and retrotranscription were performed as already reported. Relative cDNA quantification was done using a fluorescence-based real-time detection method with measurements done in triplicate and the comparative Ct method used. Quantitative real-time polymerase chain reaction (qPCR) was performed with an ABI PRISM 7900HT Sequence Detection System (Life Technologies, Applied Biosystems Division, Carlsbad, CA, USA) in a 384-well plate. All qPCR mixtures contained 1 ul of cDNA template (approximately 20 ng of retrotranscribed total RNA) diluted in 9 ul of distilled-sterile water, 1200 nM of each primer, 200 nM of internal probe and TaqMan Gene Expression Master Mix (Life Technologies Thermo Fisher Scientific) to a final volume of 20 ul. Cycling conditions were 50°C for 2 minutes, 95°C for 10 minutes followed by 46 cycles at 95°C for 15 seconds and 60°C for 1 minute. Baseline and threshold for cycle threshold (Ct) calculation were set manually with ABI Prism SDS 2.1 Software. A mixture containing Human Total RNA (Stratagene, La Jolla, CA) was used as control calibrator on each plate. beta-Actin was used as internal reference gene. The fold change in gene expression levels, expressed in unitless values, was evaluated using the 2-∆∆Ct method.

Statistical analysis

A descriptive statistical analysis was performed. Proportion agreement analysis was made using Cohen’s unweighted kappa (k). A k value from 0.61 to 0.8 was considered as substantial and between 0.81 and 1.0 as excellent, according to Landis and Koch. Comparison between TS expression in FISH-positive ALK rearranged specimens and a control cohort of ALK FISH-negative ones was performed with the Mann-Whitney and Kruskal-Wallis tests or the Spearman’s test.
Results

IHC was performed on 37 FISH-positive ALK locally advanced or metastatic NSCLC specimens from 7 different Italian Oncology Centres. Diagnoses were made between 2010 and 2015. Patients’ main characteristics are reported in Table I. Median age at diagnosis was 60 years (range 22-81), 22 patients were male (62%) and all patients had adenocarcinoma histology. Data on smoking habits were available for 19 patients: 12 (63%) were current or former smokers, while 7 (37%) were neve-smokers. Seven were cell-blocks (19%), while the others were biopsies. 31 patients (84%) had stage IV disease at diagnosis, 3 (8%) stage IIIA, while initial staging was unknown for 3 (8%).

Immunohistochemistry

The scoring distribution for each antibody is represented in Figure 1 and Figure 2. Three cases (8.1%) showed strong (3+) and 13 (35%) mild (2+) staining intensity with ALK1 antibody, while 17 (45.9%) had

| Characteristics                  | Total (N = 37) |
|----------------------------------|---------------|
| Age at diagnosis (years)         |               |
| Median                           | 60            |
| Range                            | 22-81         |
| Gender, n (%)                    |               |
| Male                             | 22 (62)       |
| Female                           | 15 (38)       |
| Smoking habits, n (%)            |               |
| Current/Former smoker            | 12 (32.5)     |
| Never smoker                     | 7 (19)        |
| Unknown                          | 18 (48.5)     |
| Histology, n (%)                 |               |
| Adenocarcinoma                   | 37 (100)      |
| Other                            | 0 (0)         |
| Stage at diagnosis, n (%)        |               |
| IV                               | 31 (84)       |
| IIIA                             | 3 (8)         |
| Other                            | 0 (0)         |
| Unknown                          | 3 (8)         |

* Score value between 0+ and 3+ for ALK1 and 5A4; positive (3+) or negative (0) for D5F3

Figure 1. Distribution of immunohistochemical scores with the 3 different clones (ALK1, 5A4 and D5F3).
Figure 2. Detailed distribution of immunohistochemical staining score along the entire series of 37 ALK FISH-positive cases according to the 3 different clones (ALK1, 5A4, D5F3).

Figure 3. Example of invasive lung adenocarcinoma with acinar pattern on surgical specimen (A, haematoxylin-eosin staining) showing weak positivity (score 1+) (B, immunohistochemistry) with clone ALK1, moderate positivity (score 2+) (C, immunohistochemistry) with clone 5A4 and strong positivity (score 3+) (D, immunohistochemistry) with clone D5F3.
weak intensity (1+) and 4 were considered to be negative. When using 5A4 antibody, 19 (51.3%) samples showed strong (3+) staining, 14 (37.9%) mild (2+) and 4 (10.8%) were considered to be negative (0+). None of the samples showed a weak intensity. Results with D5F3 were scored as positive or negative only, as previously indicated. 33 positive (89.1%) and 4 negative (10.9%) cases were found. When considering 2+ and 3+ scored samples as positive, 16/37 cases (43%) were positive with ALK1, as compared to 33/37 (89%) for 5A4. The concordance between each pair of antibodies is reported in Table II, Figure 3 and Figure 4. The proportions of agreement between ALK1 and 5A4 was 0.1691 (95% CI 0-0.4595), 0.1691 for ALK1 and D5F3 (95% CI 0-0.4595), and 1 for D5F3 and 5A4. When considering 3+ cases only as positive, three cases were positive using ALK1 as

Table II. Crosstabulation considering 2+ and 3+ cases as positive.

|          | ALK1 |          |          |
|----------|------|----------|----------|
|          | Positive | Negative |          |
| 5A4      | 16      | 17       |          |
| 5A4      | 0       | 4        |          |

|          | ALK1 |          |          |
|----------|------|----------|----------|
|          | Positive | Negative |          |
| D5F3     | 16      | 17       |          |
| D5F3     | 0       | 4        |          |

|          | 5A4 |          |          |
|----------|-----|----------|----------|
|          | Positive | Negative |          |
| D5F3     | 33   | 0        |          |
| D5F3     | 0    | 4        |          |

Figure 4. Example of metastatic adenocarcinoma on cell-block from pleural effusion (A, haematoxylin-eosin staining) showing weak positivity (score 1+) (B, immunohistochemistry) with clone ALK1, moderate positivity (score 2+) (C, immunohistochemistry) with clone 5A4 and strong positivity (score 3+) (D, immunohistochemistry) with clone D5F3.
compared to 19 with 5A4. The concordance between each pair of antibodies using this cut-off is reported in Table III. The proportion of agreement using this score was 0.1543 (95% CI 0-0.4665) between ALK1 and 5A4, 0.0212 (95% CI 0-0.1736) for ALK1 and D5F3, and 0.2269 (95% CI 0-0.5462) for 5A4 and D5F3.

**Thymidylate Synthase Expression Analysis**

TS expression analysis was done in 36 samples. Only in one sample the analysis was not performed due to material exhaustion. Median TS expression of the whole ALK-positive case series was 6.07 (1.28-14.94). When compared with the ALK negative advanced population who had a median value of TS expression of 8.59 (range 1.04-27.3), our ALK positive cases had significant lower TS expression (p = 0.0053, Fig. 5).

**Table III.** Crosstabulation considering only 3+ cases as positive.

|          | ALK1 Positive | ALK1 Negative | 5A4 Positive | 5A4 Negative | D5F3 Positive | D5F3 Negative | 5A4 Positive | 5A4 Negative | D5F3 Positive | D5F3 Negative |
|----------|---------------|---------------|--------------|--------------|---------------|---------------|--------------|--------------|---------------|---------------|
| ALK1     |               |               |              |              |               |               |              |              |               |               |
| Positive | 3             | 16            | 19           | 14           | 19            | 14            |              |              |               |               |
| Negative | 0             | 18            | 19           | 14           | 0             | 4             |              |              |               |               |

**Figure 5.** Distribution of thymidylate synthase (TS) relative expression levels in ALK-positive and ALK-negative lung adenocarcinomas.
**Response to therapy**

Clinical data on prescribed therapies were available for 30 patients (81%). Twenty-four of them received at least one ALK TKI, being crizotinib in 23 patients (96%) and ceritinib in 1 (4%). Among these patients, the best response with the first TKI administered was: partial response (PR) in 16 cases (67%), stable disease (SD) in 3 (12%) and a progressive disease (PD) in 5 (21%). Interestingly, 2 of these latter patients were IHC negative with all of the 3 tested clones, while the other showed IHC positivity (all 5A4 3+ and D5F3 positive with different ALK1 scores). Among those who obtained disease control (SD or PR), all but one was IHC positive with 5A4 and D5F3 clones. The only IHC negative patient (patient #36) had a partial response on crizotinib treatment.

Data about pemetrexed-based chemotherapy were available for 14 patients. Among these patients, 10 obtained disease control (7 SD, 3 PR), while 4 had disease progression as best response to treatment. The median number of chemotherapy cycles was 4 (range 1 to 6). Nine patients obtaining disease control with pemetrexed-containing regimens were ALK IHC positive with D5F3 and 5A4 clones while only 5 were also positive with ALK1. One patient (number 36) who achieved SD with cisplatin-pemetrexed was ALK IHC negative; interestingly, the same patient derived benefit from crizotinib second-line treatment.

**Discussion**

ALK rearrangements in NSCLC have been initially diagnosed using break apart FISH assay, a time-consuming and operator-dependent technique often unlikely applicable in small biopsies with artifacts or cell blocks. Following the demonstration of high concordance with the break apart ALK FISH assay, IHC with D5F3 clone using Ventana platform and Optiview amplification system has been approved by the US FDA as an alternative diagnostic tool for ALK rearrangements detection and TKI treatment. In contrast, the European label of TKI requires only proof of an “advanced ALK-positive NSCLC” without any statement about a preferred assay, allowing each pathology laboratory to use either FISH, IHC or molecular biology assays such as RT-PCR or NGS alone or in combination. The main advantages of IHC are its wide distribution among pathology laboratories, easy interpretation and lower cost. Moreover, IHC requires far less material than FISH, an important issue considering the prevalence of small biopsies and the vast number of tests currently needed for NSCLC diagnosis and correct management. For such reasons, many groups investigated different IHC clones with or without amplification systems and compared them with other assays.

Of note, in the era of an NGS approach to NSCLC predictive biomarkers, ALK gene fusion is the only targetable oncogenic driver for which IHC may authorise the treatment with specific TKIs in case of positivity.

As previously proposed, we considered positive those specimens scored as 2+ and 3+ with any clone. The agreement between 5A4 and D5F3 clones, when considering both 2+ and 3+ as positive, was excellent. On the contrary, as previously demonstrated, the agreement was poor when considering ALK1, a clone with very low sensitivity.

A major limitation of this analysis is the absence of a FISH negative control, since differences in specificity could make the agreement between the antibodies even weaker. Being limited to FISH positive cases, our analysis is able to describe the sensitivity of the 3 different antibodies in classifying as eligible for treatment all the cases that would have been selected by FISH, without the possibility to describe their specificity performance in FISH negative cases.

However, literature data suggest that all tested clones are characterised by high specificity (approaching 100%), but variable sensibility compared to the FISH assay, supporting the current study design. We also repeated concordance agreement analysis considering only 3+ cases as positive. Using this scoring system, the agreement between ALK1 and 5A4 or ALK1 and D5F3 was poor, and it became poorer also between 5A4 and D5F3. In our series, 4 cases would have been considered negative (score 0) by all IHC clones despite being FISH positive. Interestingly, among the 3 patients that received crizotinib treatment, 2 experienced rapid progression and death within 1 month after the start of treatment, while the other patient had a partial response lasting for 11 months. Of note, both the early progressors had a borderline FISH positivity (16% and 18% of positive cells, respectively).

Using different ALK detection methods, discordant cases have been increasingly reported. A recent review of the literature shows a response rate of 100% in IHC positive / FISH negative as compared to 46% in IHC negative / FISH positive cases. Such data underline the role of IHC analysis in the selection of patients for ALK inhibitors. For such reason, a recently published algorithm by Marchetti and colleagues suggests to screen all specimens with a high IHC sensitive assay (such as D5F3 clone with OptiView system or Novocastra 5A4), using FISH for doubtful cases such as those scored as 1+ or 2+ or nega-
tive cases with certain clinical or pathological characteristics often reported in ALK-rearranged lung adenocarcinomas (young age at diagnosis, light/no smoking habit, adenocarcinoma with signet ring cell features) 24. In other words, the use of 2 different ALK detection techniques when facing with a patient showing clinico-pathological characteristics suggestive of ALK rearrangement may significantly reduce the chance of missing an ALK-positive case receiving clinical benefit from specific inhibitors.

Apart from new generation ALK inhibitors, chemotherapy still represents an option in daily clinical practice when ALK-positive patients experience disease progression. Of note, the role of pemetrexed in association with cisplatin has proven to be particularly active in lung ADC 25, particularly when harbouring ALK rearrangement due to the low levels of thymidylate synthase (TS), a key enzyme in folate metabolism. In light of this finding, we performed TS analysis in 36 of our specimens aimed at further confirming this observation and supporting the role of TS as possible predictive marker in ALK-positive patients. According to few similar investigations 19,20, we observed that mean expression value is statistically lower as compared to a series of ALK-negative ADC. Although the low number of TS-positive and TS-negative cohorts partly limited the consistency of our results, we support the possible role of TS levels in the choice of pemetrexed-cisplatin chemotherapy in ALK-positive patients during disease progression after ALK-inhibitors.

Conclusions

IHC is a reliable tool for the diagnosis of ALK-rearranged lung ADC. D5F3 and 5A4 clones have the higher percentage of agreement. We also confirmed previous studies that IHC could be used as a screening tool, solely authorising the adoption of ALK TKIs when detecting positivity. Moreover, since FISH positive / IHC negative cases rarely respond to TKI, IHC has a role to confirm a positive FISH result in patients with a borderline number of positive cells. Finally, we confirmed the role of TS expression in this setting, highlighting a significantly lower level in ALK rearranged patients, possibly explaining the higher sensitivity to pemetrexed-based chemotherapy and resulting a promising predictive marker of chemotherapy efficacy in disease progression during TKIs.

Conflict of interest

The authors declare no conflicts of interest.

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Ethical consideration

The study was conducted in accordance with the precepts of the Helsinki declaration and approved by the ethical committee of each centre involved.

Author contributions

Writing: PB, LM, GR; Data curation and investigation: PB, LM, GR, SN; Analysis and interpretation of data: PB, LR, PG, GR, SN; Critical revision: VM, FB, EM, MM, AL, MT; Review and editing: LM, DT, PB, GR.

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