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Does immunohistochemistry represent a robust alternative technique in determining drugable predictive gene alterations in non-small cell lung cancer ?

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Short title: Predictive immunohistochemistry in NSCLC

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Abstract

Immunohistochemistry (IHC) is a widely-tested, low-cost and rapid ancillary technique available in all laboratories of pathology. This method is generally used for diagnostic purposes, but several studies have investigated the sensitivity and specificity of different immunohistochemical antibodies as a surrogate test in the determination of predictive biomarkers in non-small cell lung cancer (NSCLC), particularly for *Epidermal Growth Factor Receptor (EGFR)* gene mutations, *Anaplastic Lymphoma Kinase (ALK)* gene and *ROS1* rearrangements. In this review, a critical examination of the works comparing the consistency of IHC expression and conventional molecular techniques to identify genetic alterations with predictive value in NSCLC is discussed.

Summarizing, data on sensitivity and specificity of antibodies against ALK and ROS1 are very consistent and time is comes to trust in IHC at least as a cost-effective screening tool to identify patients with rearranged tumors in clinical practice. On the other hand, mutant-specific antibodies against EGFR demonstrate a good specificity but a low-to-fair sensitivity, then raising some cautions on their employment as robust predictive biomarkers. A brief comment on preliminary experiences with antibodies against BRAF, RET, HER2 and c-MET is also included.

Introduction

More than half of non-small cell lung cancer (NSCLC), in particular adenocarcinoma histologic type, show genetic alterations involving oncogenic drivers leading to aberrant activation of tyrosine kinases acting as molecular targets for selective inhibitors.^{1,2}

The presence of these genetic alterations is the unique most important finding in predicting effectiveness of specific targeted therapies.³ Most important, patients with adenocarcinomas harbouring epidermal growth factor receptor (*EGFR*) mutations or Anaplastic Lymphoma Kinase (*ALK*) and *ROS1* rearrangements have a significant disease-free survival when compared with conventional chemotherapy.⁴⁻¹⁰ This revolutionary therapeutic approach in patients with NSCLC generally requires a drug specific “companion diagnostic” test to select patients with tumors harboring genetic alterations.¹¹ Of note, some “companion diagnostics” have been approved differently in various countries.¹²⁻¹⁴

In brief, approximately 10% to 40% of lung adenocarcinomas (mainly depending on ethnicity with highest frequency in Asiatic patients) harbor a *EGFR* mutation and about 90% of these mutations occur in exons 19 (E746-A750 deletion and other short in-frame variants of this deletions in exon 19) and 21 (leucine to arginine substitution at amino acid 858, L858R).^{1,2} Less common mutations predicting a minor clinical response to *EGFR* tyrosine kinase inhibitors (TKI) occur in exons 18 and 20.¹⁵⁻¹⁷ Overall, *EGFR* mutations are the best predictors of efficacy when adopting selective TKIs, namely gefitinib, erlotinib and afatinib.⁴⁻⁶ Several PCR-based DNA extractive methods are available and accepted to detect *EGFR* mutations, although there is a wide range in terms of sensitivities among various methodologies, significantly leading to quite different results.^{13,14,18,19}

About 5-7% of lung adenocarcinomas harbor a rearrangement of *ALK* gene, particularly through an intrachromosomal inversion with *EML4-ALK* gene and other genes, then leading to a fusion product associated with *ALK* protein overexpression.¹² Clinical features, (e.g., young age,

non/light-smoking habit, adenocarcinoma with cribriform/signet ring cell pattern) cannot reliably identify patients with ALK-positive tumors.²⁰ Fluorescence in-situ hybridization (FISH) using break-apart probe is currently considered the gold standard for detection of *ALK* rearrangement, since clinical trials with the dual MET and ALK-inhibitor crizotinib are based on *ALK* FISH detection.⁷⁻⁹

More recently, the rearrangement of *ROS1* gene has been identified in a small subset (about 1-2%) of lung adenocarcinomas.²¹ Detection of *ROS1* rearrangement in the recent clinical trial by Shaw et al.¹⁰ was performed using a break apart probe with FISH assay.

Other promising gene alterations involving *BRAF*, *HER2*, *RET* and *c-MET* seem to predict novel therapeutic scenarios in NSCLC.^{2,22}

Although immunohistochemistry (IHC) is the main ancillary technique in pathology labs and it has been largely demonstrated that some antibodies against EGFR, ALK and ROS1 can indirectly detect the presence of gene alterations with high specificity and fair-to-high sensitivity, this technique is not validated to directly select patients undergoing specific targeted therapies in NSCLC, as instead happen in breast and gastric cancer with HER2 detection.²³

Although generally poorly-appreciated in the molecular biology community, IHC is a rapid, cheap and well-known assay that does not require huge tumor cell content and perform quite well even in degraded tissue (e.g., decalcified bone tissue) or cytology samples (**Table 1**).

In the present review, we aimed at summarizing the characteristics and the performance rate in terms of sensitivity and specificity of the antibodies acting as possible surrogates to predictive molecular tests, also discussing the future landscape in which IHC could be adequately adopted as screening as well as confirmatory test to detect predictive biomarkers in routine clinical practice.

1. *EGFR* mutations

The finding of an activating *EGFR* mutation in NSCLC is the best single predictor of efficacy using selective tyrosine kinase inhibitors (TKI), while total *EGFR* expression at immunohistochemistry and *EGFR* FISH assay did not demonstrate to be reliable predicting biomarkers.³ Various activating *EGFR* mutations have been reported in exons 18, 19, 20 and 21, but about 90% of *EGFR* mutations in NSCLC involve in-frame deletions of exon 19 (E746-A750 del and variants) and point mutation L858R in exon 21.^{13,18,19} These mutations are also significantly related to a better and prolonged clinical response to *EGFR* TKI (particularly exon 19 deletions),¹⁶ although other less drug-sensitive mutations may occur in exon 18 (G719X), exon 20 (insertions) and exon 21 L861X at a much lower frequency. Despite the great variability of the detection rate sensitivity, all PCR-based tumor DNA extractive methods are basically accepted in determination of *EGFR* mutations and represent validated techniques in all guidelines proposed from different scientific societies in various countries.^{13,18,19}

Detection of *EGFR* mutations significantly increase when using more sensitive detection methodologies.¹³ Supporting this view, Won et al.²⁴ recently demonstrated the presence of additional activating *EGFR* mutations when passing from direct sequencing to mutant-enriched Next Generation Sequencing (NGS) in a population of *ALK*-positive tumors, finally evidencing *EGFR* mutations in 15.4% of *ALK*-rearranged NSCLC (14 out of 91). The presence of minor clones of *EGFR* mutated tumor cells in *ALK*-positive NSCLC might explain the occurrence of dual-positive tumors and the different detection rate of *EGFR* mutations due to variable sensitivity of various methods used in the study. Again, Zhou et al.²⁵ detected 30% of false negative *EGFR* mutations using the Scorpion amplification refractory mutation system (ARMS) in a population of wild-type cases originally investigated with direct sequencing.

In 2009, Yu et al.²⁶ developed two rabbit monoclonal antibodies selectively directed against E746-A750 deletion in exon 19 (clone 6B6, Cell Signaling Technology; also known as clone SP111 from Ventana Medical Systems, Inc., Tucson, AZ, USA) and the L858R point mutation in exon 21 (clone 43B2 from Cell Signaling Technology; also known as clone SP125 from Ventana Medical Systems) demonstrating high sensitivity (88% and 92%, respectively) and specificity (100% and 99%, respectively) of these antibodies in recognizing the relevant *EGFR* mutations. However, discordant results have been reported in different studies investigating the adequacy of IHC in detecting *EGFR* mutations when compared with standard DNA sequencing methods.²⁷⁻⁵³

Provided that in normal routine practice DNA sequencing analysis should always be considered the gold standard,⁵⁴ the main advantages to use mutation-specific antibodies in detecting *EGFR* mutations may be the following: 1. in critical patients requiring a rapid determination of *EGFR* mutations; 2. in tiny biopsy or scarce cytologic specimens; 3. in poorly-preserved or degraded tumor tissue (e.g., decalcified bone tissue), precluding reliable results, and then false negative cases, when using low-sensitive DNA extractive techniques.

As summarized in **Table 2**, the sensitivity of these antibodies is very variable (ranging from 30% to 100% with a mean value between 60% and 70%), mainly depending on the cut-off scoring system to quote immunoreactivity, while the specificity is excellent (overall >90%). Of course, these mutant-specific antibodies do not stain tumors harboring *EGFR* mutations different from E746-A750del and L858R.

Basically, the sensitivity of mutant-specific antibodies to detect all *EGFR* mutations range from about 40% to 60%. Thus, *EGFR* mutant-specific antibodies cannot replace conventional DNA sequencing molecular methods in detecting this predictive biomarker. Nevertheless, these antibodies should represent a valid adjunct to molecular techniques when specimens contain a low cellularity or poorly-preserved tumor cells. In fact, tumor cell showing consistent expression

(score 2+/3+) for these antibodies firmly indicate the presence of the relevant *EGFR* mutation (Figures 1 and 2).

2. *ALK* rearrangement

In 2007, Soda et al.⁵⁵ first documented a small inversion within chromosome 2p resulting in the formation of a fusion gene comprising *EML4* and *ALK* in NSCLC. The chromosomal rearrangement of the 3' kinase domain of *ALK* most often involve the N-terminal portion of Echinoderm Microtubule-associated protein-Like 4 (*EML4*) gene, but less common 5' fusion partners have been identified including Kinesin Family member 5B (*KIF5B*), *TRK*-fused gene (*TFG*), Kinesin Light Chain 1 (*KLC1*) and others.²⁰

This gene alteration represented the molecular target for the double *MET* and *ALK* inhibitor PF-02341066 (crizotinib). The clinical efficacy of crizotinib in patients with *ALK* rearranged NSCLC was then demonstrated in various clinical trials,⁷⁻¹⁰ both in heavily-pretreated and naïve (first line) settings. Despite patients with *ALK*-rearranged tumor have some peculiar characteristics (never/light smokers, younger age, adenocarcinomas histotype with mixed patterns including acinar and cribriform architecture and signet-ring cells), none of these clinico-pathologic features may reliably predict the response to *ALK* inhibitors (crizotinib, ceritinib).^{20,56,57} According to the Food and Drug Administration (FDA), in United States the use of crizotinib is limited to *ALK*-positive tumors when detected by the Abbott Vysis *ALK* break apart Fluorescent in situ Hybridization (FISH) probe kit. Tumors are considered positive when $\geq 15\%$ of cells (counting at least 50 tumor cells) show a positive signal (either as single red spot or break apart of the colored probes indicating deletion or inversion of *ALK* gene, respectively). Although clinically validated, FISH test is laborious, relatively expensive and requires a good expertise. In addition, a not

insignificant rate of tumors also show borderline results with 10-14% of positive cells.⁵⁸⁻⁶⁰ The European Medicines Agency (EMA) approved the use of crizotinib in all ALK-positive patients tested with a validated method without restriction to FISH technique. While the presence of more than twenty *ALK* gene fusions with *EML4* and other *ALK* gene fusion partners seems to preclude the use of Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) in routine practice, the demonstration of ALK protein expression by immunohistochemistry (IHC) seems to open an important screening test to detect ALK-positive NSCLC.¹²

Since ALK protein is not expressed in normal adult lung tissue, IHC appears a robust alternative method in identifying lung cancers harbouring *ALK* rearrangement. Provided that IHC is not expensive and of rapid execution, it has the great advantage to be the most used ancillary technique in all laboratories of pathology. The main problem is that the cellular content of ALK protein in *ALK* rearranged NSCLC tumor cells is much lower than that observed in ALK-lymphomas.⁶¹ Then, the concentration of the primary antibody against ALK should be increased and the positive signal needs improvement by using appropriate detection and visualization kits to increase the inter-observer reproducibility. Despite different protocols and primary antibody clones, the results comparing ALK detection by IHC and FISH are quite good, if not excellent (**Table 3**).⁶²⁻¹⁰⁰

In particular, it is important to underline that consistent positivity (score of intensity 2+ and 3+) with ALK at IHC is almost always associated with a positive FISH test and, most important, all negative cases at IHC are basically negative at FISH analysis (**Figure 3**). While it is quite exceptional to have ALK IHC negative and FISH positive cases, about 1-2% of IHC positive and FISH negative cases have been well-documented and these cases do respond to ALK inhibitor.¹⁰¹⁻¹⁰⁴

The main concerns about the use of IHC as screening tool in detecting ALK-positive lung tumors rely on the fact that: 1. clinical trials with crizotinib refer only to positive patients with FISH test; 2.

the sensitivity in detecting ALK tumors may depend on different clones and detection systems; 3. the lack of complete interobserver reproducibility among pathologists when using 4-tier scoring systems and low sensitivity antibody clones.

However, several experiences with FISH also indicate that this assay may give false positive and negative results as well as a significant rate of not interpretable results.^{65,69,76} Among different ALK antibodies (clones ALK-1, 5A4 and D5F3) investigated to detect ALK by IHC, the clone 5A4 has been validated by the European Thoracic Oncology Platform (ETOP) consortium,⁸⁵ while the clone D5F3 has been included in the validated test by Ventana Medical System. Inc. and confirmed in several works.^{105,106}

The use of antibodies with high sensitivity to ALK protein is entirely cost-effective as screening test, saving at least 95% of usefulness FISH tests with the certainty to do not left behind true ALK-positive patients.^{81,97,107,108} In addition, ALK IHC test is very helpful in small biopsies characterized by a limited number of tumor cells.

The limited sensitivity reported in preliminary reports is overridden by more recent works that highlight a almost complete correlation between IHC and FISH methods, particularly when using clones 5A4 and D5F3 coupled to an adequate detection kit.^{85,105,106,108} Needless to say that since lung cancer generally has a low expression of ALK protein at a cytoplasmic and/or membrane level when compared with ALK lymphomas, and weak staining (score 1+) may be associated with a ALK-rearranged tumor, all these cases require a confirmatory determination using FISH assay.

Finally, some novel ALK fusion gene (e.g., KIF5B-ALK) were identified by IHC in cases originally tested negative at FISH analysis.⁶⁸

Application of adequate external controls in each bath, the use of standardized protocols incorporating the correct choice of antibody clone and detection kit as well as the participation to external quality control assurance programs should always considered to ensure rigorous

immunohistochemical results in routine practice, providing an effective integration of IHC with FISH testing.^{76,106,108}

Selinger et al.⁷⁰ recently reported 100% specificity between IHC and FISH testing 594 resected NSCLCs with either ALK1, 5A4, D5F3 clones. IHC also evidenced 6, 11 and 6 ALK + cases using ALK1 (5 with 1+ and 1 with 2+ score), 5A4 (10 with 1+, 1 with 2+ score) and D5F3 (3 with 1+, 2 with 2+ and 1 with 1+ score), respectively.

Minca et al.⁷⁶ demonstrated 100% sensitivity and specificity of D5F3 clone using the Ventana automated immunostainer BechMark XT with the OptiView detection system on 249 informative NSCLCs diagnosed on formalin-fixed paraffin-embedded and Thin-Prep matched specimens. In addition, in 55 not informative FISH samples, IHC was the sole method detecting ALK positivity in 3 cases. Narrow signal separation of probes, uninformative cases due to scarce number or poor preservation of tumor cells, challenging identification of tumor cell morphology in biopsy with confounding features (i.e., crash artifacts, the presence of a rich inflammatory infiltrate, a close admixture of normal salivary gland structures) (**Figure 4**) do represent important factors precluding a reliable examination of specimens submitted to FISH test. Uninformative results in this study were significantly ($p < 0.001$) lower in IHC-tested samples (4%) than in FISH-tested cases (26%),⁷⁶ similarly to what observed in the study by McLeer-Florin et al.⁶⁵

While all used ALK clones show an excellent specificity, the clone ALK1 is characterized by a moderate sensitivity ranging from 60% to 90% with a relatively low intensity of staining. When using a less sensitive clone, the results could be improved by “high-sensitivity” pathologists carefully noticing all staining shadows (**Figure 5**). As a rule, the less sensitive clone is adopted, the more sensitive should be the pathologist examining ALK test on IHC.

By contrast, sensitivity and specificity of studies with clone 5A4 are quite consistent ranging from 95% to 100%. Studies employing clones ALK1 and 5A4 used a four-tier scoring system (from 0-

negative to 1+-weak, 2+-moderate, 3+-strong intensity staining), somehow complicating the inter-observer reproducibility. The binary system adopted when using the high-sensitivity D5F3-based kit proposed by Ventana clearly facilitate ALK determination by IHC even in the hands of less expert pathologists.^{105,106,108}

Reviewing the last 153 consecutive screened lung adenocarcinomas (unpublished observations), the sensitivity and specificity of the clone ALK1 adopting the OptiView detection kit and the automated immunostainer BenchMark XT (Ventana Medical Instruments, Tucson, AZ, USA) are 100% and 96%, respectively. Eighteen ALK-positive cases (11.8%) were identified at IHC and 12 at FISH assay (7.8%). All FISH positive cases showed moderate-to-strong expression at IHC level, while among the 6 discordant cases 5 revealed a weak intensity (score 1+) and 1 with a moderate (2+ score) intensity showed gene amplification at FISH test. In addition, other 27 randomly selected ALK negative cases at IHC were entirely negative also with FISH. Of note, 3 out of 12 ALK-positive cases had a concomitant EGFR (1 case) or KRAS (2 cases) mutation using MALDI-TOF methodology, according to other experiences.^{24,86}

Practically speaking, IHC is a likely accessible, low-cost, rapid and widely informative technique to detect ALK status in NSCLC. High-sensitivity IHC protocols (particularly when using the clone D5F3 and OptiView amplification kit) are quite consistent when compared to FISH results and should be introduced as validated screening test to detect ALK positive patients requiring a confirmatory FISH test in a limited NSCLC population.

3. ROS1

ROS1 is a receptor tyrosine kinase involved in tumor cancerogenesis of different tumors. The rearrangement of *ROS1* with other fusion genes, such as *CD74*, *FIG*, *EZR* and solute carrier protein

34A2 (*SLC34A2*), is the oncogenic driver in a subset (1-2%) of NSCLC, particularly of adenocarcinoma subtype.¹⁰⁹⁻¹¹⁰ Identification of patients with NSCLC harbouring *ROS1* rearrangement in a recent clinical trial with the TKI crizotinib has been performed with a break apart FISH assay, demonstrating a dramatic objective clinical response in 72% of cases.¹⁰ However, as with *ALK*, all *ROS1* rearrangements may be easily detected at the immunohistochemical level with the specific rabbit monoclonal antibody D4D6.¹¹¹⁻¹¹³ *ROS1* expression seems to be mutually exclusive with *ALK*, but a subgroup of *ALK* and *ROS1* positive NSCLC may show *EGFR* activating mutations detected in the same tumor cells using molecular techniques and *EGFR* mutant-specific antibodies.¹¹⁴⁻¹¹⁶

Recent studies have confirmed the validity of the clone D4D6 to detect *ROS1* rearrangement when IHC assay was compared to FISH test (**Table 4**).¹¹⁷⁻¹²⁰ Basically, all *ROS1* FISH-positive cases showed a consistent (moderate to strong and diffuse) expression of *ROS1* protein.^{111-113,117-120} On one side, all FISH-positive NSCLC are well-recognized by IHC assay reaching 100% of sensitivity, but a subset of non-rearranged tumors are IHC-positive leading to a moderate specificity (from 80% to 95%).^{111-113,117-120} This is particularly true when quoting as positive also tumor showing weak immunoreactivity (score 1+) in a limited fraction of tumor cells. Adopting more rigorous and solid scoring systems considering IHC-positivity only tumors with moderate-to-strong intensity signal (score 2+/3+) in more than 50% of tumor cells, the specificity of *ROS1* clone D4D6 becomes quite consistent (>95%).¹¹⁷⁻¹²⁰

While different previous studies have demonstrated the validity of IHC method in terms of specificity and sensitivity to identify *ROS1*-rearranged NSCLC, a recent work by Cha et al.¹¹⁹ highlighted some false positive tumors in ever-smokers. By contrast, Boyle et al.¹¹¹ recently demonstrated that *ROS1* IHC assay has an absolute sensitivity and specificity when compared with

FISH and reverse transcriptase polymerase chain reaction (RT-PCR) using an optimized scoring system (H-score >100) (**Figure 6**).

Taking into account all previous data, IHC screening with ROS1 clone D4D6 seems to be reasonably robust to be adopted as a practical, rapid and cost-effective screening tool in detecting *ROS1*-rearranged NSCLC, then limiting the number of cases to undergo confirmatory FISH assay.

4. Others (*BRAF* mutations, *RET* rearrangement, *HER2* mutations, c-MET expression)

BRAF mutations occur in 1-3% of patients with lung adenocarcinoma and occur more commonly in current and former smokers.^{121,122} *BRAF* encodes for a nonreceptor serine/threonine kinase, activated downstream of the Ras protein. The great majority of *BRAF* mutations occur in the hotspot transversion mutation T1799A at exon 15, leading to the amino acidic substitution of V600E.^{121,122} However, in NSCLC a wide range of other missense mutations (non-V600E) have been detected in exons 11 and 15.^{121,122} The incidence of *BRAF* mutations other than V600E is significantly higher in lung cancer than in other extrapulmonary malignancies.^{121,122}

The reported *BRAF* mutations by Paik et al.¹²¹ were V600E (50%), G469A (39%), and D594G (11%). In the work by Marchetti et al.¹²² 56.8% mutations were V600E, and 43.2% were non-V600E. All non-V600E mutations were found in smokers.

The V600E mutation-specific antibody (clone VE1) has been recently developed.¹²³ This clone perfectly match with V600E *BRAF* mutations in melanoma and papillary thyroid carcinoma.

There is a unique study by Ilie et al.¹²⁴ demonstrating VE1 expression in 19 out of 21 (90%) *BRAF* V600E mutated adenocarcinomas. As expected, this mutant-specific antibody failed to stain all other 10 non-V600E mutations identified in the work.

HER2/EGFR-2 mutations characterize a subset of NSCLC (about 3%), particularly in non-smoking younger patients with adenocarcinoma subtype.¹²⁵

To date, most of *HER2* mutations detected in NSCLC were in-frame insertions of exon 20 with duplication of amino acids YVMA at codon 775 (A775_G776insYVMA).^{125,126} However, neither FISH test nor IHC consistently matched with *HER2* mutations, then appearing inconsistent tools in predicting *HER2* mutated adenocarcinomas. Given the similarity of mutational events, it could be that a mutant-specific antibody will be developed in the next future to identify *HER2* mutations in lung adenocarcinoma at IHC level.

The *RET* (rearranged during transfection) proto-oncogene is located at 10q11.2 and encodes a receptor tyrosine kinase.¹²⁷ The *RET* protein is lacking in lung tissue and *RET* rearrangement has been recently identified in NSCLC.¹²⁸⁻¹³³

A subset (1-2%) of lung adenocarcinomas harbour fusion of the *KIF5B* (kinesin family member 5B) gene to the *RET* (rearranged during transfection) gene or other *RET* gene fusions, namely *TRIM33-RET*, *NCOA4-RET*, and *CCDC6-RET*. A clinical response has been demonstrated using *RET* inhibitors in *RET* fusion-positive lung adenocarcinomas.^{134,135}

In a recent study, Lee et al.¹³³ showed that 16% (15 out of 94) of *EGFR/KRAS/ALK* triple-negative lung adenocarcinomas harboured *RET* rearrangement. In contrast to previous studies,^{129,132} the authors demonstrated moderate-to-strong immunoreactivity for *RET* at IHC (clone 134100, Abcam) in all 15 *RET* fusion-positive cases (100% of sensitivity), while a focal staining was observed also in 10 out of 79 (12%) *RET* fusion-negative cases (89% of specificity).

Adenocarcinoma of the lung harboring *RET*-rearrangement seem to have a peculiar morphology with cribriform pattern, lymphangitic spread and psammoma bodies.¹³⁶

While the phase III trial with Ornatuzumab (Genentech/Roche) failed to demonstrate a significant prolonged progression-free survival in the group of patients treated with Ornatuzumab plus

erlotinib versus erlotinib alone, IHC with the clone SP44 still remain the most promising biomarker in predicting clinical response to humanized monoclonal antibody MetMAb, particularly when tumor express MET with at least 2+ intensity in more than 50% of tumor cells.¹³⁷

At the moment, FISH assay demonstrated a poor prognosis in amplified NSCLC, but it is inferior to IHC in this setting and *c-MET* activating mutations does occur only in sporadic NSCLC.

Closing remarks

- DNA sequencing and FISH assay are universally considered the gold-standard for selecting patients for TKI therapy, although often characterized by long turn-around time, tissue insufficiency, difficulty in interpretation and equivocal results
- IHC represents a fast and cost-effective “in-situ” proteomic assay that can reversely and effectively detect mutations and rearrangements
- EGFR mutant-specific antibodies have a high specificity (strongly expression/score 3+ does not require molecular confirmation) but low/fair sensitivity (negative staining requires molecular testing, also microdissecting IHC-stained tumor cells)
- IHC is a cost-effective widely available method that could be used to screen patients with lung cancer for ALK and ROS1 rearrangements
- Standardized protocols clearly defining the best antibody clone, detection kit and scoring system to be adopted should be organized and widely shared
- Practical algorithms including IHC screening for ALK and ROS1 and subsequent FISH assay confirmation actually represent the best way in terms of adequacy, rapidity and cost-effectiveness to specifically identify tumors harbouring these genetic rearrangements without losing patients with equivocal FISH setup

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Figure Legend

Figure 1. A case of liver metastasis from lung adenocarcinoma (**A**, H&E stain) harboring exon 19 E746-A750 del EGFR mutation detected using mutant-specific antibody (**B** and insert, immunohistochemistry with clone 6B6) and negative for mutant-specific antibody against L858 EGFR mutation (**C**, immunohistochemistry, clone 43B2)

Figure 2. A lung adenocarcinoma diagnosed on cell block (**A**, H&E stain) expressing mutant-specific antibody against L858 EGFR mutation (**B**, immunohistochemistry, clone 43B2), but not with mutant-specific antibody against exon 19 E746-A750 del EGFR mutation (**C**, immunohistochemistry with clone 6B6).

Figure 3. Pulmonary adenocarcinoma (**A**, H&E stain) strongly expressing ALK protein with clone D5F3 (**B**, immunohistochemistry) and clone 5A4 (**C**, immunohistochemistry).

Figure 4. A small aggregate of tumor cells (< than 50 did not permit a reliable FISH test) of adenocarcinoma in a bronchial biopsy (**A**, H&E stain) strongly expressing ALK (**B**, clone ALK1 immunohistochemistry). Few tumor cells of adenocarcinoma intermingled with inflammatory cells in a cell block (**C**, H&E stain) and bone metastasis from adenocarcinoma (**D**, H&E stain) may complicate determination of ALK rearrangement by FISH test).

Figure 5. A poorly-differentiated lung adenocarcinoma showing a weak positivity for ALK (clone ALK1, immunohistochemistry) resulted ALK rearranged at FISH assay (insert).

Figure 6. A lung adenocarcinoma (**A**, H&E stain) consistently expressing ROS1 (**B**, clone D4D6 immunohistochemistry).

Table 1. Summary of immunohistochemical antibodies possibly acting as surrogates to recognize predictive biomarkers conventionally detected by DNA sequencing or FISH

Antibody	Clone	Biomarker	Main Pro	Main Cons	Validation in clinical practice
EGFR	6B6	EGFR mutations	- rapid - application poor material - low cost	- restricted to exon 19 delE746-A750 - not clinically validated	Not yet
EGFR	43B2	EGFR mutations	- rapid - application on poor material	- restricted to exon 21 L858R - not clinically validated	Not yet
ALK	ALK1	ALK rearrangement	- rapid - application on poor material - screening tool - good specificity - low cost	- low/moderate sensitivity - not clinically validated	Possible use as screening method
ALK	5A4	ALK rearrangement	- rapid - application on poor material - screening tool - good sensitivity & specificity - low cost	- not clinically validated	Possible use as screening method
ALK	D5F3	ALK rearrangement	- rapid - application on poor material - screening tool - high sensitivity & specificity - low cost	- not clinically validated	Possible use as screening method
ROS1	D4D6	ROS1 rearrangement	- rapid - application on poor material - screening tool - high sensitivity & specificity - low cost	- not clinically validated	Possible use as screening method
BRAF	VE1	BRAF mutations	- rapid - application on poor material - screening tool - low cost	- restricted to exon 15 V600E - limited experience in lung cancer - not clinically validated	Not yet
RET	-	RET rearrangement		- not clinically validated	Not yet

Table 2. Summary of all previous studies using EGFR E746-A750 deletion specific (clone 6B6) and EGFR L858R mutant-specific (clone 43B2) (Cell Signaling Technology, Inc.)

Reference	Case n	Molecular test of comparison	Sensitivity & Specificity Exon 19	Sensitivity & Specificity Exon 21	Overall Sensitivity & Specificity
Yu et al. 2009	40 adc (h)	Direct Sequencing	N.A. N.A.	N.A. N.A.	92% 99%
Brevet et al. 2010	218 adc (h/TMA)	PCR-RFLP	85% 99%	95% 99%	75% 99%
Simonetti et al. 2010	78 NSCLC (h)	Direct Sequencing	63% 100%	93% 100%	78% 100%
Kawahara et al. 2010	60 NSCLC (h)	Direct Sequencing	77% 100%	91.5% 100%	85% 100%
Nakamura et al. 2010	20 adc (h)	PNA-LNA	90% 100%	100% 100%	90% 100%
Kato et al. 2010	70 NSCLC (h/TMA)	Direct Sequencing	50% 100%	75% 97%	44% 99%
Ilie et al. 2010	61 adc (h/TMA)	Direct Sequencing	89% N.A.	No exon 21 mutations	89% N.A.
Ambrosini-Spaltro et al. 2012	33 adc (h)	Direct Sequencing	50% 100%	83% 100%	61% 100%
Tsai et al. 2012	78 (cell block)	Direct sequencing	58% 95%	71 86%	69% 79%
Hasanovic et al. 2012	145 adc (c + core biopsy and decalcified bone biopsies)	Rapid PCR-based method	N.A.	N.A.	- 100%
Kawahara et al. 2011	24 adc (c)	Direct Sequencing	100% 100%	100% 100%	100% 100%
Kitamura et al. 2010	238 NSCLC (h/TMA)	Direct Sequencing	40% 99%	36% 97%	47% 96%
Kozu et al. 2010	577 adc (h)	HRMA	42% 99.5%	76% 98%	63% 70%
Wu et al. 2011	143 adc (h)	Direct Sequencing	73% 95%	88% 77%	83% 75%
Fan et al 2013	169 adc (h)	Direct Sequencing	74% 99%	93% 100%	-
Jiang et al.	399 NSCLC (h/c)	TaqMan PCR assay	80% 85%	76% 92%	-

Hofman et al. 2012	154 adc (h)	Direct Sequencing	55% 97%	24% 98%	-
Ho et al. 2013	445 adc (h)	Direct Sequencing ARMS	57% 100%	68% 95%	-
Cooper et al. 2013	204 adc (h)	Sequenom	100% 98%	86% 98.5%	-
Xiong et al. 2013	50 adc (h)	QIAamp	59% 100%	81% 97%	-
Seo et al. 2014	240 adc (h)	Direct Sequencing / Pyrosequencing	80.5% 90%	71% 99%	-
Ping W et al. 2014	215 adc (h)	Direct Sequencing	N.P.	88% 100%	-
Bondgaard et al. 2014	210 NSCLC (h)	TheraScreen	63% 99%	80% 98%	-
Allo et al. 2014	247 adc (h)	Sequenom Direct Sequencing	83%	76%	-
Houang et al. 2014	326 non-squamous (h)	Direct Sequencing Sequenom Cobas-Roche	63% 100%	96% 92%	58% 92%
Wang et al. 2014	115 NSCLC (h/c)	ARMS	98.5% 100%	100% 100%	-

Abbreviations: adc, adenocarcinoma; NSCLC, non-small cell lung cancer; h, histologic samples; c, cytology; TMA, tissue microarray; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism assay; HRMA, high resolution melting analysis; PNA-LNA, peptide nucleic-acid locked nuclei acid-PCR; N.A., not available; N.P., not performed.

Table 3. Summary of studies comparing ALK detection by IHC and FISH

Reference	Cases n	Antibody Clone (Source)	Detection System	% FISH + & % IHC +	Sensitivity	Specificity
Boland et al. 2009	185 adc (h) 150 sqc (h)	ALK1 (Dako)	-	1.8%/1.8%	100%	100%
Mino-Kenudson et al. 2010	153 adc (h)	D5F3 (CST) ALK1 (Dako)	EnVision Flex+	14.3% / n.a.	100% 67%	99% 97%
McLeer-Florin et al. 2011	441 adc (h)	5A4 (Novocastra)	Ultraview	n.a. / 6.5%	95%	100%
Paik et al. 2011	465 NSCLC (395 adc) (h)	5A4 (Novocastra)	Ultra-view	3.8% / 7.5% 6.8% (adc)	100%	96%
Yi et al. 2011	101 adc (h)	ALK1 (Dako)	Advance	10% / 11%	90%	98%
Paik et al. 2012	735 NSCLC (h)	5A4 (Novocastra)	Ultraview	3.8%/7.5%	100%	96%
Rimkunas et al. 2012	556 NSCLC (h)	D5F3 (CST)	EnVision Flex+	4%/4%	100%	100%
Park et al. 2012	262 non-sqc (h)	5A4 (Novocastra)	Bond Polymer Refine	9.5%/10.7%	100%	99%
Martinez et al. 2013	80 NSCLC (h/c)	D5F3 (CST)	Ultraview	7%	83%	100%
Selinger et al. 2013	594 NSCLC (h)	ALK1 (Dako) 5A4 (Novocastra) D5F3 (CST)	Envision Flex + Ultraview Optiview	1%	100%	99%
Zhang et al. 2013	173 NSCLC (h)/selected	D5F3 (CST)	-	35.5%/35.7%	100%	100%
Sholl et al. 2013	186 adc (h)	5A4 (Novocastra)	EnVision Flex +	8%	93%	100%
Tuononen et al. 2013	87 NSCLC (h)	5A4 (Novocastra)	Optiview	6%/6%	100%	100%
Han et al. 2013	139 non-sqc (h) selected	D5F3 (CST)	-	32.6%/33%	98%	97%
Ying et al. 2013	196 adc (h) selected	D5F3 (CST)	Optiview	32%/33%	100%	98%
Minca et al. 2013	265 NSCLC (h/c)	D5F3 (CST)	Optiview	12.8%	100%	100%
Takamochi et al. 2013	360 NSCLC (h)	5A4 (Novocastra)	iAEP	3%	100%	100%

Savic et al. 2013	41 NSCLC (c) selected	5A4 (Novocastra)	Bond Polymer Refine	37.5%	93%	96%
To et al. 2013	373 adc (h)	5A4 (Novocastra)	-	6%	100%	100%
Conklin et al. 2013	273 NSCLC (h)	5A4 (Novocastra) D5F3 (CST) ALK1 (Dako)	Advance Advance Envision Flex +	4%/4%	100% 100% 66%	87.5% 75% 100%
Sullivan et al. 2013	110 NSCLC (h/c)	5A4 (Novocastra)	EnVision Flex +	6.4%/10%	100%	96%
Houang et al. 2014	256 NSCLC (h/c)	5A4 (Novocastra)	-	4%	100%	100%
Cabillic et al. 2014	3244 NSCLC (h)	5A4 (Novocastra)	Ultraview	4.6%	69%	83%
Conde et al. 2014	103 NSCLC (h) selected	5A4 (Novocastra) D5F3 (CST)	Optiview	-	98%	100%
Demidova et al. 2014	46 NSCLC (h) selected	D5F3 (CST) 5A4 (Novocastra) ALK1 (Dako)	Bond Polymer Refine	-	100% 91% 91%	100% 100% 100%
Hutarew et al. 2014	303 adc (h)	ALK1 (Dako) 5A4 (Novocastra) D5F3 (CST) SP8 (Abcam)	EnVision Flex EnVision Flex Optiview EnVision Flex	4.5%	50% 100% 100% 65%	100% 97% 100% 90%
Ali et al. 2014	523 NSCLC (h)	D5F3 (CST)	OptiView	3.8%/3.4%	90%	100%
Wynes et al. 2014	100 NSCLC (h) selected	D5F3 (CST)	Optiview	-	90%	95%
Cha et al. 2014	330 adc (h)	D5F3 (CST)	OptiView	6%/7%	100%	100%
Wang et al. 2014	430 adc (h)	D5F3 (CST)	Optiview	10.7%/12.3%	100%	98%
Personal experience	154 adc (h/c)	ALK1 (Ventana)	Optiview	7.8%/11.8%	100%	96%

Abbreviations: adc, adenocarcinoma; NSCLC, non-small cell lung cancer; sqc, squamous cell carcinoma ; CST, Cell Signaling Technology; h, histology; c, cytology or cell block; IHC, immunohistochemistry; FISH, fluorescence in situ hybridization.

Table 4. Summary of studies comparing ROS1 detection by IHC and FISH

Reference	Cases n	Antibody Clone (Source)	Detection System	% FISH + & % IHC +	Sensitivity	Specificity
Rimkunas et al. 2012	556 NSCLC (h)	D4D6 (CST)	EnVision Flex+	1.6%/1.6%	100%	100%
Yoshida et al. 2014	270 adc (h) TMA	D4D6 (CST)	EnVision Flex+	-	94%	98%
Sholl et al. 2013	220 adc (h)	D4D6 (CST)	-	1.2%/1.2%	100%	92%
Mescam-Mancini et al. 2014	121 adc (h)	D4D6 (CST)	UltraView	7.5% selected wild-type cases	100%	98%
Cha et al. 2014	330 adc (h)	D4D6 (CST)	OptiView	4%/8%	100%	98%

Abbreviations: adc, adenocarcinoma; NSCLC, non-small cell lung cancer; sqc, squamous cell carcinoma ; CST, Cell Signaling Technology; h, histology; c, cytology or cell block; IHC, immunohistochemistry; FISH, fluorescence in situ hybridization; TMA, tissue microarray.