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

Giulio Rossi,1 Moira Ragazzi,2 Ione Tamagnini,2 Maria Cecilia Mengoli,1 Giada Vincenzi,3 Fausto Barbieri,4 Silvia Piccioli,1 Alessandra Bisagni,2 Tiziana Vavalà,5 Luisella Righi,5 Silvia Novello,5 Francesco Gelsomino,6 and Marcello Tiseo7

1 Section of Pathologic Anatomy, University Hospital Policlinico of Modena, Modena, Italy; 2 Operative Unit of Pathologic Anatomy, Arcispedale S. Maria Nuova / I.R.C.C.S., Reggio Emilia, Italy; 3 Department of Life Sciences, University of Modena and Reggio Emilia, Modena, Italy; 4 Division of Medical Oncology, University Hospital Policlinico of Modena, Modena, Italy; 5 Department of Oncology, University of Torino at San Luigi Hospital, Orbassano, Torino, Italy; 6 Dept. Medical Oncology 1, Fondazione IRCCS - Istituto Nazionale dei Tumori, Milan, Italy; 7 Division of Medical Oncology University Hospital, Parma, Italy

Key words: lung cancer; immunohistochemistry; EGFR; ALK; ROS1; molecular biology; gene mutation

Short title: Predictive immunohistochemistry in NSCLC

Corresponding author: Dr. Giulio Rossi, Section of Pathologic Anatomy, University Hospital Policlinico of Modena, Via del Pozzo, 71- 41124 Modena (Italy); Phone: +39.059.4223890; Fax: +39.059. 4224998; email: giurossi68@gmail.com
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.\textsuperscript{1,2} The presence of these genetic alterations is the unique most important finding in predicting effectiveness of specific targeted therapies.\textsuperscript{3} Most important, patients with adenocarcinomas harbouring epidermal growth factor receptor (\textit{EGFR}) mutations or Anaplastic Lymphoma Kinase (\textit{ALK}) and \textit{ROS1} rearrangements have a significant disease-free survival when compared with conventional chemotherapy.\textsuperscript{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.\textsuperscript{11} Of note, some “companion diagnostics” have been approved differently in various countries.\textsuperscript{12-14}

In brief, approximately 10% to 40% of lung adenocarcinomas (mainly depending on ethnicity with highest frequency in Asiatic patients) harbor a \textit{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).\textsuperscript{1,2} Less common mutations predicting a minor clinical response to \textit{EGFR} tyrosine kinase inhibitors (TKI) occur in exons 18 and 20.\textsuperscript{15-17} Overall, \textit{EGFR} mutations are the best predictors of efficacy when adopting selective TKIs, namely gefitinib, erlotinib and afatinib.\textsuperscript{4-6} Several PCR-based DNA extractive methods are available and accepted to detect \textit{EGFR} mutations, although there is a wide range in terms of sensitivities among various methodologies, significantly leading to quite different results.\textsuperscript{13,14,18,19} About 5-7% of lung adenocarcinomas harbor a rearrangement of \textit{ALK} gene, particularly through an intrachromosomal inversion with \textit{EML4-ALK} gene and other genes, then leading to a fusion product associated with \textit{ALK} protein overexpression.\textsuperscript{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.\textsuperscript{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.\textsuperscript{7-9}

More recently, the rearrangement of ROS1 gene has been identified in a small subset (about 1-2\%) of lung adenocarcinomas.\textsuperscript{21} Detection of ROS1 rearrangement in the recent clinical trial by Shaw et al.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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. \textit{EGFR} mutations

The finding of an activating \textit{EGFR} mutation in NSCLC is the best single predictor of efficacy using selective tyrosine kinase inhibitors (TKI), while total \textit{EGFR} expression at immunohistochemistry and \textit{EGFR} FISH assay did not demonstrate to be reliable predicting biomarkers. Various activating \textit{EGFR} mutations have been reported in exons 18, 19, 20 and 21, but about 90\% of \textit{EGFR} mutations in NSCLC involve in-frame deletions of exon 19 (E746-A750 del and variants) and point mutation L858R in exon 21. These mutations are also significantly related to a better and prolonged clinical response to \textit{EGFR} TKI (particularly exon 19 deletions), 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 \textit{EGFR} mutations and represent validated techniques in all guidelines proposed from different scientific societies in various countries.

Detection of \textit{EGFR} mutations significantly increase when using more sensitive detection methodologies. Supporting this view, Won et al. recently demonstrated the presence of additional activating \textit{EGFR} mutations when passing from direct sequencing to mutant-enriched Next Generation Sequencing (NGS) in a population of \textit{ALK}-positive tumors, finally evidencing \textit{EGFR} mutations in 15.4\% of \textit{ALK}-rearranged NSCLC (14 out of 91). The presence of minor clones of \textit{EGFR} mutated tumor cells in \textit{ALK}-positive NSCLC might explain the occurrence of dual-positive tumors and the different detection rate of \textit{EGFR} mutations due to variable sensitivity of various methods used in the study. Again, Zhou et al. detected 30\% of false negative \textit{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.\textsuperscript{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 \textit{EGFR} mutations. However, discordant results have been reported in different studies investigating the adequacy of IHC in detecting \textit{EGFR} mutations when compared with standard DNA sequencing methods.\textsuperscript{27-53}

Provided that in normal routine practice DNA sequencing analysis should always be considered the gold standard,\textsuperscript{54} the main advantages to use mutation-specific antibodies in detecting \textit{EGFR} mutations may be the following: 1. in critical patients requiring a rapid determination of \textit{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 \textit{EGFR} mutations different from E746-A750del and L858R.

Basically, the sensitivity of mutant-specific antibodies to detect all \textit{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. 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.

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, 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). 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 ≥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 delection or inversion of ALK gene, respectively). Although clinically validated, FISH test is laborious, relatively expensive and requires a good expertise. In addition, a not
The insignificant rate of tumors also show borderline results with 10-14% of positive cells. 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.

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 that that observed in ALK-lymphomas. 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).

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.

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. 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, while the clone D5F3 has been included in the validated test by Ventana Medical System, Inc. and confirmed in several works.

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. 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. 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. 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.\textsuperscript{76,106,108}

Selinger et al.\textsuperscript{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.\textsuperscript{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) (\textbf{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%),\textsuperscript{76} similarly to what observed in the study by McLeer-Florin et al.\textsuperscript{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 (\textbf{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.\textsuperscript{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.\textsuperscript{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. \textit{ROS1}

\textit{ROS1} is a receptor tyrosine kinase involved in tumor cancerogenesis of different tumors. The rearrangement of \textit{ROS1} with other fusion genes, such as \textit{CD74}, \textit{FIG}, \textit{EZR} and solute carrier protein
34A2 (SLC34A2), is the oncogenic driver in a subset (1-2%) of NSCLC, particularly of adenocarcinoma subtype. Identification of patients with NSCLC harbouring \textit{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. However, as with ALK, all \textit{ROS1} rearrangements may be easily detected at the immunohistochemical level with the specific rabbit monoclonal antibody D4D6. \textit{ROS1} expression seems to be mutually exclusive with \textit{ALK}, but a subgroup of ALK and ROS1 positive NSCLC may show \textit{EGFR} activating mutations detected in the same tumor cells using molecular techniques and EGFR mutant-specific antibodies.

Recent studies have confirmed the validity of the clone D4D6 to detect \textit{ROS1} rearrangement when IHC assay was compared to FISH test (Table 4). Basically, all ROS1 FISH-positive cases showed a consistent (moderate to strong and diffuse) expression of ROS1 protein. 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%). 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%).

While different previous studies have demonstrated the validity of IHC method in terms of specificity and sensitivity to identify \textit{ROS1}-rearranged NSCLC, a recent work by Cha et al. highlighted some false positive tumors in ever-smokers. By contrast, Boyle et al. 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.\textsuperscript{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).\textsuperscript{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.\textsuperscript{127} The RET protein is lacking in lung tissue and RET rearrangement has been recently identified in NSCLC.\textsuperscript{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.\textsuperscript{134,135}

In a recent study, Lee et al.\textsuperscript{133} showed that 16\% (15 out of 94) of EGFR/KRAS/ALK triple-negative lung adenocarcinomas harboured RET rearrangement. In contrast to previous studies,\textsuperscript{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 crobroform pattern, lymphangitic spread and psammoma bodies.\textsuperscript{136}

While the phase III trial with Ornatumzumab (Genentech/Roche) failed to demonstrate a significant prolonged progression-free survival in the group of patients treated with Ornatumzumab 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.\textsuperscript{137}

At the moment, FISH assay demonstrated a poor prognosis in amplified NSCLC, but it is inferior to IHC in this setting and \textit{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 loosing patients with equivocal FISH setup.
References


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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

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Biomarker</th>
<th>Main Pro</th>
<th>Main Cons</th>
<th>Validation in clinical practice</th>
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<td>6B6</td>
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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.)

<table>
<thead>
<tr>
<th>Reference</th>
<th>Case n</th>
<th>Molecular test of comparison</th>
<th>Sensitivity &amp; Specificity Exon 19</th>
<th>Sensitivity &amp; Specificity Exon 21</th>
<th>Overall Sensitivity &amp; Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yu et al. 2009</td>
<td>40 adc (h)</td>
<td>Direct Sequencing</td>
<td>N.A.</td>
<td>N.A.</td>
<td>92%</td>
</tr>
<tr>
<td>Brevet et al. 2010</td>
<td>218 adc (h/TMA)</td>
<td>PCR-RFLP</td>
<td>85%</td>
<td>95%</td>
<td>75%</td>
</tr>
<tr>
<td>Simonetti et al. 2010</td>
<td>78 NSCLC (h)</td>
<td>Direct Sequencing</td>
<td>63%</td>
<td>93%</td>
<td>78%</td>
</tr>
<tr>
<td>Kawahara et al. 2010</td>
<td>60 NSCLC (h)</td>
<td>Direct Sequencing</td>
<td>77%</td>
<td>91.5%</td>
<td>85%</td>
</tr>
<tr>
<td>Nakamura et al. 2010</td>
<td>20 adc (h)</td>
<td>PNA-LNA</td>
<td>90%</td>
<td>100%</td>
<td>90%</td>
</tr>
<tr>
<td>Kato et al. 2010</td>
<td>70 NSCLC (h/TMA)</td>
<td>Direct Sequencing</td>
<td>50%</td>
<td>75%</td>
<td>44%</td>
</tr>
<tr>
<td>Ilie et al. 2010</td>
<td>61 adc (h/TMA)</td>
<td>Direct Sequencing</td>
<td>89%</td>
<td>N.A.</td>
<td>89%</td>
</tr>
<tr>
<td>Ambrosini-Spaltro et al. 2012</td>
<td>33 adc (h)</td>
<td>Direct Sequencing</td>
<td>50%</td>
<td>83%</td>
<td>61%</td>
</tr>
<tr>
<td>Tsai et al. 2012</td>
<td>78 (cell block)</td>
<td>Direct Sequencing</td>
<td>58%</td>
<td>71%</td>
<td>69%</td>
</tr>
<tr>
<td>Hasanovic et al. 2012</td>
<td>145 adc (c + core biopsy and decalcified bone biopsies)</td>
<td>Rapid PCR-based method</td>
<td>N.A.</td>
<td>N.A.</td>
<td>- 100%</td>
</tr>
<tr>
<td>Kawahara et al. 2011</td>
<td>24 adc (c)</td>
<td>Direct Sequencing</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Kitamura et al. 2010</td>
<td>238 NSCLC (h/TMA)</td>
<td>Direct Sequencing</td>
<td>40%</td>
<td>36%</td>
<td>47%</td>
</tr>
<tr>
<td>Kozu et al. 2010</td>
<td>577 adc (h)</td>
<td>HRMA</td>
<td>42%</td>
<td>76%</td>
<td>63%</td>
</tr>
<tr>
<td>Wu et al. 2011</td>
<td>143 adc (h)</td>
<td>Direct Sequencing</td>
<td>73%</td>
<td>88%</td>
<td>83%</td>
</tr>
<tr>
<td>Fan et al 2013</td>
<td>169 adc (h)</td>
<td>Direct Sequencing</td>
<td>74%</td>
<td>93%</td>
<td>-</td>
</tr>
<tr>
<td>Jiang et al. 2013</td>
<td>399 NSCLC (h/c)</td>
<td>TaqMan PCR assay</td>
<td>80%</td>
<td>76%</td>
<td>-</td>
</tr>
<tr>
<td>Study</td>
<td>Tumor Type</td>
<td>Sample Size</td>
<td>Methodology</td>
<td>Sensitivity</td>
<td>Specificity</td>
</tr>
<tr>
<td>----------------------</td>
<td>------------</td>
<td>-------------</td>
<td>-------------------------------------------------</td>
<td>-------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Hofman et al. 2012</td>
<td>adc (h)</td>
<td>154</td>
<td>Direct Sequencing</td>
<td>55%</td>
<td>97%</td>
</tr>
<tr>
<td>Ho et al. 2013</td>
<td>adc (h)</td>
<td>445</td>
<td>Direct Sequencing</td>
<td>57%</td>
<td>100%</td>
</tr>
<tr>
<td>Cooper et al. 2013</td>
<td>adc (h)</td>
<td>204</td>
<td>Sequenom</td>
<td>100%</td>
<td>98%</td>
</tr>
<tr>
<td>Xiong et al. 2013</td>
<td>adc (h)</td>
<td>50</td>
<td>QIAamp</td>
<td>59%</td>
<td>100%</td>
</tr>
<tr>
<td>Seo et al. 2014</td>
<td>adc (h)</td>
<td>240</td>
<td>Direct Sequencing / Pyrosequencing</td>
<td>80.5%</td>
<td>90%</td>
</tr>
<tr>
<td>Ping W et al. 2014</td>
<td>adc (h)</td>
<td>215</td>
<td>Direct Sequencing</td>
<td>N.P.</td>
<td>88%</td>
</tr>
<tr>
<td>Bondgaard et al. 2014</td>
<td>NSCLC (h)</td>
<td>210</td>
<td>TheraScreen</td>
<td>63%</td>
<td>99%</td>
</tr>
<tr>
<td>Allo et al. 2014</td>
<td>adc (h)</td>
<td>247</td>
<td>Sequenom</td>
<td>83%</td>
<td>76%</td>
</tr>
<tr>
<td>Houang et al. 2014</td>
<td>non-squamous (h)</td>
<td>326</td>
<td>Direct Sequencing / Sequenom Cobas-Roche</td>
<td>63%</td>
<td>100%</td>
</tr>
<tr>
<td>Wang et al. 2014</td>
<td>NSCLC (h/c)</td>
<td>115</td>
<td>ARMS</td>
<td>98.5%</td>
<td>100%</td>
</tr>
</tbody>
</table>

**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

<table>
<thead>
<tr>
<th>Reference</th>
<th>Cases n</th>
<th>Antibody Clone (Source)</th>
<th>Detection System</th>
<th>% FISH + &amp; % IHC +</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boland et al. 2009</td>
<td>185 adc (h)</td>
<td>ALK1 (Dako)</td>
<td>-</td>
<td>1.8%/1.8%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Mino-Kenudson et al. 2010</td>
<td>153 adc (h)</td>
<td>D5F3 (CST) ALK1 (Dako)</td>
<td>EnVision Flex+</td>
<td>14.3% / n.a.</td>
<td>100%</td>
<td>99%</td>
</tr>
<tr>
<td>McLeer-Florin et al. 2011</td>
<td>441 adc (h)</td>
<td>5A4 (Novocastra)</td>
<td>Ultraview</td>
<td>n.a. / 6.5%</td>
<td>95%</td>
<td>100%</td>
</tr>
<tr>
<td>Paik et al. 2011</td>
<td>465 NSCLC (395 adc (h))</td>
<td>5A4 (Novocastra)</td>
<td>Ultra-view</td>
<td>3.8% / 7.5%</td>
<td>100%</td>
<td>96%</td>
</tr>
<tr>
<td>Yi et al. 2011</td>
<td>101 adc (h)</td>
<td>ALK1 (Dako)</td>
<td>Advance</td>
<td>10% / 11%</td>
<td>90%</td>
<td>98%</td>
</tr>
<tr>
<td>Paik et al. 2012</td>
<td>735 NSCLC (h)</td>
<td>5A4 (Novocastra)</td>
<td>Ultraview</td>
<td>3.8%/7.5%</td>
<td>100%</td>
<td>96%</td>
</tr>
<tr>
<td>Rimkusnas et al. 2012</td>
<td>556 NSCLC (h)</td>
<td>D5F3 (CST)</td>
<td>EnVision Flex+</td>
<td>4%/4%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Park et al. 2012</td>
<td>262 non-sqc (h)</td>
<td>5A4 (Novocastra)</td>
<td>Bond Polymer Refine</td>
<td>9.5%/10.7%</td>
<td>100%</td>
<td>99%</td>
</tr>
<tr>
<td>Martinez et al. 2013</td>
<td>80 NSCLC (h/c)</td>
<td>D5F3 (CST)</td>
<td>Ultraview</td>
<td>7%</td>
<td>83%</td>
<td>100%</td>
</tr>
<tr>
<td>Selinger et al. 2013</td>
<td>594 NSCLC (h)</td>
<td>ALK1 (Dako) 5A4 (Novocastra) D5F3 (CST)</td>
<td>Envision Flex + Ultraview Optiview</td>
<td>1%</td>
<td>100%</td>
<td>99%</td>
</tr>
<tr>
<td>Zhang et al. 2013</td>
<td>173 NSCLC (h)/selected</td>
<td>D5F3 (CST)</td>
<td>-</td>
<td>35.5%/35.7%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Sholl et al. 2013</td>
<td>186 adc (h)</td>
<td>5A4 (Novocastra)</td>
<td>EnVision Flex +</td>
<td>8%</td>
<td>93%</td>
<td>100%</td>
</tr>
<tr>
<td>Tuononen et al. 2013</td>
<td>87 NSCLC (h)</td>
<td>5A4 (Novocastra)</td>
<td>Optiview</td>
<td>6%/6%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Han et al. 2013</td>
<td>139 non-sqc (h) selected</td>
<td>D5F3 (CST)</td>
<td>-</td>
<td>32.6%/33%</td>
<td>98%</td>
<td>97%</td>
</tr>
<tr>
<td>Ying et al. 2013</td>
<td>196 adc (h)</td>
<td>D5F3 (CST)</td>
<td>Optiview</td>
<td>32%/33%</td>
<td>100%</td>
<td>98%</td>
</tr>
<tr>
<td>Minca et al. 2013</td>
<td>265 NSCLC (h/c)</td>
<td>D5F3 (CST)</td>
<td>Optiview</td>
<td>12.8%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Takamochi et al. 2013</td>
<td>360 NSCLC (h)</td>
<td>5A4 (Novocastra)</td>
<td>Optiview</td>
<td>iAEP 3%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Study</td>
<td>Tissue Type</td>
<td>Target</td>
<td>Primary Stain</td>
<td>Bonding Method</td>
<td>IHC</td>
<td>FISH</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>-------------</td>
<td>----------</td>
<td>---------------</td>
<td>----------------</td>
<td>-------</td>
<td>--------</td>
</tr>
<tr>
<td>Savic et al. 2013</td>
<td>41 NSCLC (c) selected</td>
<td>5A4 (Novocastra)</td>
<td>Bond Polymer Refine</td>
<td>37.5%</td>
<td>93%</td>
<td>96%</td>
</tr>
<tr>
<td>To et al. 2013</td>
<td>373 adc (h)</td>
<td>5A4 (Novocastra)</td>
<td>-</td>
<td>6%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Conklin et al. 2013</td>
<td>273 NSCLC (h)</td>
<td>5A4 (Novocastra)</td>
<td>Advance</td>
<td>4%/4%</td>
<td>100%</td>
<td>87.5%</td>
</tr>
<tr>
<td>Sullivan et al. 2013</td>
<td>110 NSCLC (h/c)</td>
<td>5A4 (Novocastra)</td>
<td>EnVision Flex +</td>
<td>6.4%/10%</td>
<td>100%</td>
<td>96%</td>
</tr>
<tr>
<td>Houang et al. 2014</td>
<td>256 NSCLC (h/c)</td>
<td>5A4 (Novocastra)</td>
<td>-</td>
<td>4%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Cabilik et al. 2014</td>
<td>3244 NSCLC (h)</td>
<td>5A4 (Novocastra)</td>
<td>Ultraview</td>
<td>4.6%</td>
<td>69%</td>
<td>83%</td>
</tr>
<tr>
<td>Conde et al. 2014</td>
<td>103 NSCLC (h) selected</td>
<td>5A4 (Novocastra)</td>
<td>Optiview</td>
<td>-</td>
<td>98%</td>
<td>100%</td>
</tr>
<tr>
<td>Demidova et al. 2014</td>
<td>46 NSCLC (h) selected</td>
<td>D5F3 (CST)</td>
<td>Bond Polymer Refine</td>
<td>-</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Hutarew et al. 2014</td>
<td>303 adc (h)</td>
<td>ALK1 (Dako) 5A4 (Novocastra) D5F3 (CST)</td>
<td>EnVision Flex EnVision Flex</td>
<td>4.5%</td>
<td>50%</td>
<td>100%</td>
</tr>
<tr>
<td>Ali et al. 2014</td>
<td>523 NSCLC (h)</td>
<td>D5F3 (CST)</td>
<td>OptiView</td>
<td>3.8%/3.4%</td>
<td>90%</td>
<td>100%</td>
</tr>
<tr>
<td>Wynes et al. 2014</td>
<td>100 NSCLC (h) selected</td>
<td>D5F3 (CST)</td>
<td>Optiview</td>
<td>-</td>
<td>90%</td>
<td>95%</td>
</tr>
<tr>
<td>Cha et al. 2014</td>
<td>330 adc (h)</td>
<td>D5F3 (CST)</td>
<td>Optiview</td>
<td>6%/7%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Wang et al. 2014</td>
<td>430 adc (h)</td>
<td>D5F3 (CST)</td>
<td>Optiview</td>
<td>10.7%/12.3%</td>
<td>100%</td>
<td>98%</td>
</tr>
<tr>
<td>Personal experience</td>
<td>154 adc (h/c)</td>
<td>ALK1 (Ventana)</td>
<td>Optiview</td>
<td>7.8%/11.8%</td>
<td>100%</td>
<td>96%</td>
</tr>
</tbody>
</table>

**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

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

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.