

# MOLECULAR PATHOLOGY IN LUNG CANCER

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## Abstract

Increasing understanding of genomic changes in cancer is transforming the diagnosis and treatment of a subset of lung cancers. A significant proportion of lung adenocarcinomas harbour biologically relevant or targetable somatic genetic changes such as mutations, amplifications or translocations in a range of genes, including KRAS, EGFR, ALK, ROS1, MET and BRAF. This review highlights the key actionable somatic changes seen in lung cancer, with particular emphasis on epidermal growth factor receptor mutations and ALK gene rearrangements in adenocarcinoma, as well as identifying promising new targets in squamous cell carcinoma of the lung. Accurate and sensitive molecular testing is essential to ensure patients with this poor prognosis disease receive the correct therapy, but mutation testing in lung cancer poses particular challenges. As the majority of patients with lung cancer present with advanced disease that is unsuitable for resection, many biopsies submitted for molecular testing are small biopsies such as core biopsies and fine needle aspirate biopsies, often with only a very small amount of diagnostic material available for mutation analysis. This paper highlights the need for good communication between clinicians, radiologists and pathologists to ensure optimal samples for molecular testing and the benefits of testing for multiple genes in one assay.

While optimal treatment of lung cancer in the past depended largely on histological classification and tumour stage, advancements in understanding of the molecular pathology of lung cancer has revolutionised treatment strategies and drug development. A variety of oncogenic driver mutations have been identified in just over 50% of lung adenocarcinomas and are almost always exclusive of each other.<sup>1-3</sup> Tumours harbouring driver mutations are 'addicted' to the effects of the molecular aberration in an oncogene, which singularly drives tumour transformation by exclusively regulating critical downstream signalling pathways, making them key targets for molecular inhibition. Adenocarcinomas harbouring activating EGFR (epidermal growth factor receptor) mutations are sensitive to targeted EGFR-TKIs (tyrosine kinase inhibitors) such as gefitinib and erlotinib, while adenocarcinomas with anaplastic lymphoma kinase (ALK) or ROS1 gene rearrangements are sensitive to TKIs such as crizotinib.<sup>6</sup> These predictive molecular abnormalities have had a dramatic impact on pathologic assessment of non-small cell lung cancer (NSCLC) and established somatic gene mutation testing, as a routine part of lung cancer work-up for most patients with advanced stage disease. With the development of newer targeted agents and a greater understanding of the crucial role of molecular predictive markers, we can expect molecular pathology to play an increasing role in the diagnosis and management of lung cancer. Advances in the molecular understanding of lung cancer types other than adenocarcinomas, such as through the Cancer Genome Atlas Project comprehensive genomic characterisation of squamous cell carcinoma, has revealed potential molecular targets that may also impact other tumour types.<sup>7</sup>

## EGFR mutations in NSCLC

Epidermal growth factor receptor (also known as human EGFR or HER1) belongs to a family of tyrosine kinase receptors including EGFR, HER1, HER2/neu, HER3 and HER4, and consists of an extracellular ligand-binding domain, a membrane domain and an intracellular tyrosine kinase domain.<sup>8</sup> Upon ligand binding (by EGFR or TGF- $\alpha$ ), the receptor undergoes homo- or hetero-dimerisation and autophosphorylation of intracellular tyrosine residues within the activation loops of the catalytic tyrosine kinase domain. This leads to activation of a series of downstream cell signalling pathways involved in cell proliferation, survival, angiogenesis and metastasis, including the Ras-MAPK, PI3K-Akt and Jak-STAT pathways.

Activating mutations of the EGFR gene in lung cancer lead to markedly increased affinity for ATP and increased tyrosine kinase activity with disrupted auto-inhibition.<sup>11</sup> The TKIs gefitinib and erlotinib preferentially bind the ATP-binding pocket of mutant EGFR proteins displacing ATP, thereby inhibiting phosphorylation and activation of downstream signalling pathways.<sup>11-13</sup> A systematic review and meta-analysis of EGFR mutations as potential predictive markers for EGFR-TKI sensitivity, including 3101 patients with 1020 mutations from 59 eligible studies, demonstrated mutations were effective predictive biomarkers of patient response to TKI treatment.<sup>14</sup> A study combining patient data from predominantly western patients treated with EGFR-TKIs in five trials, found a response rate of 67% in patients harbouring sensitising EGFR mutations, with slightly better responses in patients with exon 19 deletions (compared to L858R mutations).<sup>15</sup> Determination of EGFR mutation status has therefore become standard practice when patients are being considered for EGFR-TKI treatment.

In Australia, and other western countries, activating mutations in the EGFR gene are found in approximately 10-15% of NSCLC patients, while in Asian populations the frequency is higher (30-40%). EGFR mutations are more common in patients who are younger, female gender and never-smokers.<sup>18-23</sup> While EGFR mutations are negatively correlated with increasing smoking history, they can occur at a lower frequency in patients who are current or ex-smokers. The reported better prognosis of patients with EGFR mutations may relate to their association with other favourable prognostic factors such as younger age and non-smoking status, as EGFR was not a significant prognostic factor in two large multivariate analyses. While there are distinct clinical features associated with EGFR mutations, a study combining patient data from several clinical trials found clinical features were inferior to EGFR mutation status at predicting response to treatment.<sup>15</sup>

Pathologically, EGFR mutations occur almost exclusively in adenocarcinomas, or lung cancer with an adenocarcinoma component including adenosquamous carcinomas, or more rarely, pleomorphic sarcomatoid carcinomas,<sup>30</sup> or combined small cell carcinoma with adenocarcinoma.<sup>31</sup> They have also been reported very rarely in EBV-associated lymphoepithelioma-like carcinomas.<sup>23</sup> Using microdissection techniques, EGFR mutations are generally found in both the squamous and glandular components of resected adenosquamous carcinoma. Small biopsies of metastatic adenosquamous carcinomas may show pure squamous cell carcinoma. A report of two such cases in never smokers demonstrated EGFR mutations in the squamous cell carcinoma component, as well as in the glandular component identified in other specimens.<sup>33</sup> It is therefore important that a biopsy diagnosis of squamous cell carcinoma in a never smoker should raise suspicion of an incompletely sampled adenosquamous carcinoma that could potentially harbour an EGFR mutation. Many large studies have found no EGFR mutations in squamous cell carcinomas, although others have reported them in a low proportion of squamous cell carcinomas, mostly in studies including small biopsy samples with little attention to histological diagnostic criteria. However, two cases of the sensitising EGFR mutation Leu861Gln were found in 178 squamous cell carcinomas (1.1%) that underwent comprehensive genome analysis as part of the Cancer Genome Atlas project, in which rigorous pathological assessment was undertaken.<sup>7</sup>

### Different EGFR mutations in NSCLC

Activating EGFR mutations occur in the kinase domain encoded by exons 18 to 21. The commonest EGFR mutations known to be sensitive to EGFR-TKIs occur in the ATP-binding loops of the kinase domain, namely exon 19 in frame deletions, exon 21 point mutations (L858R and L861Q) and the exon 18 point mutation G719X. Together, these alterations account for approximately 85-95% of EGFR mutations in NSCLC. In a comprehensive review of 2880 patients with 569 EGFR mutations, in frame deletions in exon 19, of which there are over 20 variants, accounted for almost 50% of all EGFR mutations.<sup>34</sup> The second commonest mutation is a single amino acid substitution of a leucine with arginine, resulting from a T to G substitution

in codon 858 in exon 21. These L858R mutations make up about 40% of EGFR mutations.<sup>34</sup>

EGFR mutations associated with primary resistance to EGFR-TKIs occur in approximately 5-10% of untreated adenocarcinomas. They mostly consist of insertions or duplications in exon 20, or the T790M mutation in exon 20 that can occur de novo, but is more commonly associated with acquired TKI resistance.<sup>36</sup> The T790M mutation restores the affinity of the EGFR receptor for ATP rather than TKIs,<sup>37</sup> thus conferring resistance to first generation TKIs. These primary resistance mutations can occur in isolation or in combination with more common sensitising EGFR mutations. In addition, genetic alterations in other genes that may coexist with activating EGFR mutations, such as PIK3CA mutations,<sup>1</sup> or rarely, primary MET amplification,<sup>38</sup> may circumvent sensitivity to TKIs by activating downstream signalling pathways.<sup>36</sup>

Acquired resistance occurs after an initial response to EGFR-TKI treatment and in 50% or more of cases is associated with development of a secondary EGFR mutation, usually T790M in exon 20,<sup>39-41</sup> (or selective expansion of previously undetected resistant clones). The T790M point mutation results in a single amino acid substitution of methionine for threonine in the ATP-binding pocket, which interferes with binding of EGFR-TKIs, in preference for ATP binding.<sup>37</sup> Second generation EGFR-TKIs with different binding sites to EGFR show potential to overcome this resistance mechanism.<sup>42</sup> The second commonest cause of acquired resistance results from MET oncogene amplification, which occurs in about 10-20% of cases and enables activation of the AKT pathway through ERBB3. More rarely, patients may relapse with small cell carcinoma. Strategies aimed at simultaneously inhibiting EGFR mutations associated with sensitivity and known resistance mechanisms are required to overcome the problem of acquired resistance.

In view of the range of possible activating mutations and the potential for coexistent sensitising and resistance EGFR mutations, it is important comprehensively assess the EGFR kinase domain including exons 18 to 21.

### EGFR mutation detection techniques

A variety of molecular genetic techniques may be used to identify EGFR (and other mutations) in lung cancer and they all have different strengths and limitations. Tissue based biopsies and cytology specimens are both adequate for mutations analysis.<sup>46</sup> A study of EGFR testing accuracy across 15 different centres in France found sample quality was more important than the type of molecular genetic techniques utilised.<sup>47</sup> Direct sequencing is limited by low sensitivity, requiring the mutation to be present in 20% of tumour cells in the sample and is also labour intensive. Sensitivity can be improved by enriching for tumour cells with dissection techniques.<sup>48</sup> Screening techniques such as high resolution melting analysis and denaturing high-performance liquid chromatography can improve analytic sensitivity. Targeted methods such as ARMS™ (Amplification Refractory Mutation System), PCR-Invader®, peptide nucleic acid-locked nucleic acid PCR clamp and Cycleave™, generally have higher sensitivity and are more rapid than direct DNA sequencing, but only identify

specific targeted mutations and are unable to detect rarer or novel mutations. Multiplex platforms that enable concurrent testing of multiple genetic abnormalities, such as Sequenom MassArray and SNaPshot, enable a more comprehensive genotype to be efficiently established with relatively small amounts of DNA, and are likely to be more clinically beneficial as greater numbers of targeted agents become available.

EGFR mutation is often associated with EGFR gene amplification, particularly amplification of the mutant allele,<sup>53</sup> however several studies have shown EGFR gene copy number is inferior to EGFR mutation at predicting response to EGFR-TKIs. While EGFR mutation specific immunohistochemistry is relatively fast and cheap and can be undertaken in routine histopathology laboratories, it only detects two specific mutations (L858R in exon 21 and the E746-A750 exon 19 deletion) and is inferior to DNA molecular analysis at identifying mutations.

### KRAS mutations in NSCLC

KRAS mutations occur in about 38% of lung adenocarcinomas in an Australian population,<sup>1</sup> similar to that observed in other studies of western populations.<sup>58</sup> By contrast, KRAS mutations are less frequent in Asian populations with a frequency of 10-15%. KRAS mutations in NSCLC mostly occur at codons 12 and less often at 13 and 61.<sup>19</sup> KRAS mutations occur in adenocarcinomas, particularly poorly differentiated tumours, and are associated with mucinous and solid predominant tumour types.<sup>16</sup> They are more common in males and are strongly related to a history of smoking, with 30-43% of smokers harbouring KRAS mutations compared to 0-7% of non-smokers.<sup>62</sup>

A meta-analysis of KRAS mutations in NSCLC found these patients have a worse prognosis than patients with KRAS wild type tumours.<sup>63</sup> As expected of driver mutations, EGFR and KRAS mutations are almost always mutually exclusive in NSCLC, although rare cases of patients harbouring both activating EGFR mutations and KRAS mutations have been reported.<sup>58</sup> Not surprisingly, KRAS mutations predict insensitivity to EGFR-TKI treatment.

### ALK rearrangements in NSCLC

ALK is a receptor tyrosine kinase that belongs to the insulin receptor family and undergoes constitutive activation in a small subset of NSCLC through chromosomal rearrangement.<sup>65</sup> ALK is located on chromosome 2p and undergoes inversion leading to formation of an oncogenic fusion gene, most commonly EML4-ALK (echinoderm microtubule-associated protein-like 4), encoding a constitutively activated tyrosine kinase that stimulates cell proliferation, survival and migration pathways.<sup>66-68</sup> These oncogene addicted tumours are highly sensitive to inhibition, with a clinical trial of crizotinib demonstrating a response rate of 57%.<sup>6</sup> While ALK rearrangements have been reported to occur in 0.4-13.5% of NSCLC, in most unselected studies they are found in about 4% of cases.<sup>65</sup> More recent data suggests the incidence is closer to 1% of lung adenocarcinomas in Australian populations.

Clinical features associated with ALK rearrangements include younger patient age and non-smoking status,<sup>69-73</sup> similar to the typical EGFR clinical picture, although racial and gender associations are less apparent for ALK. Pathologically, ALK rearrangements are found almost always in adenocarcinomas,<sup>73</sup> particularly with solid, acinar, cribriform with extracellular mucin, or signet ring cell morphology.

As expected for a driver mutation, ALK rearrangements are almost always mutually exclusive with EGFR and KRAS mutations. ALK rearrangements have also rarely been reported to occur in combination with EGFR mutation, including a patient who was resistant to erlotinib treatment.<sup>79</sup> More recently, there has been a case report of a combined small cell carcinoma and adenocarcinoma harbouring EML4-ALK fusion in the small cell component, and EGFR exon 19 deletion in the adenocarcinoma component.<sup>80</sup>

As with EGFR-TKIs, patients treated with crizotinib develop acquired resistance, the mechanism of which includes secondary ALK gene mutations, and activation of signalling pathways that bypass the inhibited pathway, including activation of EGFR signalling.<sup>82</sup>

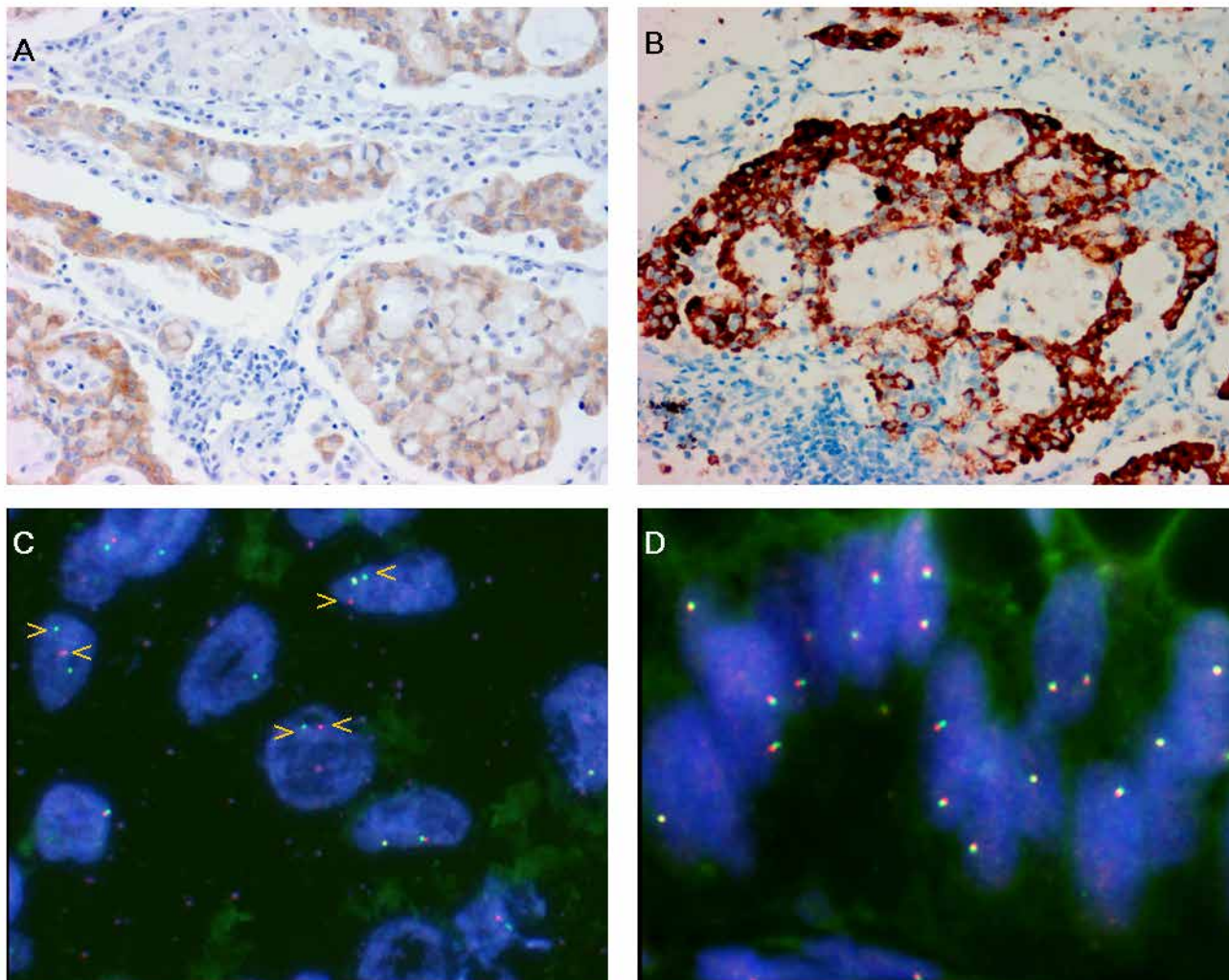
Fluorescence in situ hybridisation (FISH), using a break-apart probe that targets the breakpoint of the ALK gene, is the standard method for identifying ALK rearrangements in clinical samples and has been validated in clinical trials.<sup>6</sup> In the US, ALK FISH is currently the only Food and Drug Administration approved technique for detecting ALK rearranged NSCLC, however this method is relatively labour intensive and costly, and can be technically challenging to interpret due to the inversion resulting in a subtle split in the FISH signal. There is increasing evidence that immunohistochemistry has very high sensitivity for detecting rearranged ALK using both the 5A4 clone and the newly available D5F3 clone, and could be used to screen for cases that could then be confirmed with FISH (figure 1). This is of particular importance in populations with low prevalence of the genetic abnormality, where it may not be cost-effective to undertake FISH in all cases. In addition, some crizotinib sensitive tumours may only demonstrate ALK alteration by immunohistochemistry and not FISH. While reverse-transcriptase PCR is highly accurate and can also be used to detect ALK rearrangements,<sup>88</sup> this technique is not practical in a routine clinical diagnostic setting, as there are 13 different breakpoints in EML4 from exon2 to 20, as well as some rarer non-EML4 fusion partners. All require different primers to target the fusion variants, some of which generate large amplicons not ideal for identification from paraffin embedded tissue in which the RNA is often quite degraded.

### Other potentially targetable mutations

Other rare, but potentially targetable mutations in lung adenocarcinomas, include BRAF mutations which have been reported to occur in about 3% of lung adenocarcinomas,<sup>89</sup> and in limited data to date are likely to be sensitive to the selective kinase inhibitors vemurafenib and dabrafenib.<sup>90</sup> Primary MET gene amplifications occur in approximately 4% of NSCLC,<sup>38</sup> and can potentially be



**Figure 1:** Immunohistochemistry can be used to identify ALK overexpression in lung adenocarcinomas, with ALK rearrangement using the 5A4 clone (A) and the D5F3 clone (B). ALK rearrangement can be confirmed with FISH using a breakapart probe demonstrating (C) rearranged signals (arrowheads show split red and green signals). By contrast, non-rearranged ALK shows a normal pattern of fused red and green signals (D).



targeted by MET inhibitors such as crizotinib. ROS1 gene rearrangements were discovered at the same time as ALK-rearranged NSCLC.<sup>66</sup> ROS1 encodes a transmembrane tyrosine kinase receptor and is located on chromosome 6.<sup>91</sup> It has high homology with the intracellular kinase domain and ATP binding site of ALK.<sup>92</sup> Activation of ROS1 leads to signalling through downstream oncogenic pathways, including PI3K/Akt, MTOR and RAS-MAPK/ERK pathways.<sup>93</sup> ROS1 rearrangements have been found to occur in up to 4% of lung adenocarcinomas,<sup>94-98</sup> and are mutually exclusive with other driver mutations. Patients harbouring ROS1 rearrangements have overlapping clinical features with ALK rearranged tumours. There is in vitro evidence of sensitivity to crizotinib in a NSCLC cell line harbouring ROS1 rearrangement, although it is unclear if the growth inhibition related to inhibition of ROS1 or MET amplification, which is also present in the HCC78 cell line.<sup>100</sup> One young non-smoker patient with a ROS1 rearrangement, showed near complete response to crizotinib treatment as part of a clinical trial.<sup>94</sup>

## Squamous cell carcinoma

While current targeted molecular therapies in lung cancer have almost exclusively been in adenocarcinomas, increasing knowledge and interest in the molecular genetics of other lung cancer types, particularly squamous cell carcinoma,<sup>101</sup> will hopefully have a clinical impact in the future. Recently the Cancer Genome Atlas Research Network, as part of the Cancer Genome Atlas project, published the first comprehensive assessment of genomic alterations in squamous cell carcinomas after profiling 178 tumours.<sup>7</sup> They found TP53 mutations in almost all cases and potential therapeutic targets in the majority (64%) of squamous cell carcinomas (64%). Promising new targetable oncogenes in squamous cell carcinoma include fibroblast growth factor receptor amplifications, PIK3CA mutations and DDR2 mutations.<sup>102</sup>

## Approach to molecular genetic testing in NSCLC

With an increasing range of molecular targeted treatments for selected NSCLC patients, there is increasing need for testing of multiple genes. An algorithmic approach exploiting the mutually exclusive nature of most of the genetic alterations can be used for efficiencies of time and cost (figure 2). The optimal algorithm in each centre will largely depend on local resources and expertise, as well as the nature of the samples. Adequate sample quality and tumour DNA quantity is essential to ensure accurate testing, as low quality samples can compromise results and impact patient care. As the majority of patients with lung cancer are inoperable at diagnosis, many of the biopsies submitted for mutation testing are extremely limited, posing significant challenges, including the risk of false negative and positive results. Clinicians, radiologists and pathologists need to have a co-ordinated multidisciplinary approach, with good lines of communication to ensure optimal specimens are submitted for testing, as inappropriate specimens may lead to unnecessary delays and repeat testing. Given the generally limited amount of biopsy material in NSCLC, multi-gene testing is cost and time effective to rapidly identify patients with targetable changes, while helping to exclude the need for unnecessary FISH or other molecular testing, for example in patients harbouring KRAS mutations. In our experience, assessment of EGFR, KRAS and other major driver mutations in parallel with immunohistochemistry for ALK with confirmatory FISH if required, is cost and time effective and makes the best use of limited tissue samples.

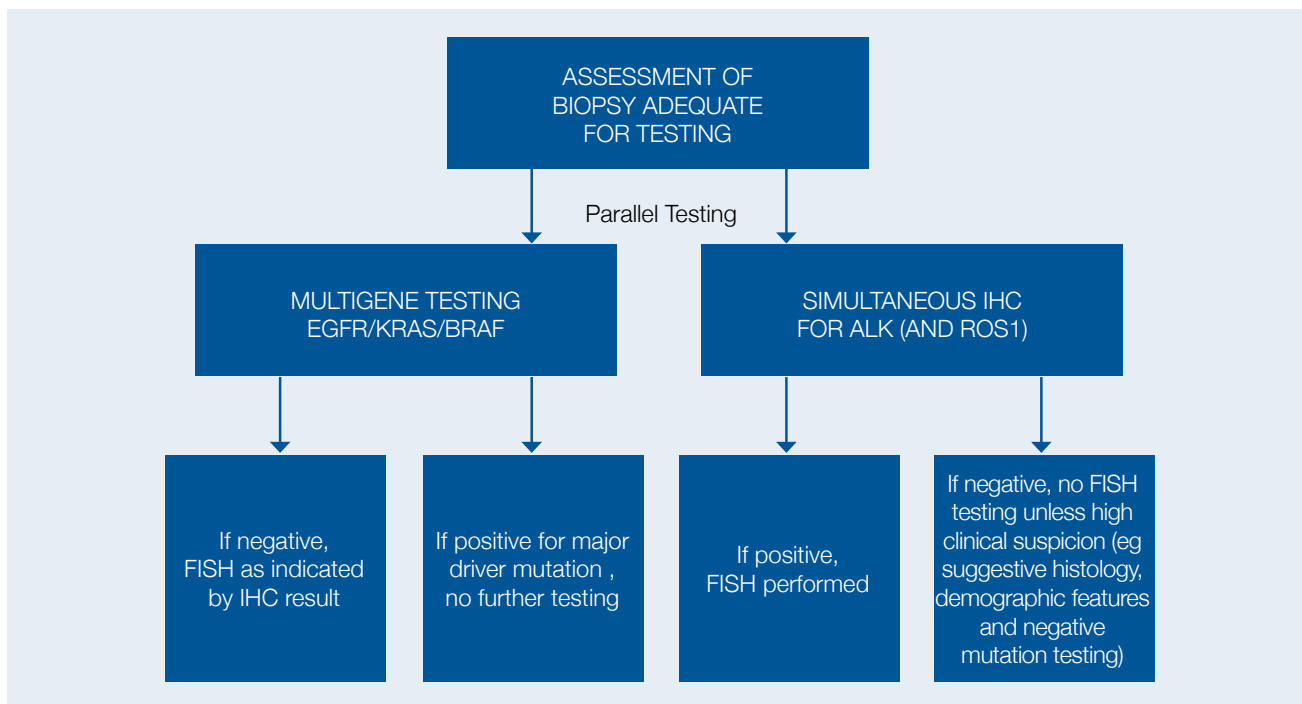
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## References

1. Yip PY, Yu B, Cooper WA, Selinger CI, Ng CC, Kennedy CW, et al. Patterns of DNA Mutations and ALK Rearrangement in Resected Node Negative Lung Adenocarcinoma. *J Thorac Oncol.* 2013; 8: 408-14
2. Kris M, Johnson B, Kwiatkowski Dea. Identification of driver mutations in tumor specimens from 1000 patients with lung adenocarcinoma: the NCI's Lung Cancer Mutation Consortium (LCMC). *J Clin Oncol.* 2011; 29 (Suppl, abstract CRA 7506).
3. Cardarella S, Ortiz TM, Joshi VA, Butaney M, Jackman DM, Kwiatkowski DJ, et al. The Introduction of Systematic Genomic Testing for Patients with Non-Small-Cell Lung Cancer. *J Thorac Oncol.* 2012; 7: 1767-74.
4. Hirsch FR, Jänne PA, Eberhardt WE, Cappuzzo F, Thatcher N, Pirker R, et al. Epidermal Growth Factor Receptor Inhibition in Lung Cancer: Status 2012. *J Thorac Oncol.* 2013; 8: 373-84.
5. Yoshida K, Yatabe Y, Park JY, Shimizu J, Horio Y, Matsuo K, et al. Prospective Validation for Prediction of Gefitinib Sensitivity by Epidermal Growth Factor Receptor Gene Mutation in Patients with Non-Small Cell Lung Cancer. *J Thorac Oncol.* 2007; 2: 22-8.
6. Kwak E, Bang Y, Camidge DR, Shaw A, Solomon B, Maki R, et al. Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. *N Engl J Med.* 2010; 363: 1693-703.
7. Network TCGAR. Comprehensive genomic characterization of squamous cell lung cancers. *Nature.* 2012; 489: 519-25.
8. Prenzel N, Fischer OM, Streit S, Hart S, Ullrich A. The epidermal growth factor receptor family as a central element for cellular signal transduction and diversification. *Endocr Relat Cancer.* 2001;8:11-31.
9. Scagliotti GV, Selvaggi G, Novello S, Hirsch FR. The Biology of Epidermal Growth Factor Receptor in Lung Cancer. *Clin Cancer Res.* 2004;10: 4227s-32s.
10. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol.* 2001;2: 127-37.
11. Yun C, Boggon T, Li Y, Woo M, Gleulich H, Meyerson M. Structures of lung cancer-derived EGFR mutants and inhibitor complexes: mechanism of activation and insights into differential inhibitor sensitivity. *Cancer Cell.* 2007;11:217-27.

**Figure 2:** Flow chart showing an algorithm for molecular genetic testing in lung adenocarcinomas.



12. Carey KD, Garton AJ, Romero MS, Kahler J, Thomson S, Ross S, et al. Kinetic analysis of epidermal growth factor receptor somatic mutant proteins shows increased sensitivity to the epidermal growth factor receptor tyrosine kinase inhibitor, erlotinib. *Cancer Res.* 2006;66:8163-71.
13. Mulloy R, Ferrand A, Kim Y, Sordella R, Bell DW, Haber DA, et al. Epidermal Growth Factor Receptor Mutants from Human Lung Cancers Exhibit Enhanced Catalytic Activity and Increased Sensitivity to Gefitinib. *Cancer Res.* 2007;67:2325-30.
14. Dahabreh IJ, Linardou H, Siannis F, Kosmidis P, Bafaloukos D, Murray S. Somatic EGFR Mutation and Gene Copy Gain as Predictive Biomarkers for Response to Tyrosine Kinase Inhibitors in Non-Small Cell Lung Cancer. *Clin Cancer Res.* 2010;16:291-303.
15. Jackman DM, Miller VA, Cioffredi L-A, Yeap BY, Jänne PA, Riely GJ, et al. Impact of Epidermal Growth Factor Receptor and KRAS Mutations on Clinical Outcomes in Previously Untreated Non-Small Cell Lung Cancer Patients: Results of an Online Tumor Registry of Clinical Trials. *Clin Cancer Res.* 2009;15:5267-73.
16. Russell PA, Barnett SA, Walkiewicz M, Wainer Z, Conron M, Wright GM, et al. Correlation of Mutation Status and Survival with Predominant Histologic Subtype According to the New IASLC/ATS/ERS Lung Adenocarcinoma Classification in Stage III (N2) Patients. *J Thorac Oncol.* 2013;8:461-8.
17. Eberhard DA, Giaccone G, Johnson BE. Biomarkers of Response to Epidermal Growth Factor Receptor Inhibitors in Non-Small-Cell Lung Cancer Working Group: Standardization for Use in the Clinical Trial Setting. *J Clin Oncol.* 2008;26:983-94.
18. Shigematsu H, Lin L, Takahashi T, Nomura M, Suzuki M, Wistuba II, et al. Clinical and Biological Features Associated With Epidermal Growth Factor Receptor Gene Mutations in Lung Cancers. *J Natl Cancer Inst.* 2005;97:339-46.
19. Kosaka T, Yatabe Y, Endoh H, Kuwano H, Takahashi T, Mitsudomi T. Mutations of the Epidermal Growth Factor Receptor Gene in Lung Cancer: Biological and Clinical Implications. *Cancer Res.* 2004;64:8919-23.
20. Tokumo M, Toyooka S, Kiura K, Shigematsu H, Tomii K, Aoe M, et al. The Relationship between Epidermal Growth Factor Receptor Mutations and Clinicopathologic Features in Non-Small Cell Lung Cancers. *Clin Cancer Res.* 2005;11:1167-73.
21. Ding L, Getz G, Wheeler D, Mardis ER, McLellan MD, Cibulskis, K et al. Somatic mutations affect key pathways in lung adenocarcinoma. *Nature.* 2008;455:1069-75.
22. Marchetti A, Ardzizoni A, Papotti M, Crinò L, Rossi G, Gridelli C, et al. Recommendations for the Analysis of ALK Gene Rearrangements in Non-Small-Cell Lung Cancer: A Consensus of the Italian Association of Medical Oncology and the Italian Society of Pathology and Cytopathology. *J Thorac Oncol.* 2013;8:352-8.
23. Tam IYS, Chung LP, Suen WS, Wang E, Wong MCM, Ho KK, et al. Distinct Epidermal Growth Factor Receptor and KRAS Mutation Patterns in Non-Small Cell Lung Cancer Patients with Different Tobacco Exposure and Clinicopathologic Features. *Clin Cancer Res.* 2006;12:1647-53.
24. Kim YT, Seong YW, Jung YJ, Jeon YK, Park IK, Kang CH, et al. The Presence of Mutations in Epidermal Growth Factor Receptor Gene Is Not a Prognostic Factor for Long-Term Outcome after Surgical Resection of Non-Small-Cell Lung Cancer. *J Thorac Oncol.* 2013;8:171-8.
25. Kosaka T, Yatabe Y, Onozato R, Kuwano H, Mitsudomi T. Prognostic implication of EGFR, KRAS, and TP53 gene mutations in a large cohort of Japanese patients with surgically treated lung adenocarcinoma. *J Thorac Oncol.* 2009;4:22-9.
26. Wu JY, Wu SG, Yang CH, Gow CH, Chang YL, Yu CJ, et al. Lung Cancer with Epidermal Growth Factor Receptor Exon 20 Mutations Is Associated with Poor Gefitinib Treatment Response. *Clin Cancer Res.* 2008;14:4877-82.
27. Kang S, Kang H, Shin J, Kim H, Shin D, Kim S, et al. Identical epidermal growth factor receptor mutations in adenocarcinomatous and squamous cell carcinomatous components of adenocarcinoma of the lung. *Cancer.* 2007;109:581-7.
28. Rekhman N, Paik P, Arcila M, Tafe L, Oxnard G, Moreira A, et al. Clarifying the spectrum of driver oncogene mutations in biomarker-verified squamous carcinoma of lung: lack of EGFR/KRAS and presence of PIK3CA/AKT1 mutations. *Clin Cancer Res.* 2012;18:1167-76.
29. Tochigi N, Dacic S, Nikiforova M, Ciepily KM, Yousem SA. Adenosquamous Carcinoma of the Lung: A Microdissection Study of KRAS and EGFR Mutational and Amplification Status in a Western Patient Population. *Am J Clin Pathol.* 2011;135:783-9.
30. Kaira K, Horie Y, Ayabe E, Murakami H, Takahashi T, Tsuya A, et al. Pulmonary Pleomorphic Carcinoma: A Clinicopathological Study Including EGFR Mutation Analysis. *J Thorac Oncol.* 2010; 5: 460-5.
31. Tatematsu A, Shimizu J, Murakami Y, Horio Y, Nakamura S, Hida T, et al. Epidermal Growth Factor Receptor Mutations in Small Cell Lung Cancer. *Clin Cancer Res.* 2008;14:6092-6.
32. Ohtsuka K, Ohnishi H, Fujiwara M, Kishino T, Matsushima S, Furuyashiki G, et al. Abnormalities of epidermal growth factor receptor in lung squamous-cell carcinomas, adenosquamous carcinomas, and large-cell carcinomas: tyrosine kinase domain mutations are not rare in tumors with an adenocarcinoma component. *Cancer.* 2007;109:741-50.
33. Baik CS, Pritchard CC, Eaton KD, Chow LQ. EGFR Mutations in Squamous Cell Lung Cancer in Never-Smokers. *J Thorac Oncol.* 2013;8:e6-e7.
34. Yamamoto H, Toyooka S, Mitsudomi T. Impact of EGFR mutation analysis in non-small cell lung cancer. *Lung cancer.* 2009;63:315-21.
35. Oxnard GR, Lo PC, Nishino M, Dahlberg SE, Lindeman NI, Butaney M, et al. Natural History and Molecular Characteristics of Lung Cancers Harboring EGFR Exon 20 Insertions. *J Thorac Oncol.* 2013;8:179-84.
36. Pao W, Chmielecki J. Rational, biologically based treatment of EGFR mutant non-small-cell lung cancer. *Nat Rev Cancer.* 2010;10:760-74.
37. Yun CH, Mengwasser KE, Toms AV, Woo MS, Greulich H, Wong KK, et al. The T790M mutation in EGFR kinase causes drug resistance by increasing the affinity for ATP. *Proc Natl Acad Sci USA* 2008;105:2070-5.
38. Cappuzzo F, Marchetti A, Skokan M, Rossi E, Gajapathy S, Felicioni L, et al. Increased MET Gene Copy Number Negatively Affects Survival of Surgically Resected Non-Small-Cell Lung Cancer Patients. *J Clin Oncol.* 2009;27:1667-74.
39. Arcila ME, Oxnard GR, Nafa K, Riely GJ, Solomon SB, Zakowski MF, et al. Rebiopsy of Lung Cancer Patients with Acquired Resistance to EGFR Inhibitors and Enhanced Detection of the T790M Mutation Using a Locked Nucleic Acid-Based Assay. *Clin Cancer Res.* 2011;17:1169-80.
40. Balak MN, Gong Y, Riely GJ, Somwar R, Li AR, Zakowski MF, et al. Novel D761Y and Common Secondary T790M Mutations in Epidermal Growth Factor Receptor-Mutant Lung Adenocarcinomas with Acquired Resistance to Kinase Inhibitors. *Clin Cancer Res.* 2006;12:6494-501.
41. Kosaka T, Yatabe Y, Endoh H, Yoshida K, Hida T, Tsuboi M, et al. Analysis of Epidermal Growth Factor Receptor Gene Mutation in Patients with Non-Small Cell Lung Cancer and Acquired Resistance to Gefitinib. *Clin Cancer Res.* 2006;12:5764-9.
42. Hammerman PS, Jänne PA, Johnson BE. Resistance to Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitors in Non-Small Cell Lung Cancer. *Clin Cancer Res.* 2009;15:7502-9.
43. Engelman J, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park J, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. *Science.* 2007;306:1039-43.
44. Zakowski M, Ladanyi M, Kris M. EGFR mutations in small-cell lung cancers in patients who have never smoked. *N Engl J Med.* 2006;355:213-5.
45. Morinaga R, Okamoto I, Furuta K, Kawano Y. Sequential occurrence of non-small cell and small cell lung cancer with the same EGFR mutation. *Lung cancer.* 2007;58:411-3.
46. Goto K, Satouchi M, Ishii G, Nishio K, Hagiwara K, Mitsudomi T, et al. An evaluation study of EGFR mutation tests utilized for non-small-cell lung cancer in the diagnostic setting. *Ann Oncol.* 2012;23:2914-9.
47. Beau-Faller M, Degeorges A, Rolland E, Mounawar M, Antoine M, Poulot V, et al. Cross-validation study for epidermal growth factor receptor and KRAS mutation detection in 74 blinded non-small cell lung carcinoma samples: a total of 5550 exons sequenced by 15 molecular French laboratories (evaluation of the EGFR mutation status for the administration of EGFR-TKIs in non-small cell lung carcinoma [ERMETIC] project-part 1). *J Thorac Oncol.* 2011; 6:1006-15.
48. Ellison G, Zhu G, Moulis A, Dearden S, Speake G, McCormack R. EGFR mutation testing in lung cancer: a review of available methods and their use for analysis of tumour tissue and cytology samples. *J Clin Pathol.* 2013;66:9-89.
49. Do H, Krypuy M, Mitchell P, Fox S, Dobrovic A. High resolution melting analysis for rapid and sensitive EGFR and KRAS mutation detection in formalin fixed paraffin embedded biopsies. *BMC Cancer.* 2008;8: 142.
50. Sueoka N, Sato A, Eguchi H, Komiya K, Sakuragi T, Mitsuoka M, et al. Mutation profile of EGFR gene detected by denaturing high-performance liquid chromatography in Japanese lung cancer patients. *J Cancer Res Clin Oncol.* 2007;133:93-102.
51. Ellison G, Donald E, McWalter G, Knight L, Fletcher L, Sherwood J, et al. A comparison of ARMS and DNA sequencing for mutation analysis in clinical biopsy samples. *J Exp Clin Cancer Res.* 2010; 29:132.
52. Sequist LV, Heist RS, Shaw AT, Fidias P, Rosovsky R, Temel JS, et al. Implementing multiplexed genotyping of non-small-cell lung cancers into routine clinical practice. *Ann Oncol.* 2011;22:2616-24.
53. Li AR, Chitale D, Riely GJ, Pao W, Miller VA, Zakowski MF, et al. EGFR Mutations in Lung Adenocarcinomas: Clinical Testing Experience and Relationship to EGFR Gene Copy Number and Immunohistochemical Expression. *J Mol Diagn.* 2008;10:242-8.
54. Bell DW, Lynch TJ, Haslerat SM, Harris PL, Okimoto RA, Brannigan BW, et al. Epidermal Growth Factor Receptor Mutations and Gene Amplification in Non-Small-Cell Lung Cancer: Molecular Analysis of the IDEAL/INTACT Gefitinib Trials. *J Clin Oncol.* 2005;23:8081-92.
55. Fukuoaka M, Wu YL, Thongprasert S, Sunpaweravong P, Leong SS, Sriuranpong V, et al. Biomarker Analyses and Final Overall Survival Results From a Phase III, Randomized, Open-Label, First-Line Study of Gefitinib Versus Carboplatin/Paclitaxel in Clinically Selected Patients With Advanced Non-Small-Cell Lung Cancer in Asia (IPASS). *J Clin Oncol.* 2011;29:2866-74.
56. Brevet M, Arcila M, Ladanyi M. Assessment of EGFR mutation status in lung adenocarcinoma by immunohistochemistry using antibodies specific to the two major forms of mutant EGFR. *J Mol Diagn.* 2010;12:169-76.
57. Kawahara A, Azuma K, Sumi A, Taira T, Nakashima K, Aikawa E, et al. Identification of non-small-cell lung cancer with activating EGFR mutations in malignant effusion and cerebrospinal fluid: rapid and sensitive detection of exon 19 deletion E746-A750 and exon 21 L858R mutation by immunocytochemistry. *Lung Cancer.* 2011; 74:35-40.



58. Schmid K, Oehl N, Wrba F, Pirker R, Pirker C, Filipits M. EGFR/KRAS/BRAF Mutations in Primary Lung Adenocarcinomas and Corresponding Locoregional Lymph Node Metastases. *Clin Cancer Res.* 2009;15:4554-60.
59. Mao C, Qiu LX, Liao RY, Du FB, Ding H, Yang WC, et al. KRAS mutations and resistance to EGFR-TKIs treatment in patients with non-small cell lung cancer: A meta-analysis of 22 studies. *Lung cancer.* 2010;69:272-8.
60. Yoshizawa A, Sumiyoshi S, Sonobe M, Kobayashi M, Fujimoto M, Kawakami F, et al. Validation of the IASLC/ATS/ERS Lung Adenocarcinoma Classification for Prognosis and Association with EGFR and KRAS Gene Mutations: Analysis of 440 Japanese Patients. *J Thorac Oncol.* 2013; 8: 52-61.
61. Zhang Y, Sun Y, Pan Y, Li Cea. Frequency of driver mutations in lung adenocarcinoma from female never-smokers varies with histologic subtypes and age at diagnosis. *Clin Cancer Res.* 2012; 18: 1947-53.
62. Subramanian J, Govindan R. Molecular genetics of lung cancer in people who have never smoked. *Lancet Oncol.* 2008;9:676-82.
63. Mascaux C, Iannino N, Martin Bea. The role of RAS oncogene in survival of patients with lung cancer: a systematic review of the literature with meta-analysis. *Br J Cancer.* 2005;92:131-9.
64. Linardou H, Dahabreh I, Kanaloupiti D, Siannis F, Bafaloukos D, Kosmidis P, et al. Assessment of somatic k-RAS mutations as a mechanism associated with resistance to EGFR-targeted agents: a systematic review and meta-analysis of studies in advanced non-small-cell lung cancer and metastatic colorectal cancer *Lancet Oncol.* 2008; 9:962-72.
65. Solomon B, Varella-Garcia M, Camidge R. ALK gene rearrangements. A new therapeutic target in a molecularly defined subset of non-small cell lung cancer. *J Thorac Oncol.* 2009;4:1450-4.
66. Rikova K, Guo A, Zeng Q. Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. *Cell.* 2007;131:1190-203.
67. Soda M, Choi Y, Enomoto M. Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. *Nature.* 2007;448:561-6.
68. Chiarle R, Voena C, Ambrogia C, Piva R, Inghirami G. The anaplastic lymphoma kinase in the pathogenesis of cancer. *Nat Rev Cancer.* 2008;8:11-23.
69. Selinger C, Rogers T, Russell P, O'Toole S, Yip P, Wright G, et al. Testing for ALK rearrangement in lung adenocarcinoma - a multicenter comparison of immunohistochemistry and fluorescent in situ hybridization. *Mod Pathol.* 2013; In Press.
70. Rodig SJ, Mino-Kenudson M, Dacic S, Yeap BY, Shaw A, Barletta JA, et al. Unique clinicopathologic features characterize ALK-rearranged lung adenocarcinoma in the Western population. *Clin Cancer Res.* 2009; 15:5216-23.
71. Sakairi Y, Nakajima T, Yasufuku K, Ikebe D, Kageyama H, Soda M, et al. EML4-ALK Fusion Gene Assessment Using Metastatic Lymph Node Samples Obtained by Endobronchial Ultrasound-Guided Transbronchial Needle Aspiration. *Clin Cancer Res.* 2010;16:4938-45.
72. Shaw A, Yeap B, Mino-Kenudson M, Digumarthy S, Costa D, Heist R, et al. Clinical features and outcome of patients with non-small-cell lung cancer who harbor EML4-ALK. *J Clin Oncol.* 2009;27: 4247-53.
73. Wong DW, Leung EL, So KK-T, Tam IYS, Sihoe ADL, Cheng LC, et al. The EML4-ALK fusion gene is involved in various histologic types of lung cancers from nonsmokers with wild-type EGFR and KRAS. *Cancer.* 2009;115:1723-33.
74. Inamura K, Takeuchi K, Togashi Y, Hatano S, Ninomiya Hea. EML4-ALK lung cancers are characterized by rare other mutations, a TTF-1 cell lineage, an acinar histology, and young onset. *Mod Pathol.* 2009;22:508-15.
75. Jokoji R, Yamasaki T, Minami Sea. Combination of morphological feature analysis and immunohistochemistry is useful for screening of EML4-ALK-positive lung adenocarcinoma. *J Clin Pathol.* 2010;63:1066-70.
76. Yoshida A, Tsuta K, Nakamura Hea. Comprehensive histologic analysis of ALK-rearranged lung carcinomas. *Am J Surg Pathol.* 2011;35:1226-34.
77. Koivunen JP, Mermel C, Zejnullahu K, Murphy C, Lifshits E, Holmes AJ, et al. EML4-ALK Fusion Gene and Efficacy of an ALK Kinase Inhibitor in Lung Cancer. *Clin Cancer Res.* 2008; 14: 4275-83.
78. Sholl LM, Weremowicz S, Gray SW, Wong K-K, Chirieac LR, Lindeman NI, et al. Combined Use of ALK Immunohistochemistry and FISH for Optimal Detection of ALK-Rearranged Lung Adenocarcinomas. *J Thorac Oncol.* 2013;8:322-8.
79. Tiseo M, Gelsomino F, Boggiani D, Bortesi B, Bartolotti M, Bozzetti C, et al. EGFR and EML4-ALK gene mutations in NSCLC: A case report of erlotinib-resistant patient with both concomitant mutations. *Lung cancer.* 2011;71:241-3.
80. Toyokawa G, Taguchi K, Ohba T, Morodomi Y, Takenaka T, Hirai F, et al. First Case of Combined Small-Cell Lung Cancer with Adenocarcinoma Harboring EML4-ALK Fusion and an Exon 19 EGFR Mutation in Each Histological Component. *J Thorac Oncol.* 2012;7:e39-e41.
81. Choi Y, Soda M, Yamashita Y, Yueno Tea. EML4-ALK mutations in lung cancer that confer resistance to ALK inhibitors. *N Engl J Med.* 2010;363:1734-9.
82. Sasaki T, Koivunen J, Ogino A, Yanagita M, Nikiforow S, Zheng W, et al. A Novel ALK Secondary Mutation and EGFR Signaling Cause Resistance to ALK Kinase Inhibitors. *Cancer Res.* 2011;71:6051-60.
83. McLeer-Florin A, Moro-Sibilot D, Mellis A, Salameire D, Lefebvre C, Ceccaldi F, et al. Dual IHC and FISH Testing for ALK Gene Rearrangement in Lung Adenocarcinomas in a Routine Practice: A French Study. *J Thorac Oncol.* 2012;7:348-54.
84. Paik JH, Choe G, Kim H, Choe J-Y, Lee HJ, Lee CT, et al. Screening of Anaplastic Lymphoma Kinase Rearrangement by Immunohistochemistry in Non-small Cell Lung Cancer: Correlation with Fluorescence In Situ Hybridization. *J Thorac Oncol.* 2011;6:466-72.
85. Conklin C, Craddock K, Have C, Laskin J, Couture C, Ionescu D. Immunohistochemistry is a reliable screening tool for identification of ALK rearrangement in non-small-cell lung carcinoma and is antibody dependent. *J Thorac Oncol.* 2013 8:45-51.
86. Peled N, Palmer G, Hirsch FR, Wynes MW, Ilouze M, Varella-Garcia M, et al. Next-Generation Sequencing Identifies and Immunohistochemistry Confirms a Novel Crizotinib-Sensitive ALK Rearrangement in a Patient with Metastatic Non-Small-Cell Lung Cancer. *J Thorac Oncol.* 2012;7:e14-e6.
87. Sun J-M, Choi Y-L, Won J-K, Hirsch FR, Ahn JS, Ahn M-J, et al. A Dramatic Response to Crizotinib in a Non-Small-Cell Lung Cancer Patient with IHC-Positive and FISH-Negative ALK. *J Thorac Oncol.* 2012;7:e36-e8.
88. Takeuchi K, Choi Y, Soda M, Inamura K, Togashi Y, Hatano Sea. Multiplex reverse-transcription-PCR screening for EML4-ALK fusion transcripts. *Clin Cancer Res.* 2008;14:6618-24.
89. Paik PK, Arcila ME, Fara M, Sima CS, Miller VA, Kris MG, et al. Clinical Characteristics of Patients With Lung Adenocarcinomas Harboring BRAF Mutations. *J Clin Oncol.* 2011; 29: 2046-51.
90. Falchook G, Long G, Kurzrock R, Kim K, Arkenau T, Brown M, et al. Dabrafenib in patients with melanoma, untreated brain metastases, and other solid tumours: a phase 1 dose-escalation trial. *Lancet.* 2012;379:1893-901.
91. Nagarajan L, Louie E, Tsujimoto Y, Balduzzi P, Heubner K, Croce C. The human c-ros gene (ROS) is located at chromosome region 6q16—6q22. *Proc Natl Acad Sci USA.* 1986; 83: 6568-72.
92. Ou S, Tan J, Yen Y, Soo R. ROS1 as a 'druggable' receptor tyrosine kinase: lessons learned from inhibiting the ALK pathway. *Expert Rev Anticancer Ther.* 2012;12:447-56.
93. Acquaviva J, Wong R, Charest A. The multifaceted roles of the receptor tyrosine kinase ROS in development and cancer. *Biochim Biophys Acta.* 2009;1795:37-52.
94. Bergeth K, Shaw AT, Ignatius Ou S-H, Katayama R, Lovly CM, McDonald NT, et al. ROS1 Rearrangements Define a Unique Molecular Class of Lung Cancers. *J Clin Oncol.* 2012;30:863-70.
95. Chin LP, Soo RA, Soong R, Ou S-HI. Targeting ROS1 with Anaplastic Lymphoma Kinase Inhibitors: A Promising Therapeutic Strategy for a Newly Defined Molecular Subset of Non-Small-Cell Lung Cancer. *J Thorac Oncol.* 2012;7:1625-30.
96. Yoshida A, Kohno T, Tsuta K, Wakai S, Arai Y, Shimada Y, et al. ROS1-Rearranged Lung Cancer: A Clinicopathologic and Molecular Study of 15 Surgical Cases. *Am J Surg Pathol.* 2013;37: 554-62.
97. Rimkunas V, Crosby K, Li D, Hu Y, Kelly M, Gu T, et al. Analysis of receptor tyrosine kinase ROS1-positive tumors in non-small cell lung cancer: identification of a FIG-ROS1 fusion. *Clin Cancer Res.* 2012; 18: 449-57.
98. Takeuchi K, Soda M, Togashi Y, Suzuki R, Sakata S, Hatano S, et al. RET, ROS1 and ALK fusions in lung cancer. *Nature Med.* 2012;18:378-81.
99. Yasuda H, de Figueiredo-Pontes LL, Kobayashi S, Costa DB. Preclinical Rationale for Use of the Clinically Available Multitargeted Tyrosine Kinase Inhibitor Crizotinib in ROS1-Translocated Lung Cancer. *J Thorac Oncol.* 2012;7:1086-90.
100. Komiya T, Thomas A, Khozin S, Rajan A, Wang Y, Giaccone G. Response to Crizotinib in ROS1-Rearranged Non-Small-Cell Lung Cancer. *J Clin Oncol.* 2012;30:3425-6.
101. Heist RS, Sequist LV, Engelman JA. Genetic Changes in Squamous Cell Lung Cancer: A Review. *J Thorac Oncol.* 2012;7:924-33
102. Oxnard GR, Binder A, Jänne PA. New Targetable Oncogenes in Non-Small-Cell Lung Cancer. *J Clin Oncol.* 2013;31:1097-104.