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NEW TRENDS IN CLINICAL ONCOLOGY

# Biomarker testing in advanced non-small-cell lung cancer: a National Consensus of the Spanish Society of Pathology and the Spanish Society of Medical Oncology

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**Abstract** In 2011, the Spanish Society of Medical Oncology and the Spanish Society of Pathology started a joint project to establish recommendations on biomarker testing in patients with advanced non-small-cell lung cancer based on the current evidence. Most of these recommendations are still valid, but new evidence requires some aspects to be updated. Specifically, the recommendation about which biomarkers to test in which patients is being amended, and the best way to manage tumour samples and minimum requirements for biomarker test material are defined. Suitable techniques for testing for epidermal growth factor receptor mutations and anaplastic lymphoma kinase rearrangement are also reviewed, and a consensus is reached on which situations warrant re-biopsy.

**Keywords** *ALK* · Biomarkers · *EGFR* · Lung cancer

## Introduction

Lung cancer is the leading cause of cancer deaths, and therefore represents a major health problem. Smoking is the main cause of lung cancer and only 10–15 % of these tumours are diagnosed in non-smokers. To decide how to treat patients with non-small-cell lung cancer (NSCLC) and metastatic disease, the histological subtype and, in most cases, the result of biomarker analysis, must be known. A joint project between the Spanish Society of Medical Oncology (SEOM) and the Spanish Society of

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Pathology (SEAP) was therefore set up in 2011, with the aim of defining consensus guidelines on biomarker testing in patients with NSCLC and advanced disease in Spain. This first SEOM/SEAP consensus involved five pathologists and five medical oncologists specialising in chest disease, who reviewed all the available literature and agreed a series of recommendations [1]. Most recommendations in the first SEOM/SEAP consensus on biomarkers in NSCLC are still valid today. However, the publication of new evidence, especially about the predictive value of anaplastic lymphoma kinase (*ALK*) testing, led us to write this second SEOM/SEAP consensus statement.

The same authors were involved again this time. The methodology consisted of a first face-to-face meeting to define which recommendations in the first consensus needed revising, and formulate a series of questions considered of interest. All the available literature was then reviewed and all the questions answered, including recommendations and references for each of them. Lastly, at a second face-to-face meeting, this document was discussed and approved.

## Which recommendations of the previous guidelines are still valid?

In the first consensus statement, recommendations were divided into those addressing clinical issues, those on pathology and molecular issues, and those covering common issues affecting both clinical aspects and predictive biomarkers:

- As far as first consensus guidelines on clinical issues are concerned, the recommendation to test for epidermal growth factor receptor (*EGFR*) mutation in NSCLC patients with advanced disease if they have non-squamous cell carcinoma, and in all non-smokers irrespective of histology, is still valid. However, the recommendation on *ALK* testing was thought to need updating.
- Most first consensus recommendations on pathology issues remain fully valid today. Since publication of the first consensus statement, SEAP has been implementing a quality control programme for *ALK* testing, and another for new massive sequencing technology.
- As regards recommendations about common clinicopathological issues, those on obtaining optimal specimens, preparing them, the information needed in reports, and acceptable turnaround times (getting results within 7 working days of receiving the sample) are still valid.

Also in this second SEOM/SEAP consensus statement, the recommendation about which other biomarkers to test and in which patients is updated, the best way to manage tumour samples and minimum material requirements are defined, suitable techniques for testing for *EGFR* mutations and *ALK* rearrangements are reviewed, and a consensus is reached on which situations warrant re-biopsy. Lastly, issues that may soon acquire great importance, such as molecular tests on blood and the use of massive sequencing techniques, are examined.

## Which biomarkers should be tested and in which patients?

In patients with advanced NSCLC, treatment selection based on molecular markers that predict efficacy has substantially altered the clinical focus and lines of research in the last few years.

### EGFR

In our setting, mutations are present in 10–16 % of patients with advanced NSCLC [2]. The most common (85–90 %) are deletions in exon 19 and point mutations in exon 21. At present, there are three drugs available (gefitinib, erlotinib and afatinib), which have demonstrated clear benefit in phase III randomised trials in this context [3–5]. Therefore, the main clinical guidelines recommend prescribing one of these as first-line treatment in advanced patients [6, 7]. For a while there was some debate about the "clinical profile" of patients who should be tested for these mutations. There is now enough evidence for this test to be recommended in patients with stage IV non-squamous NSCLC regardless of their smoking habits, and in non-smokers irrespective of histology (Fig. 1).

### *ALK* rearrangements

ALK is a receptor tyrosine kinase that was first identified as part of the t(2;5) translocation associated with most anaplastic lymphomas. It is calculated that 2–7 % of patients with NSCLC have *ALK* rearrangements [8], mainly translocations, and this is again more common in patients with little or no smoking history. These patients tend to be young, and there are no gender differences. Most of these tumours are adenocarcinomas, often associated with certain morphological features, e.g. "signet-ring" cells or a cribriform pattern [9]. *ALK* rearrangements tend not to coexist with *EGFR* mutations [10].

Several ALK inhibitors are in clinical development, although only crizotinib is licensed in Europe at the moment. This oral drug, with activity against *ALK*, c-*MET* and *ROS1*, demonstrated significant benefit in terms of progression-free survival (PFS) versus pemetrexed or docetaxel chemotherapy in a phase III study in 347 patients

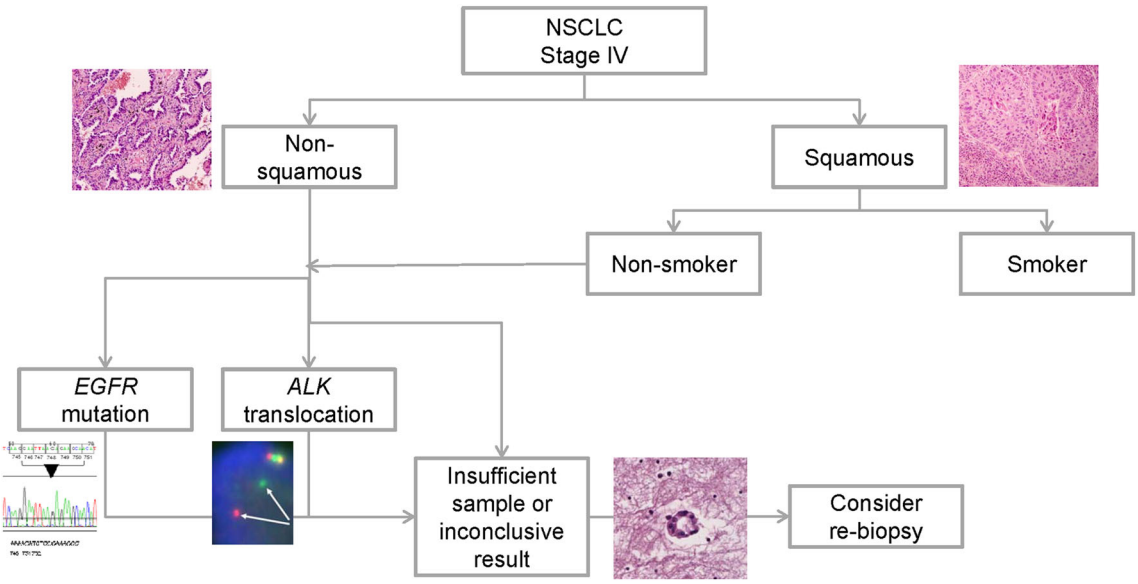


**Fig. 1** Diagnostic algorithm for advanced NSCLC patients. *ALK* anaplastic lymphoma kinase, *EGFR* epidermal growth factor receptor, *NSCLC* non-small-cell lung cancer

with locally advanced or metastatic *ALK*-positive lung cancer previously treated with platinum-based chemotherapy [11]. Median PFS was 7.7 months for patients treated with crizotinib and 3.0 months for those given chemotherapy (Hazard ratio [HR]: 0.49; $p < 0.001$). The objective response rate (ORR) was also higher for patients treated with crizotinib (65 vs. 20 %; $p < 0.001$). The most common adverse effects were visual disturbances, raised transaminases, nausea and vomiting.

Results were recently announced from a phase III study in 343 previously untreated patients with advanced *ALK*-positive non-squamous cell lung cancer randomised to crizotinib 250 mg twice daily or chemotherapy (pemetrexed with cisplatin or carboplatin) [12]. This study demonstrated crizotinib superiority over chemotherapy in terms of PFS (10.9 vs. 7.0 months; HR: 0.454; $p < 0.0001$). The ORR was also higher in patients treated with crizotinib (74 % vs. 45 %; $p < 0.0001$).

Lastly, results are also available from a phase I study evaluating the activity of ceritinib, another tyrosine kinase inhibitor (TKI), for the treatment of patients with advanced *ALK*-positive NSCLC [13]. In this study [14], 255 patients were treated with 750 mg/day of this drug. The most common adverse effect was diarrhoea (84 %). The most common grade 3/4 adverse effects included elevation of the hepatic enzymes alanine aminotransferase (ALT) (21 %) and aspartate aminotransferase (AST) (8 %). In this same clinical trial, ceritinib activity was tested in 121 patients previously treated with crizotinib, who achieved a PFS of 6.9 months and an ORR of 55.4 %. This ORR was unaffected by the number of previous treatment regimens received (1–3). This study also analysed the progress of 59 previously untreated *ALK*-positive patients. These patients did not reach the median PFS, and the ORR was 69.5 %.

Testing for *ALK* rearrangements is probably indicated in patients with stage IV non-squamous NSCLC regardless of their smoking habits, and in non-smokers irrespective of histology (Fig. 1), i.e. the indications are probably the same as for *EGFR* mutation testing.

## What is the optimal sample type and how should it be managed? Should tests be done simultaneously or sequentially?

The tumour specimen is very precious material for which two objectives must be defined. The first includes obtaining an accurate pathological diagnosis, and the second involves preserving enough material for subsequent biomarker tests. Tissue obtained by surgery, bronchoscopy or needle biopsy is equally valid, as the most important thing is the number of tumour cells present in the sample. Cytology is considered useful if a suitable cell block is obtained, because tests on smears only work well at highly specialised centres. The first thing to stress, although it may seem obvious, is that it is essential to review all of a patient's tumour specimens when deciding which one to use for conducting (or repeating) tests [7].

The pathologist should use the smallest amount of tissue for tumour typing, which means being careful with the morphological examination and using no more than two immunohistochemical markers, in cases without any clear

morphological differentiation. The first marker needed at the current time is TTF-1, which defines up to 77 % of adenocarcinomas, with negligible positivity rates for squamous cell carcinomas (0 %) [15]. The second recommended marker is p40, given its greater specificity than p63, which is still useful. However, it must be stressed that p63 marks up to 18 % of adenocarcinomas [15]. The performance of additional histochemical techniques is not recommended. Having made a diagnosis of advanced non-squamous NSCLC, biomarker tests should be done.

For all the above reasons, paraffin-embedded material, whether from a biopsy or a cell block, should be processed according to a tissue-sparing procedure (protocol) that allows not only pathological diagnosis, but also testing for multiple predictive biomarkers. It is worth restating two obvious but often overlooked principles: (1) the fewer times the paraffin block is placed in the microtome, the more tissue is spared; and (2) the order of biomarker prioritisation is important, as the tissue will be running out [16, 17]. We propose the following sequence of steps: an initial stained section (haematoxylin/eosin [H&E]) to obtain the first diagnosis, allowing two sections to be scheduled for immunohistochemical tests (only if glandular or squamous cell differentiation is not apparent); a series of sections for deoxyribonucleic acid (DNA) extraction (the number and thickness of which will depend on the laboratory's DNA extraction protocol, although it should be stressed that a real possibility already exists of doing mutation studies based on a single 5-µm section); and lastly one or two sections for *ALK* tests (Fig. 2).

One issue being debated is whether the two biomarkers should be tested simultaneously or sequentially. Although they should ideally be done simultaneously, this is not possible in all cases. Therefore, a pragmatic way of saving tissue and time is to plan for simultaneous testing even though tests may be done sequentially. This means sections for both *EGFR* and *ALK* are cut at the same time, although testing for one of the biomarkers may only go ahead if the other proves negative.

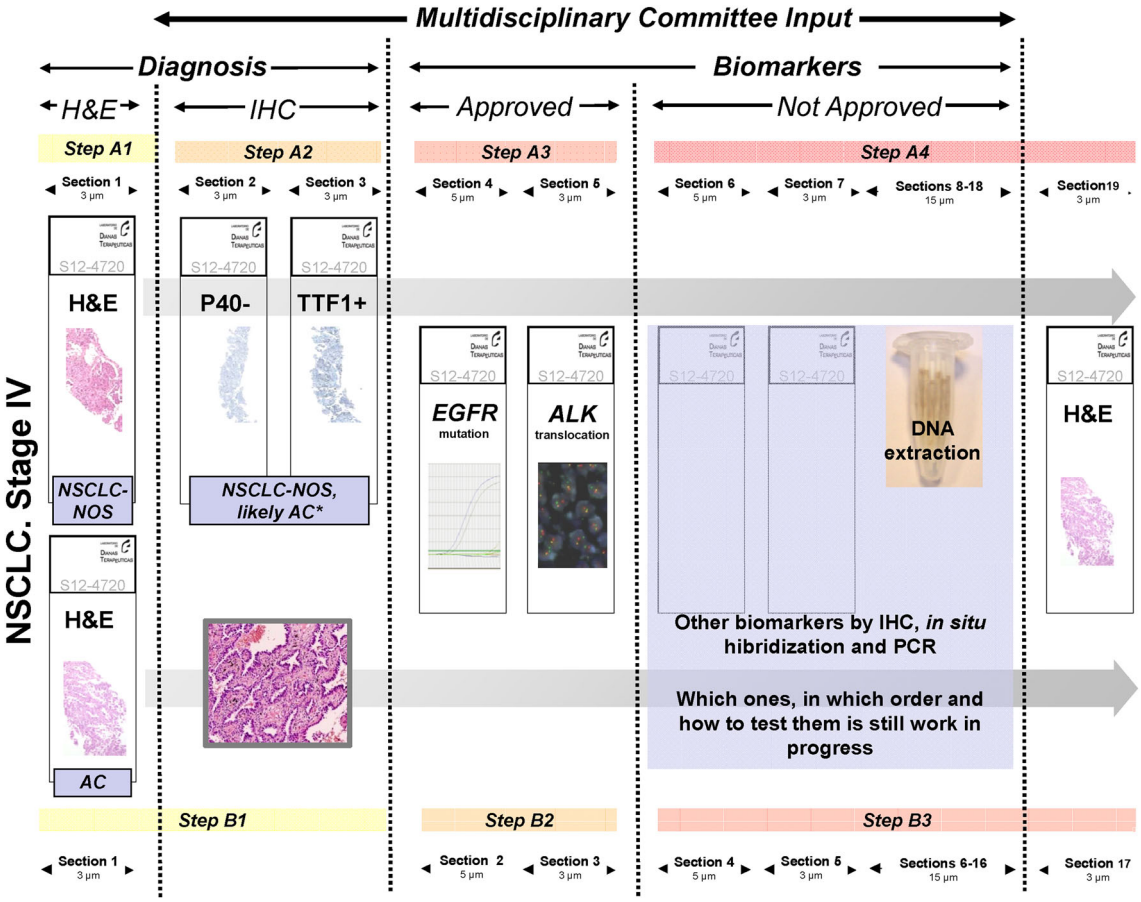


**Fig. 2** Protocol for multiple biomarker testing on samples from advanced NSCLC patients. The *upper route* (**a**) is for cases that require classificatory IHC. The *lower route* (**b**) is for samples in which this step is unnecessary because H&E clearly shows glandular differentiation. Adapted from Clin Transl Oncol 2013;15:503–508. *AC* adenocarcinoma, *ALK* anaplastic lymphoma kinase, *DNA* deoxyribonucleic acid, *EGFR* epidermal growth factor receptor, *H&E* haematoxylin/eosin, *IHC* immunohistochemistry, *NOS* (type) not otherwise specified, *NSCLC* non-small-cell lung cancer, *PCR* polymerase chain reaction

## Which *EGFR* mutations should be tested for, by which techniques?

### *EGFR* mutations to test for

In general, there is agreement with the consensus opinion of the College of American Pathologists, International Association for the Study of Lung Cancer, and Association for Molecular Pathology that clinical *EGFR* mutation tests should be able to detect all individual mutations that have been reported with a frequency of at least 1 % of *EGFR*-mutated lung adenocarcinomas [18, 19]. Performing tests to detect only the two major mutations is no longer considered acceptable (Table 1). Mutation analysis should be done on exons 18–21 of the *EGFR* tyrosine kinase domain. Two types of mutations represent 90 % of all *EGFR*-activating mutations: deletions in exon 19 (around codons 746–750) and mutation in exon 21 (L858R). Point mutations in exons 18 and 20 (including T790M), and insertions in exon 20 account for another 2–5 and 5–10 %, respectively.

Although there is a growing trend towards extensive molecular characterisation of tumours, it is recommended that unusual mutations be reported separately, with a comment about their unclear or uncertain clinical significance until proven otherwise. Some recent publications may help to understand the clinical meaning of some of these rare mutations [20, 21].

**Table 1** *EGFR* mutations

| Exon | Codon | Mutations | % of all *EGFR* mutations |
|---|---|---|---|
| 18 | 709 | E709K, E709A, E709G, E709V, E709D, E709Q | 1 |
| | 719 | G719S, G719C, G719A, G719D | 2–5 |
| 19 | 739–744 | Insertions | 1 |
| | 746–753 | Deletions: 9, 12, 15, 18 or 24 bp | 45 |
| 20 | 763, 764 | Insertions | 5–10 |
| | 767–774 | Insertions[a]: 3, 6, 9, 12 bp | |
| | 768 | S768I | 1–2 |
| | 790 | T790M[a] | 2 |
| 21 | 858 | L858R | 40 |
| | | L858M | Rare |
| | 861 | L861Q, L861R | 2–5 |

*EGFR* epidermal growth factor receptor

[a] Mutations associated with tyrosine kinase inhibitor (TKI) resistance

### Techniques for testing for *EGFR* mutations

A distinction can be made between systematic/comprehensive detection methods, which detect all mutations in exons 18–21, including new variants, and "targeted" methods, which detect specific mutations. Although this is a rapidly changing field, some practical recommendations can be made. If possible, a highly sensitive method should ideally be used (<5 %), such as real-time polymerase chain reaction (PCR) (recent guidelines from the National Institute for Health and Care Excellence [NICE] select two specific methods). Direct sequencing should only be used for samples containing at least 50 % tumour cells. Laboratory reports should always specify which mutations were detected systematically and the sensitivity of detection methods used.

## Which techniques should be used to test for *ALK*?

At the moment there are basically three techniques that enable rearrangement of the *ALK* gene to be detected in clinical samples. These are immunohistochemistry, fluorescence in situ hybridization (FISH) and reverse transcription PCR (RT-PCR). The only test approved by the United States Food and Drug Administration (FDA) is FISH with the commercial probe Vysis® ALK Break-Apart FISH Probe Kit (Abbott Molecular, Inc.), which was approved as a companion test for the drug crizotinib [22]. Likewise, it is worth noting that a FISH scanner recently received FDA approval for reading the above-mentioned *ALK* probe [23]. On the other hand, there are copy number alterations in the *ALK* gene that seem unrelated to a good response to the specific drug, although cases in which that copy number is increased must be studied in depth so as not to miss possible cases of atypical presentation [24, 25].

The advent of clones of the right sensitivity to be employed in immunohistochemistry, such as D5F3 and 5A4, and the use of polymer-based amplification techniques to enhance the immunohistochemical signal, have led to these being proposed as a screening method prior to confirmation by FISH [19, 26]. Most published series show very high concordance between FISH and immunohistochemical techniques [22, 27–29], although false positives and negatives exist with both methodologies. Reduced sensitivity is probably the greatest risk, so it is very important to: (1) try to control pre-analytical parameters, which particularly affect immunohistochemistry; and (2) be aware of the difficulties involved in interpreting FISH (it is sensible to verify results in cases of unusual patterns). As a practical rule, we recommend examining the *ALK* gene by two methods when any uncertainties of any kind exist regarding the test result. In our experience, the most common have been: dubious treatment response in a case reported as positive, tests in squamous cell carcinomas, immunohistochemical stain that is not clearly granular, and polysomies and monosomies in FISH assays. As regards

the molecular technique RT-PCR, although it may display appropriate sensitivity and specificity, it is not recommended by the College of American Pathologists [19].

## In which situations should the patient be re-biopsied?

Taking another sample of tumour material is usually contemplated in two situations: (1) at the time of initial diagnosis; and (2) under certain circumstances when disease progression occurs. In both cases, the purpose is to obtain guidance as to the best treatment option.

### Performing a re-biopsy when beginning diagnosis

The decision to re-biopsy should be based on three fundamental aspects that will provide an estimate of the real benefit: (1) the patient's clinical features; (2) tumour pathology; and (3) the risks of re-biopsy. From the indication perspective, an assessment should be made as to whether re-biopsy is going to be essential for making the best treatment decision. With regard to the risks of this procedure, the technical difficulty of performing it must be considered along with its possible morbidity and the potential delay entailed in starting treatment. It is therefore essential to inform the patient properly of the pros and cons of obtaining more tumour material, and the reasons and aims behind this decision. With all these considerations, it is recommended that the tumour be re-biopsied when any of the following circumstances apply: (1) whenever molecular tests yield uninformative results or there is not enough tumour material to do them, and the clinicopathological features of patient and tumour point to the possible presence of a therapeutic target. Examples of this might be patients below the usual age of disease presentation or who have never smoked, or suggestive pathology such as signet-ring cells [30] or a lepidic or micropapillary patterns [31]; (2) when there is a discrepancy between the patient's clinical profile and the pathology result obtained. For example, when the diagnosis is of squamous or small-cell carcinoma in a patient who has never smoked or is very young, the pathology tests should be reviewed or, in any case, consideration given to conducting the established molecular tests for tumours of non-squamous histology. Similarly, when the initial pathological diagnosis is consistent with non-small-cell carcinoma not otherwise specified (NOS) this same approach can be taken.

### Performing a re-biopsy on disease progression

In the event of disease progression, a re-biopsy may be performed in two situations: (1) when the course of the disease is abnormal and confirmation of the initial diagnosis is desired; and (2) when further molecular tests are wanted, to provide guidance on the treatment to follow [32]. It is advisable for molecular tests to be ordered on re-biopsies to find mutations after resistance has been acquired to EGFR-TKIs [33]. The *EGFR* T790M mutation is the most common (50–70 %) and drugs exist with proven efficacy against this mutation, such as AZD9291 and CO-1686 [34, 35]. In patients harbouring an *ALK* rearrangement who progress on an inhibitor, biopsy may be helpful to determine the cause of progression, although the treatment to follow in this situation has not yet been defined [36].

## Which other biomarkers are currently of interest?

In NSCLC, other biomarkers are being investigated for their potential interest as predictors of efficacy for certain drugs in the clinical trial setting, but there is as yet no scientific evidence to warrant recommending them in clinical practice [19, 37, 38].

### *KRAS* mutation

The *KRAS* gene appears mutated in about 20 % of all cases of NSCLC, especially in adenocarcinomas and smokers. It is the most common oncogenic mutation and its prognostic value has not been clearly proven. Various drugs are currently under investigation, especially those that act downstream in the RAS signalling pathway, such as MEK1/MEK2 inhibitors, which are also being tested for efficacy in the *KRAS* wild-type population, and inhibitors of the PI3 K/AKT/mTOR signalling pathway [39].

### *MET* alteration

*MET,* located on chromosome 7q21-q31, codes for a receptor tyrosine kinase activated by its specific natural ligand, hepatocyte growth factor (HGFR). *MET* can be mutated rarely in NSCLC (1 %), amplified (3–7 %) or over-expressed (25–75 %), implying a worse prognosis. These alterations occur with any NSCLC histology irrespective of the presence of *KRAS* or *EGFR* mutations. Ten to twenty per cent of patients with *EGFR*-mutated tumours acquire EGFR-TKI resistance through *MET* amplification. Some monoclonal antibody or TKI MET inhibitors are under investigation, administered in combination with other targeted therapies or chemotherapy, or as monotherapy.

### *ROS1* translocation

*ROS1* codes for a receptor tyrosine kinase and appears translocated in 1–2 % of patients with NSCLC, especially

non-smokers, in young patients with adenocarcinomas and no *EGFR*, *KRAS*, *BRAF* or *HER2* mutations or *ALK* translocation [40]. Crizotinib has shown activity in these patients. Shaw et al. [41] conducted a study in 50 patients with advanced NSCLC who were positive for *ROS1* rearrangement. This study was an expansion cohort of the phase I trial of crizotinib. ORR was 72 % (95 % confidence interval: 58–84), with 3 patients in complete response and 33 patients with partial response. The median duration of response was 17.6 months (95 % confidence interval: 14.5-not reached). The result in terms of median PFS was 19.2 months (95 % confidence interval: 14.4-not reached) and 50 % of patients are still in follow-up for progression. No correlation was detected between the type of *ROS1* rearrangement and the clinical response to crizotinib. The authors concluded that crizotinib presented noticeable antitumor activity in patients with advanced *ROS1*-rearranged NSCLC and that *ROS1* rearrangement represents other molecular subgroup of NSCLC for which crizotinib is very active.

*BRAF* mutation

BRAF is a specific serine/threonine protein kinase located downstream in the RAS signalling pathway. This mutation is present in 1–3 % of NSCLCs, as a V600E mutation in more than half of cases, and appears especially in adenocarcinomas, and in smokers and ex-smokers [42]. Various BRAF inhibitors that have shown activity in initial studies are being investigated, and the strategy of combining them with MEK inhibitors is also being considered, especially for patients with non-V600E mutations.

*RET* translocation

*RET*, which encodes a receptor tyrosine kinase, is rearranged with an incidence of 1.4–2 % in NSCLCs. This is detected mainly in adenocarcinomas, non-smokers and young patients, in the absence of other genetic alterations (*EGFR*, *KRAS*, *BRAF*, *HER2* or *ALK* translocation) [43]. Various RET inhibitors are under investigation at present. Most of these agents are active against multiple kinases.

*HER2* mutation

HER2 is a membrane tyrosine kinase in the ERBB family over-expressed in 20 % of NSCLCs, but the gene is only amplified or mutated in 2–4 % of cases. Mutation is seen particularly in women, non-smokers, adenocarcinomas and Asian patients. It generally involves an insertion in exon 20 and is mutually exclusive with *EGFR* or *KRAS* mutations [44]. In early studies, irreversible HER2 and EGFR-TKIs especially showed activity in NSCLC patients, and combining them with other targeted therapies, such as mTOR inhibitors, is being evaluated, because *HER2* mutations depend on the AKT/mTOR pathway.

Other potential biomarkers

Other potential biomarkers of interest, particularly in squamous cell carcinomas, are *PI3KCA* mutations or amplification, *FGFR1* amplification, PD-L1 expression and *DDR2* mutations.

## Which other techniques are currently of interest?

Faced with the challenge of needing to test for multiple biomarkers to optimise the efficacy of novel therapies, recent technical advances with next-generation systems that allow massive gene sequencing in a single test provide a good opportunity to tackle this new scenario. It must be stressed that these tests involve dozens of genes and thousands of mutations (colloquially termed "targeted massive sequencing"), but we are not talking about exome or whole genome sequencing. These technologies are highly robust for mutation testing, but can present problems of sensitivity and specificity for looking at insertions or small deletions. In general, it is highly advisable, or even essential in many tests, to have accompanying normal tissue (or blood) available. Although technically feasible, it is not clear how translocation studies perform on formalin-fixed, paraffin-embedded samples, or how robust they are. A good option would be to do the most prevalent and/or really essential tests by conventional methods and reserve these more massive approaches for pan-negative cases, e.g. patients who test negative for *EGFR, ALK, KRAS, RET, ROS1, BRAF* and *HER2*. Interestingly, druggable amplifications (i.e. *MET* or *HER2*) typically occur in oncogene-negative lung adenocarcinomas [45]. Therefore, we must ensure in this latter subgroup that amplifications are adequately studied with next-generation sequencing or FISH.

## What is the minimum amount of material needed for biomarker testing?

The minimum number of cancer cells a sample must contain for lung cancer to be diagnosed and molecular tests done is variable, because it depends on many factors, such as: (1) the type of diagnostic method to be used; (2) the presence of abundant stromal/inflammatory elements; (3) the existence of extensive necrosis, bleeding or fibrosis; (4) tissue quality, antigen preservation or DNA/ribonucleic acid (RNA) integrity; (5) the existence of genomic alterations such as aneuploidies, polysomies and amplifications.

Depending on the case, it is important either to have an absolute number of representative tumour cells or for the proportion of these to reach a certain value. For cytogenetic studies, a minimum of 100 tumour cells is recommended, for technical reasons, whereas molecular tests require a proportion of at least 30 % tumour cells (for direct sequencing assays) or 5 % tumour cells (for real-time PCR assays). Technology exists that allows mutations to be detected when the tumour population represents 10 % or even less (1–0.1 %), but care must be taken with these ultra-sensitive methods, because they can yield artefacts and false positives more often [19]. It is assumed that, with the technologies available, 250–500 ng of DNA/RNA is enough to conduct molecular tests that examine different genes simultaneously [46].

It is crucial for institutions to establish the necessary strategies to optimise biomarker studies [47], and for each laboratory to determine its own threshold of analytical sensitivity, or limit of detection, to validate its procedures. For cases of low yield, the possibility of performing another biopsy should be assessed [19]. If the resulting test proves negative and the tumour percentage is close to the method's limit of detection, it is more sensible for the diagnosis to be "inconclusive", to provide an opportunity for the test to be repeated if re-biopsy takes place, as described above.

## What are the applications of liquid biopsies?

The molecular profile of interest for solid tumours is currently obtained, as stated above, from surgical specimens or biopsies. However, the biopsy procedure cannot always be carried out routinely because of its invasive nature. Moreover, information obtained from a single biopsy provides a snapshot of a tumour, at a fixed time and place, and might not reflect its heterogeneity. As an alternative to tissue samples, there is growing interest in studying liquid biopsies or samples of the patient's blood, by means of the molecular characterisation of circulating tumour cells (CTCs) and examining circulating free DNA (cfDNA) in serum [48, 49].

The study of CTCs in lung cancer has been less thorough than with other tumour types [50]. Counting them might be useful for prognosis and in monitoring treatment response. In the last few years, some studies have demonstrated the possibility of genetic and cytogenetic characterisation of CTCs captured using sophisticated technologies such as microfluidic filters ("CTC-Chip"). A study conducted at Massachusetts General Hospital (MGH) showed 95 % sensitivity for detecting *EGFR*-activating mutations using the Scorpion Amplification Refractory Mutation System (SARMS) in 20 patients, 11 of whom also had the T790M resistance mutation [51].

The potential usefulness of serum cfDNA analysis includes early detection, monitoring treatment response and detecting recurrence, and particularly testing for molecular abnormalities that affect treatment aimed at molecular targets, and analysing changes in tumour genotype after treatment pressure [4, 49]. With the latter, sensitivity depends on stage and tumour burden, as well as technical factors. Sensitivity for testing for specific alterations such as *EGFR* or *KRAS* mutations in NSCLC with advanced technologies (digital PCR, Beads, Emulsions, Amplification and Magnetics [BEAMing] and next-generation sequencing [NGS]) is less than 0.01 %, so genetic alterations of interest could be identified in $\geq$90 % of patients with Stage IV disease [4, 52].

At the present time, the characterisation of liquid biopsies must be regarded as belonging essentially to the research setting. In exceptional cases, the usefulness of these procedures might be considered, particularly for identifying *EGFR* mutations in cfDNA, in the event of incomplete molecular typing of advanced NSCLC and technical difficulties or medical contraindications preventing conventional sampling.

## Conclusions

It is already a fact that patients with advanced NSCLC necessitate not just pathology data but also the results of predictive biomarker tests. There is now sufficient evidence for tests for *EGFR* mutations and *ALK* translocation to be recommended in patients with Stage IV non-squamous NSCLC regardless of smoking habits, and in non-smokers irrespective of histology. Although the two biomarkers should ideally be tested simultaneously, this is not possible in all cases. Therefore, a pragmatic way of saving tissue and time is to plan for simultaneous testing even though tests may be done sequentially. Technological advances now make it possible to work with sample prioritisation protocols to achieve these objectives, always supported by quality controls and training courses. Performing the whole process of analysing sample morphology and pathology plus predictive biomarkers quickly and to high quality standards is only feasible by working together efficiently in a multidisciplinary fashion.

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