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SPECIAL ARTICLE

Guidelines for biomarker testing in advanced non-small-cell lung cancer. A National Consensus of the Spanish Society of Medical Oncology (SEOM) and the Spanish Society of Pathology (SEAP)

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Abstract Patients with advanced non-small-cell lung cancer (NSCLC) carrying epidermal growth factor receptor *(EGFR)* mutations can now have specific treatment based on the result of biomarker analysis and patients with rearrangements of the anaplastic lymphoma kinase *(ALK)* gene will probably soon be able to. This will give them better quality of life and progression-free survival than conventional chemotherapy. This consensus statement was conceived as a joint initiative of the Spanish Society of Medical Oncology (SEOM) and the Spanish Society of Pathology (SEAP), and makes diagnostic and treatment recommendations for advanced NSCLC patients based on the scientific evidence on biomarker use. It therefore provides an opportunity to improve healthcare efficiency and resource use, which will undoubtedly benefit these patients. Although this field is in continuous evolution, at present, with the available data, this panel of experts recommends that all patients with advanced NSCLC of non-squamous cell subtype, or non-smokers regardless of the histological subtype, should be tested for *EGFR* gene mutations within a maximum of 7 days from the pathological diagnosis. Involved laboratories must participate in external quality control programmes. In contrast, *ALK* gene rearrangements should only be tested in the context of a clinical trial, although the promising data obtained will certainly justify in the near future its routine testing in patients with no *EGFR* mutations. Lastly, routine testing for other molecular abnormalities is not considered necessary in the current clinical practice.

Keywords Non-small-cell lung cancer · EGFR · ALK · KRAS · HER-2 · BRAF · Biomarkers

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Introduction

Lung cancer poses a considerable health challenge. Of more than 11 million new cancer cases worldwide, one in every eight is lung cancer, and over 1.1 million people in the world die each year of this disease. In Spain, it is estimated that there will be 24,500 incident cases of lung cancer in 2012, and this is the largest cause of cancer deaths in men (19,681 cases) and the third largest in women (4,011 cases). Similar data are obtained from analysis of the European, the North American or even the worldwide figures [1].

In the last few years, there have been many changes in the management of lung cancer, particularly non-small-cell lung cancer (NSCLC), notably the recognition of biomarkers allowing treatment selection in some subgroups of patients with advanced disease. The decision as to which biomarkers should be tested, and in which patient subgroup, is critical and must also be made in a timely fashion, because it is the first treatment a cancer patient has that gives him or her the best chance. Moreover, biomarker tests must be conducted in a healthcare setting that ensures proper quality controls and availability of the results within the recommended timescale.

There is no question that the complexity of cancer management increasingly demands close collaboration between different professionals, and the successful implementation

of individually tailored treatment strategies based on the use of biomarkers in the healthcare setting requires coordination between pathologists and oncologists.

The first biomarker, and hence the basis on which this relationship should be built, is the appropriate collection and handling of the specimen, and the resulting pathological diagnosis. With this in mind, this consensus statement was conceived as a joint initiative of the Spanish Society of Medical Oncology (SEOM) and the Spanish Society of Pathology (SEAP). It has been written by 10 experts (5 clinical pathologists and 5 medical oncologists) with the purpose of making diagnostic and treatment recommendations for advanced NSCLC patients based on the scientific evidence.

This document is intended to serve as a framework to enable the cancer committee at each site, in agreement with the hospital management team, to make appropriate arrangements to ensure that advanced NSCLC patients eligible for biomarker tests have access to them and that those tests are conducted as recommended by the scientific societies, with proven quality procedures and within a clinically reasonable timescale.

This document fosters standardisation in hospitals and promotes quality in healthcare, encouraging a reduction in clinical variability for organisational reasons. It therefore provides a great opportunity to improve healthcare efficiency and optimise the use of available resources, which will undoubtedly result in a clear benefit to patients.

Clinical issues

Despite the progress made, it has only proved possible so far to identify molecular abnormalities in approximately 50% of adenocarcinomas [2]. Although *KRAS* mutation is the most common, epidermal growth factor receptor *(EGFR)* mutations and anaplastic lymphoma kinase *(ALK)* rearrangements deserve special mention because of their clinical significance.

Clinical importance of *EGFR* mutation

EGFR is a transmembrane glycoprotein composed of an extracellular amino-terminal ligand-binding domain, a hydrophobic transmembrane helix, a cytoplasmic portion containing the tyrosine kinase domain and a carboxy-terminal region containing tyrosine residues and receptor-regulating elements. Ligand binding to the extracellular domain leads to oligomerisation of the receptor, which activates the tyrosine kinase portion of the molecule and causes autophosphorylation of both receptor domains.

Although various *EGFR*-related abnormalities exist, such as gene amplification and protein over-expression, only the presence of gene mutations is currently regarded as a predictive factor for efficacy of treatment with EGFR tyrosine kinase inhibitors (EGFR-TKIs). These mutations were first identified in NSCLC patients in 2004 and their discovery represented recognition of a molecular subgroup of clinically different carcinomas [3, 4]. Mutation leads to increased growth factor activity and brings about conformational changes causing the mutant cell to become 'addicted' to EGFR signals so that, when an EGFR-TKI is administered, activation ceases and cell death is triggered. In our setting, *EGFR* mutations are found to be present in 5–15% of NSCLC cases [5].

The most common mutations (85–90%) are in-frame deletions of 9, 12, 15, 18 or 24 nucleotides in exon 19 and CTG/CGG point mutations in exon 21 (L858R). Other less common ones exist (L861Q in exon 21, G719A/C/S in exon 18 and S768I in exon 20), but the behaviour of these is less well understood. Resistance mutations have also been described, such as T790M in exon 20.

The importance of EGFR-TKI treatment in patients with tumours harbouring activating mutations stems from many studies, although it was the results of Phase III trials with gefitinib or erlotinib that demonstrated its benefit compared with standard chemotherapy treatment (Table 1). As well as these two EGFR-TKIs, there are others, the final role of which in clinical practice will depend on the results of studies currently in progress.

The IPASS study

The IPASS study involved 1217 patients with advanced NSCLC selected on the basis of clinical criteria who were randomised to gefitinib treatment or carboplatin and paclitaxel [6]. The study demonstrated gefitinib non-inferiority, with median progression-free survivals (PFS) of 5.7 months for gefitinib and 5.8 months for the chemotherapy arm. A retrospective efficacy analysis performed on 437 patients (36%) who had a sample available for *EGFR* mutation testing, which proved positive in 261 cases, demonstrated the superiority of gefitinib in terms of PFS (HR=0.48; $p<0.0001$) and response rate (71.2% vs. 47.3%; $p=0.0001$). The interaction between *EGFR* mutation and treatment was significant (interaction test $p<0.001$). In contrast, in patients with wild-type carcinomas, chemotherapy was significantly more effective. On the other hand, tolerance and quality of life were better in the gefitinib arm.

The First-SIGNAL study

This study recruited 309 non-smoking Korean patients with advanced adenocarcinoma of the lung [7]. The main objective, to demonstrate a 40% increase in overall survival (OS) comparing gefitinib with cisplatin and gemcitabine, was not met (HR=1.02; $p=0.42$), but statistically significant differences were observed in terms of PFS, quality of life and tolerance in favour of gefitinib. In the subgroup analysis, performed on 31% of patients enrolled, *EGFR* mutations were found in 44% of them. In this subgroup, patients treated with gefitinib achieved superior PFS (HR=0.61; $p=0.084$).

Table 1 Randomised phase III studies comparing gefitinib or erlotinib with chemotherapy as first-line treatment in advanced NSCLC patients with *EGFR* mutation

Study	No. of patients	Patient screening	EGFR-TKI	CT	Response rate (%)		PFS	
					TKI	CT	HR	*p*
IPASS [6]	1217 (261 EGFR+)	Clinical	Gefitinib	Carboplatin/paclitaxel	71.2[a]	47.3[a]	0.48[a]	0.000[a]
First-SIGNAL [7]	309 (42 EGFR+)	Clinical	Gefitinib	Cisplatin/gemcitabine	84.6[a]	37.5[a]	0.61[a]	0.084[a]
WJTOG3405 [8]	177	*EGFR* mutation	Gefitinib	Cisplatin/docetaxel	62.1	32.2	0.48	0.0001
NEJ002 [9]	230	*EGFR* mutation	Gefitinib	Carboplatin/paclitaxel	73.7	30.7	0.30	0.001
OPTIMAL [10]	165	*EGFR* mutation	Erlotinib	Carboplatin/gemcitabine	83	36	0.16	0.0001
EURTAC [11]	174	*EGFR* mutation	Erlotinib	Platinum doublets	54.5	10.5	0.42	0.0001

[a]In patients with *EGFR* mutation
CT, chemotherapy; HR, hazard ratio

Study WJTOG3405

In this Japanese study gefitinib was compared with cisplatin and docetaxel in 177 advanced NSCLC patients carrying *EGFR* mutations [8]. The results showed a significant difference in PFS, which was the primary endpoint of the study, in favour of gefitinib (medians of 9.2 vs. 6.3 months; HR=0.48; *p*<0.0001), in response rate and in toxicity profile.

Study NEJ002

This Japanese study, in 230 advanced NSCLC patients with *EGFR* mutations, aimed to demonstrate superiority of gefitinib vs. carboplatin and paclitaxel in terms of PFS [9]. The interim analysis originally planned detected a significant difference in favour of gefitinib (medians of 10.8 vs. 5.4 months; HR=0.30; *p*<0.001), and in response rate too, so the study was closed. Treatment tolerance was again better for gefitinib.

The OPTIMAL study (CTONG 0802)

This study, conducted in China, compared first-line erlotinib treatment with carboplatin and gemcitabine in 165 advanced NSCLC patients carrying *EGFR* mutations [10]. It found a statistically significant difference in PFS, the primary endpoint, in favour of erlotinib (medians of 13.1 vs. 4.6 months; HR=0.16; *p*<0.0001), as well as a better response rate and more favourable toxicity profile.

The EURTAC study

This study, coordinated by the Spanish Lung Cancer Group (GECP), and the only one conducted in a Caucasian population, compared erlotinib with platinum-based chemotherapy in 174 advanced NSCLC patients with *EGFR* mutation [11]. The study favoured erlotinib in terms of the primary endpoint, PFS (medians of 9.4 vs. 5.2 months; HR=0.42, *p*<0.0001), and response rate and tolerability.

These studies thus support the use of EGFR-TKIs as the treatment of choice in advanced NSCLC patients carrying an *EGFR*-activating mutation, as stated in national and international treatment guidelines [12–14]. It is important to stress that none of the studies described demonstrated a statistically significant benefit in terms of OS. This may be explained by the large numbers of patients who receive EGFR-TKI treatment after progressing on chemotherapy.

Clinical importance of *ALK* rearrangement

ALK is a 1620 amino acid transmembrane protein consisting of an extracellular domain with amino-terminal signal peptide, an intracellular domain with a juxtamembranous segment harbouring a binding site for insulin receptor substrate-1 and a carboxy-terminal kinase domain. ALK is a member of the insulin receptor tyrosine kinases and its physiological function remains unclear. This tyrosine kinase receptor was first identified as part of the t(2;5) chromosomal translocation associated with most anaplastic lymphomas and a subset of non-Hodgkin lymphomas [15]. It has recently become clear that a subset of human cancers, like NSCLC, activate ALK signalling by creating oncogenic fusions of the *ALK* gene with a variety of partners [16]; these oncogenic fusion proteins lead to the activation of the ALK kinase domain [17, 18].

Echinoderm microtubule-associated protein-like 4 (EML4) is a cytoplasmic protein, which involves the formation of microtubules. *EML4-ALK* is a fusion of genes arising from an inversion on the short arm of chromosome 2 [Inv (2)(p21p23)] that join exons 1–13 of *EML4* to exons 20–29 of *ALK* [18].

Several variations of *EML4-ALK* fusions have been identified in NSCLC, as well as other rare fusion partners like *TFG-11* and *KIF5B* [19]. Equivalent to EGFR mutations, *ALK* rearrangements result in a constant tyrosine kinase activity and dependence of the cancer cell on activated downstream mitogenic pathways, and an exquisite sensitivity to ALK inhibition represents another case of 'oncogene addiction' [19].

ALK gene rearrangements can be identified by fluorescent *in situ* hybridisation (FISH), immunohistochemistry (IHC) and reverse transcription-polymerase chain reaction (RT-PCR). Of these, FISH appears to be the most clinically applicable diagnostic procedure. The presence of *EML4-ALK* fusion is identified in 2–7% of adenocarcinomas [2].

In general, patients with metastatic NSCLC harbouring *ALK* rearrangements tend to be younger and have little (<10 pack-years) to no smoking history [20, 21]. Most cases are adenocarcinomas with intracellular mucin pro-

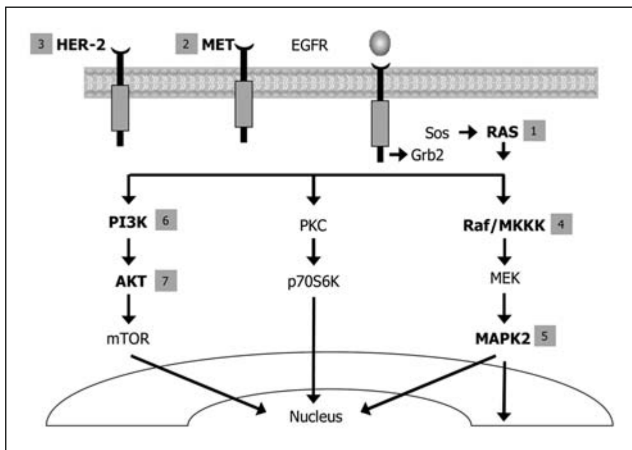

Fig. 1 Main molecular abnormalities in NSCLC patients

duction. The presence of ALK rearrangements appears to be mutually exclusive of EGFR and KRAS mutations.

Currently, several ALK inhibitors are being studied. Crizotinib, which is also an inhibitor of mesenchymal–epithelial transition factor (MET), is the ALK inhibitor in the most advanced phase of clinical research [20]. Crizotinib is administered orally, at a dose of 250 mg, twice daily. Its toxicity profile includes mild nausea and diarrhoea, transient visual disturbances without ophthalmologic findings, as well as elevations in liver and haematologic chemistries. Initial clinical data of the efficacy of crizotinib come from a cohort of 82 patients with *ALK* rearrangements, most of them previously treated and obtained from a cohort of 1500 screened patients with metastatic NSCLC [22]. At a mean treatment duration of 6.4 months, the overall response rate was 57% and 33% of patients showed a stable disease. At the time of data cut-off, 63 patients (77%) were continuing to receive crizotinib and the estimated 6-month PFS was 72%.

These findings have led to the onset of phase III clinical trials of crizotinib compared with chemotherapy in various lines of metastatic NSCLC treatment. The recruiting period of these trials is expected to finish in 2012.

Clinical importance of other biomarkers

As well as the biomarkers described above, there are others under investigation for which a biological role has yet to be defined [2]. Apart from *KRAS* mutations, the incidence of the other molecular abnormalities characterised is less than 5% and they nearly all tend to be mutually exclusive.

RAS activation

Activation of the pathway mediated by *RAS*, especially via *KRAS*, occurs in 30% of adenocarcinomas and 5% of squamous cell carcinomas [23] (Fig. 1). *KRAS* mutations in NSCLC occur in codons 12 and 13 and are associated with smoking habits. Although many studies attribute a negative prognostic value to the presence of these mutations, there is still no conclusive scientific evidence. The search for effective specific inhibitors has so far proved unsuccessful. Therefore, although the detection of *KRAS* mutations may reasonably rule out the existence of *EGFR* or *ALK* mutations, testing for them is not a priority from a clinical point of view.

MET amplification

MET is the gene that codes for hepatocyte growth factor receptor (HGFR), located on chromosome 7q21-q31 (Fig. 1). Although *MET* mutations are rare, amplification has been described in a variable percentage of advanced NSCLC patients (1.4–21%), depending on the detection method used [24]. Unlike the abnormalities described above, this has been identified in both adenocarcinomas and squamous cell carcinomas regardless of the presence of *KRAS* or *EGFR* mutations. In fact, 20% of patients with *EGFR*-mutated tumours acquire EGFR-TKI resistance through *MET* amplification.

Various different MET inhibitors are currently at an advanced stage of clinical research, including both monoclonal antibodies and TKIs, so it is hoped that they will become available in the near future [25].

HER-2 mutation

Human epidermal growth factor receptor 2 (HER-2), also known as c-erbB-2, is over-expressed in 20% of advanced NSCLC patients, whereas amplification or mutation of the gene only occur in 2% of cases, respectively [26]. These mutations occur mainly in women, non-smokers, adenocarcinomas and the Asian population. They tend to consist of insertions in exon 20 and are not found in tumours with *EGFR* or *KRAS* mutations.

Based on the information currently available, the insertions that occur in mutations of this type are thought to cause constitutive activation of the receptor, which confers greater sensitivity to dual TKIs directed against EGFR and HER-2, such as lapatinib or BIBW 2292 [27, 28], but not to inhibitors of EGFR only [29].

BRAF mutation

In patients with advanced NSCLC, the incidence of *BRAF* mutations is 1–3%, and they are not located in the same position as the classical melanoma mutation (Fig. 1).

Unlike *EGFR* and *ALK*, mutations in *BRAF* appear in smokers or ex-smokers (p<0.001) [30]. Specific inhibitors such as PLX4032, which have demonstrated their efficacy in melanomas harbouring this mutation, are currently under development.

PI3KCA mutation

Mutations in the *PI3KCA* gene, which codes for alpha catalytic phosphatidylinositol 3-kinase enzymes, are very uncommon in NSCLC [31] (Fig. 1). They are located in exon 9, can be detected in both squamous cell carcinomas and adenocarcinomas, and may even be present in *EGFR*-mutated tumours [32]. Moreover, *PI3KCA* amplification has also been seen in advanced NSCLC, especially in squamous cell carcinomas, in males and in smokers, but is not necessarily associated with the presence of mutations [33].

Which patients need biomarker tests?

EGFR mutation

At the present time, testing for *EGFR* mutations during disease diagnosis should be considered in a group of advanced NSCLC patients. The most important factors for helping to identify those patients most likely to have an *EGFR* mutation are smoking habits and histological subtype.

In the IPASS study [6], 96% of patients had adenocarcinoma, 3% had bronchioloalveolar carcinoma by the 2004 World Health Organisation (WHO) classification [34] and in only 0.2% of patients was the histological subtype unknown. Smoking habits were another inclusion criterion. The only patients accepted were non-smokers (defined as people who had smoked fewer than 100 cigarettes in their lifetime) or 'former light smokers' (defined as people who had stopped smoking at least 15 years previously and/or smoked 10 pack-years or less). Ninety-three per cent of patients enrolled in the study were non-smokers and 6% were 'former light smokers'. With these clinical features, the frequency of mutations in the study was 59.7%.

Although there are no published guidelines that make specific recommendations as to which patient group should be tested for *EGFR* mutation, in our setting the most important information on the subject is the 2009 publication by Rosell et al. containing the GECP data [5]. In that study, 2105 patients were tested for *EGFR* mutation, which was found to be present in 350 (16.6%). When the frequency of *EGFR* mutations was analysed according to patient characteristics, it was found to be 30% in women, 8.2% in men, 38% in non-smokers, 9.5% in ex-smokers, 5.8% in smokers, 17% in adenocarcinomas, 23% in bronchioloalveolar adenocarcinomas and 11.5% in large cell carcinoma patients. No mutations were seen in any of the 37 patients for whom there was no data about histological subtype.

Thus, based on the information currently available, it is recommended that the following advanced NSCLC patients be tested for *EGFR* mutation: (i) all those diagnosed with non-squamous cell carcinoma and (ii) all non-smokers, irrespective of histological subtype.

EML4-ALK translocation

The fusion oncogene *EML4-ALK* is present in 2–7% of NSCLC cases [35, 36], but its frequency is higher in non-smokers, or in patients with a history of light smoking, and in those with adenocarcinoma. Thus, with these characteristics and in tumours with wild-type *EGFR*, the frequency of this translocation rises to 33% [21].

In the study by Kwak et al. [22], in 82 patients enrolled with ALK translocation treated with crizotinib, 96% had adenocarcinoma, 1% had squamous cell carcinoma and 2% had other types of histology. Seventy-six per cent of patients were non-smokers, 18% smoked less than 10 pack-years and 6% smoked more than 10 pack-years.

Testing for *ALK* rearrangements is currently recommended only in the context of clinical trials. However, the known data are very promising and will almost certainly very soon lead to routine *ALK* testing in some advanced NSCLC patients. In principle, this subgroup should include cases who have tested negative for *EGFR* mutation, according to the criteria discussed above.

Other molecular abnormalities

Routine testing for other molecular abnormalities is not considered necessary because the result does not affect patients' treatment in current clinical practice.

Pathology issues

Pathology departments and referral centres

Pathology departments must work in coordination with the other services involved in the diagnosis and treatment of NSCLC patients. Given the relative complexity of the procedures and the participation of different professionals, a work flow must be established at each site to allow optimisation of resources in order to provide an integrated diagnosis in a timely fashion. In the case of morphology-based studies, such as IHC or FISH, the pathologist's involvement is crucial, as non-pathological structures have to be recognised and distinguished from malignant ones in order for the right diagnosis to be made. When tests are conducted using DNA, RNA or proteins extracted from tissue or cell specimens, they must be checked under the microscope in order to select, with or without microdissection, the most representative parts of the lesion and plan the procedures to be carried out, bearing in mind that specimens tend to be small and there may be tumour heterogeneity and/or divergent differentiation.

Biomarker testing in advanced NSCLC should meet the following requirements: (i) a high level of scientific/technical quality; (ii) assured patient safety; (iii) application of efficiency criteria; and (iv) compliance with ethical principles and current legislation, bearing in mind that these are healthcare activities, not biomedical research. Issues of traceability, report writing, specimen preservation, safekeeping and management are particularly important. Pathology departments and referral centres must be duly accredited to carry out these tests and comply with the legislation (Law 14/2007, Law 44/2003, Royal Decree 1277/2003, Royal Decree 1691/1989, Law 55/2003).

Of course, complex molecular tests are not done at all healthcare institutions. There is therefore a need for referral centres to be set up that offer these tests, among other services, on an institutional basis and as a network. These referral centres should be established based on: (i) staff qualifications; (ii) the nature of the facilities; (iii) the volume of cases per year; (iv) the development of scientific or training activities; (v) participation in quality assurance programs, such as the one run by SEAP and the International Academy of Pathology (IAP) [37]; and lastly, (vi) accreditation of these sites by a competent body such as

the National Accreditation Entity (ENAC) (UNE-EN-ISO 15189:2007; UNE-EN-ISO 9001:2008).

Accurate histological diagnosis as the first biomarker

The histological classification of lung cancer was defined by the WHO in 1999, and updated in 2004. Some varieties of carcinoma have been misinterpreted or disputed, but there has been no alternative classification since 2004 [34]. In 2011, a new proposed classification for adenocarcinomas was published [38]. Although it is not yet known whether this new classification will be incorporated into daily practice, the fact that it is promoted and sponsored by the International Association for the Study of Lung Cancer (IASLC), the American Thoracic Society (ATS) and the European Respiratory Society (ERS) suggests that it will become widely used.

The aim of this document is to suggest diagnostic guidelines that enable treatment selection, minimising the use of techniques that entail loss of material.

NSCLCs should be sub-classified by separating squamous cell carcinomas from the rest, either adenocarcinomas or other uncommon variant carcinomas. The first biomarker to be obtained from a specimen is the morphological diagnosis. A generic diagnosis of NSCLC is not enough. The use of further techniques, such as IHC, is essential to enable a correct diagnosis to be reached in cases in which little material is available and in poorly differentiated tumours. Terms such as TTF-1, p63 protein expression and cytokeratin 5/6, or napsin, should be familiar to oncologists, because these are what distinguish squamous cell carcinomas from adenocarcinomas. It has been agreed that the most useful marker for distinguishing between the two carcinomas is TTF-1, which is positive in adenocarcinomas. Squamous cell carcinomas, in contrast, are positive for p63 and cytokeratin 5/6. The antibody pair most widely accepted in the literature is anti-TTF-1 and anti-p63, together with others such as anti-napsin [39].

Molecular tests are carried out on tissue material, although they can also be done on cytology material at specialist sites. The diagnosis is usually made on tissue from a bronchial biopsy or radiologically guided percutaneous needle biopsy. In these cases, it is advisable to base the diagnosis on the first histological section, using only the essential techniques, such as testing for the two immunohistochemical markers mentioned above. The rest of the material is kept for the relevant molecular tests.

Pre-test phase

Preliminary considerations

The quality of any molecular test begins before the tumour specimen is taken, so the staff responsible for obtaining the specimen, the pathology technicians and the actual pathologist must understand the variables that can affect molecular tests.

It is important always to try to obtain as much tissue as possible in the various types of specimen, provided this poses no additional risk to the patient. It is therefore essential for all the specialists on the cancer committee to be involved, particularly the pathologist, both when tests are being done at his or her own site, and when the specimen is being sent to a referral centre.

Specimen types

Various types of specimen exist, depending on the technique used to obtain them, such as endoscopic biopsies, core-needle biopsies, biopsies guided by endobronchial ultrasound (EBUS) or endoscopic oesophageal ultrasound (EUS), fine-needle aspiration (FNA), mediastinoscopy and thoracotomy. These all usually provide good tumour cellularity, although any type of specimen with sufficient tumour representation can be used, provided the minimum requirements set out in Table 2 are met.

The most accessible specimen entailing the least risk to the patient should be chosen, and no major discrepancies have been found in the literature to date to prevent the testing of specimens of metastatic lesions.

All pre-test-phase processing should take place in 'clean' laboratory zones, in which no PCR-related products are handled, following standard molecular pathology recommendations to prevent contamination or inhibition.

Pre-test-phase processing

Hypoxia time and fixation: Fixation should take place as soon as possible and always within one hour of the specimen being obtained [40–42]. It is best to fix the specimen in 10% neutral buffered formalin. Fixatives based on alcohol (B5, Penfix) or containing mercury (Bouin, Zenker) should not be used. Other fixatives are currently being tested. The optimum fixation time is 8–24 h for large surgical specimens and 6–12 h for small specimens.

Cytology specimens fixed immediately by the usual alcohol-based methods can be used and provide high-quality DNA. Cytology specimens in liquid medium or cell blocks are also useful. Cytology specimens can be frozen at –80 C in pH-buffered solutions, such as PBS or citrate.

Selection and histopathology review of specimens: The pathologist should choose the most suitable block or specimen and quantify the proportion of cancer cells in a section just before those being used for molecular testing. In the case of a cytology specimen, a representative smear should be examined.

The proportion of cancer should be stated in the molecular test report. The European consensus recommends a minimum of 50% invasive component if testing is to involve low-sensitivity techniques (direct sequencing) or 10% for high-sensitivity techniques (real-time PCR) [42].

Macrodissection or microdissection: In order to achieve the right proportion of cancer cells, various macro- or mi-

Table 2 Processing conditions according to specimen type for testing for molecular abnormalities in advanced NSCLC patients

Endoscopic biopsies
5 µm sections from the whole block, if the proportion of cancer is over 10% for real-time PCR techniques, or more than 50% for direct sequencing techniques. Number of sections from the block: 10–15. Select malignant fragments by microdissection if the proportion of cancer cells is lower than the above. Minimum recommended amount: 20–30 sections. If there is no tumour specimen left in the paraffin block or if there is not enough, a previously stained preparation can be treated with acetone for 10 min to remove the cover slip and hydrated in 96° alcohol for 24–48 h, if there is a recommended minimum cellularity of 1000 cells.[a]
EBUS, EUS or FNA cytology specimens
If the proportion of cancer is suitable, the cover slip can be removed from a cytological smear and the whole slide scraped with a knife. At sites experienced in laser or needle microdissection, groups of cancer cells should be marked with a marker pen and then with a diamond tip on the slide, and the cells picked using a 25G needle under microscopic control. There should be a recommended minimum cellularity of 500 cells.[a]
Surgical specimens
Mark the area of the tumour containing the highest proportion of tumour/stroma inflammation, avoiding areas of extensive necrosis. Cut five to ten 10 µm sections and separate out the areas of interest.

[a]The minimum number of cells depends on the DNA extraction method (use of commercial kits recommended) and/or the volume of lysis buffer to be used. Specific commercial kits exist for situations of low cellularity

crodissection procedures can be employed. Macrodissection involves separating out the part of the block with the best tumour representation in the paraffin block or sections. Microdissection, by hand or laser, should only be done at sites with extensive experience.

DNA extraction and quality control: The use of commercial kits is recommended for paraffin-embedded or cytology specimens, but manual methods may also be valid in experienced hands. It is inadvisable to use kits that rely on purification columns for specimens of low cellularity. It is best to check DNA quality and concentration by the usual techniques (e.g., 260/280 ratio >1.8 and concentration per microlitre). Various commercial kits include DNA controls, to test for *EGFR* mutations by amplifying a control gene, which perform this function.

Test phase

EGFR mutations

Mutation analysis should be carried out on four exons (18–21) of the *EGFR* gene. Where DNA quantity or quality is a limiting factor, exons 19 and 21 should be tested preferentially [5, 42–51].

Although there are many test methods for *EGFR* mutations, in our setting the most widely used are direct sequencing and real-time PCR (Table 3).

PCR and direct sequencing: Direct sequencing by the Sanger method has the following main advantages over other methods: (i) it is commonly used and available; (ii) there is no need to pool samples for testing; (iii) it has the potential to detect all possible mutations; and (iv) it can identify exactly which mutation is involved.

Conversely, its disadvantages are: (i) low sensitivity (20–50%); (ii) a high risk of contamination because of handling post-PCR products; and (iii) it is more time-consuming, as many steps are required, such as DNA extraction, PCR-based amplification, sequencing and interpretation of the latter.

If this technology is used, it is recommended that:

– PCR and post-PCR work areas should be kept apart and separate materials used exclusively in each. Reaction mixes should be prepared in a laminar-flow cabinet to ensure a sterile environment, while DNA should be added outside that cabinet to minimise the risk of contamination.

– For each PCR reaction, at least one positive control containing pre-validated genomic DNA, and one control containing no DNA, should be included.

– Amplification efficiency and specificity should be checked by agarose gel electrophoresis, so that PCR products can be seen as bands on the gel by staining with ethidium bromide or similar substances.

– PCR products should be sequenced in both directions, i.e., forward and reverse.

Real-time PCR: The most widely used of these techniques is based on Scorpion-ARMS technology, which uses a commercial kit enabling 29 *EGFR* mutations to be identified (TheraScreen® EGFR29 Mutation Test Kit). The advantages of this technique include: (i) higher sensitivity than direct sequencing, of about 5% in our experience; (ii) greater speed; and (iii) fewer cancer cells required.

On the other hand, the disadvantages of this technique are: (i) not all mutations can be identified, only those for which the kit is designed; (ii) samples must be pooled to optimise products; and (iii) it is more expensive than direct sequencing.

If this technology is used, it is recommended that:

– The same precautions are taken as for sequencing in terms of separating pre- and post-PCR areas.

Table 3 Main methods used to test for EGFR mutations

Technique	Sensitivity (% mutated DNA)	Mutations identified	Precise detection of deletions and insertions
Direct sequencing			
Sanger method	25	Known and novel	Yes
Pyrosequencing	5–10	Known and novel	Yes
Real-time quantitative PCR			
TaqMan PCR	10	Known only	No
Scorpions ARMS	1	Known only	No
Mutated allele enrichment techniques			
PNA-LNA PCR clamp	1	Known only	No
Restriction enzyme digestion	0.2	Known only	No
Smart	0.1	Known only	No
COLD-PCR	1–10	Known and novel	Yes
PCR-RFLP	5	Known only	Yes
dHPLC	1	Known and novel	Yes
Immunohistochemistry	Unknown	Known only	Yes

ARMS: amplification refractory mutation system; COLD: co-amplification at lower denaturation temperature; dHPLC: denaturing high-performance liquid chromatography; PNA-LNA: peptide nucleic acid-locked nucleic acid; RFLP: restriction fragment length polymorphism

– A positive DNA control (usually supplied with the commercial kit) and a negative control, or reaction containing no DNA, are included in each test.

ALK translocation

In our setting, the only realistic option for testing for *ALK* translocation is by FISH. The protocol for doing so is essentially no different from that used to study other genes. Only laboratories that perform more than 100 FISH tests per year should undertake this technique, according to the results obtained in comparison with referral centres. There are currently two probes on the market capable of providing evaluable results. These employ different approaches, consisting of break-apart (Vysis) and fusion (Kreatech) probes.

Post-test phase

EGFR mutations

PCR and direct sequencing: It is recommended that specific computer programs be used to enable the sequence or electropherogram obtained to be compared with the wild-type *EGFR* sequence. A sample should be considered positive when the mutation is present in at least two different sequences (one forward and another reverse) obtained from two independent PCR products. The person interpreting the sequences must have proven experience.

Real-time PCR: In principle, the test is conclusive, and cases in which the controls fail to give the expected result should not be interpreted, no matter how consistent they are.

ALK translocation

Interpretation must take probe design into account, because images of separation are generally produced, and predictive information, because signal loss may also be a marker of response. The person interpreting the images must have proven experience.

External quality control

Irrespective of their involvement in external quality control, all laboratories using these technologies must, as a control measure, validate their analytical specificity and sensitivity, as well as their predictive value. SEAP is evaluating the incorporation of other markers into quality control.

EGFR mutations

In 2010, SEAP set up a quality control programme for *EGFR* mutations [37], the main features of which are described below.

The programme is designed to evaluate the various stages, including pre- and post-test phases and interpretation of results. The lead laboratory selects a series of EGFR-mutant and wild-type cases, and these are sent to participating laboratories in the form of unstained sections of formalin-fixed, paraffin-embedded tissue. Each slide is clearly identified with specimen number and section number. Each participating laboratory must use the protocols it employs routinely in clinical practice. In order to assess the percentage tumour cellularity of the specimens sent, it is essential to stain one of the sections sent with haematoxylin-eosin (HE) for review.

Results must be sent anonymously. The ID number of each participating laboratory appears on the box of slides. Participating laboratories must also e-mail the following items to the organiser within 14 days:

– A form containing the requested information about the various stages of *EGFR* gene mutation analysis.

– A test result report for each specimen, based on the report usually sent to doctors ordering mutation analysis.

– The raw test results on which the genotype arrived at for each of the four specimens sent is based. For example, electropherograms if the test was done by direct sequencing of the PCR product or a file containing Ct values if the test was done by real-time quantitative PCR.

The results are evaluated and discussed by a working party. Participants' anonymity must be maintained at all times. Each participating laboratory receives a personal report and another general one containing the results obtained.

ALK translocation
At the moment, no external quality control for this test exists anywhere in the world. In our opinion, however, at the operational level a model should be followed resembling that used for *HER-2* hybridisation in the quality control programmes employed in Spain, the United Kingdom and Scandinavia.

Common issues

Work flow and standardisation of criteria

Where to do the tests
The tests can be done at the healthcare site itself or at a referral centre. If the technique is performed at the healthcare site itself, the laboratory must: (i) have first implemented an appropriate training programme for learning the techniques; (ii) possess the necessary technical resources; (iii) be skilled in the correct interpretation of the results obtained; and (iv) undergo regular quality checks to ensure that it is being done properly.

If the technique is performed at a referral centre, the pathology department at the healthcare site must prepare the biopsy or cytology specimen for shipment, following the existing standard criteria for specimen processing. The systems for preparing and shipping specimens, and for receiving results, should be adjusted to ensure that the process takes no longer than 7–10 days.

Patients eligible for molecular tests
Given the difficulty of obtaining the specimen, the financial cost and the increased workload, candidate patients for biomarker tests must be selected correctly. At the moment, however, at the healthcare level, and pending the results of studies in progress with crizotinib and other drugs, it is only necessary to test for *EGFR* mutations in all patients with histological evidence of non-squamous cell carcinoma, and in non-smokers regardless of their histology.

Although to date the decision as to which patients should be tested for *EGFR* mutations has mainly been taken by the oncologist, it is a good idea for the analysis to be done routinely during initial diagnostic testing of patients with advanced disease. This approach should apply equally to all biomarkers adopted into daily practice.

Optimising the taking of specimens
To enable as much tumour material as possible to be obtained for testing, communication should be encouraged between the medical staff obtaining the specimen (especially chest physicians, thoracic surgeons and radiologists) and the corresponding pathologists. Having clinical information, for example about smoking habits, will help pathologists work in accordance with the proposed algorithms. This will also require a revision of sites' existing protocols for taking and storing specimens, and for transporting them to pathology departments.

Specimen preparation
Lung cancer has three special features of note: (i) Most patients cannot be treated surgically, so the specimens available for diagnostic purposes tend to be cytology preparations or small biopsies. It is therefore essential to optimise their processing in order to obtain as much useful information as possible. (ii) Recent scientific progress is modifying diagnostic and treatment strategies. (iii) These carcinomas display substantial tumour heterogeneity.

However the patient enters the process, he or she must be evaluated by the site's cancer committee in order to decide what actions to take, including the taking of specimens for cytological, histopathological, phenotypic and molecular diagnosis.

It is advisable for the cytology specimens obtained to be evaluated and processed as soon as the laboratory receives them, so that the quality, cellularity and representative nature of the available material can be reported and a preliminary diagnosis given to assist with continuity of care. It is best to use liquid-based cytology for this purpose, as this prevents artefacts and poor smears and yields high-quality preparations for morphology and phenotype studies, as well as DNA and/or RNA from surplus cell material, and also enables storage at room temperature for long periods of time. Although molecular tests can be done with a minimum of 150 cells, reliable results are obtained from 300-1000 [52]. In the absence of liquid-based cytology, making cell pellets is a good option.

When biopsies are small in size, it is important to observe fixation (10% buffered formalin, pH 7.2 for 6–24 hours) and make use of all the tissue. This can be done by saving paraffin-block off-cuts for DNA extraction, placing them in Eppendorf tubes, and then performing serial sections on adhesive-coated slides for routine stains and IHC or FISH.

If necessary, previously stained preparations can be reused to recover tissue and/or cells, even in cases that have already been archived. The results obtained from cytology preparations are identical to those from biopsies and surgical specimens, so both types of sample are valid for molecular tests [53].

On receipt of intra-operative biopsies or fresh tissue, it is advisable to select a fragment of representative tumour material and, better still, another of non-tumour tissue too, to be frozen within 30 min of removal for cryopreservation. The use of preservatives such as RNA*later*® is helpful, to preserve the quality of RNA.

Diagnostic algorithm

A possible diagnostic algorithm for advanced NSCLC patients is suggested in Fig. 2. However, it is important

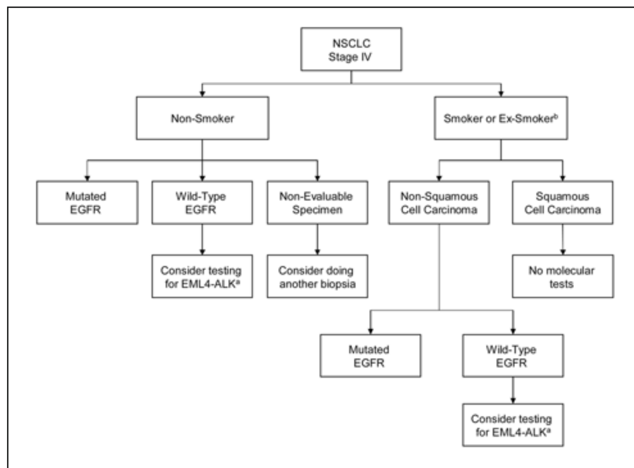

Fig. 2 Diagnostic algorithm for advanced NSCLC patients
[a]In the context of clinical trials of ALK inhibitors; [b]Consider patient's smoking burden

to bear in mind that each diagnostic process must be tailored to the individual characteristics of each patient, and this disease should be managed in a multidisciplinary fashion.

Interpreting the results

The results of tests for the presence of *EGFR* gene mutation must be unambiguous. Many different mutations in this gene have been described, but only those centred on exon 19 (deletions around the LREA motif) and point mutations in exon 21 (L858R) have proven predictive value as far as the response to EGFR-TKIs is concerned. Other much less common mutations have also been described, particularly in codons 718 and 719 of exon 18, in exon 21 (L861Q) and in exon 20 (deletions/insertions). Their significance is uncertain and they are under constant review in the literature.

It is also worth noting that the range of mutations detectable by molecular tests depends on the technique used. These limitations should be stated in the report, to give an indication of the real significance of the result being evaluated.

Moreover, the potential clinical impact of detecting mutations by highly sensitive techniques, such as co-amplification at lower denaturation temperature (COLD-PCR, COLD-pyrosequencing, etc.), is not known, and their use in the healthcare setting is not advisable at present.

Reporting the results

The test results report for any biomarker should contain at least the following information:

– Identification of the patient and the doctor who ordered the test (or, failing that, the authorised person).

– The pathological diagnosis.

– The specimen submitted, with the date on which it was taken, if possible.

– The external code in the case of referral centres.

– The medium in which the specimen was received (fresh, frozen, paraffin-embedded, etc.).

– The anatomical origin of the specimen.

– The order date, the specimen receipt date and the date on which the results were issued.

– The biomarker test method used, specifying detectable mutations and/or other abnormalities. In the case of commercial kits, the commercial name, the batch number and whether they are an approved '*In-vitro* diagnostics' (IVD) product should be stated.

– The quality of the sample, specifying the percentage of cancer cells and whether the sample was enriched by micro- or macrodissection, as well as DNA concentration and purity.

– Comments about the adequate or inadequate nature of the sample.

– The test result, defining the type of molecular abnormality detected or the absence of molecular abnormalities.

– Identification of the professional responsible for the test.

– Identification of the laboratory supervisor.

– Any additional information or comments of interest to the doctor who ordered the test.

– Accreditation or participation in quality programmes.

Recommended and acceptable timescales

The speed of molecular testing is of the essence for the patient so, based on the European consensus, it is recommended that it should take less than 7 days [42].

Two to three days are considered sufficient for taking the specimen, reviewing it histologically to check the amount of tissue and the proportion of available cancer cells, and performing macro- or microdissection techniques. Then, 1 2 days is recommended for DNA extraction, 1–2 days for the test phase and 1 day for interpreting and sending the result. The entire process could therefore be completed in 5 days, allowing 2 extra days in case any tests need to be repeated, which happens in 5–20% of cases. If molecular analysis is going to take more than 7 days, it is important to contact the oncologist and say so.

Future prospects

The opportunities for lung cancer patients to receive individually tailored treatment are expected to increase in the next few years. To date, only a minority of the abnormalities implicated in lung carcinogenesis have been exploited therapeutically. Also, some of the studies currently in progress with different inhibitors directed against targets of potential interest (e.g., Her2, PI3K, mTor, akt, Mek, LKB1, etc.) will come to fruition and they may be added to our therapeutic arsenal. Moreover, it seems likely that the search for new targets will result in the inclusion of new molecules in the future. In this respect, it is worth noting that the actual process of developing new anti-cancer drugs

will, in most cases, entail the concurrent inclusion and development of a biomarker defining the patient population likely to benefit from the drug in question.

This will undoubtedly lead to every effort being made to obtain high-quality tumour tissue with which to make the diagnosis and therapeutic prediction. The involvement of all professionals concerned in caring for lung cancer patients will be crucial for this, particularly those responsible for obtaining specimens (endoscopists and surgeons) and those in charge of optimising and assuring the quality of the information (pathologists and molecular biologists).

The addition of new drugs and biomarkers will also require the algorithms for prioritising specimen use to be reviewed frequently, to take account of criteria such as the usefulness of each biomarker and the benefit offered by each drug, as well as clinical/epidemiological parameters, availability and technological accessibility. Also anticipated in the near future is the development of technologies allowing the use of markers based on specimens easily accessible in clinical practice, such as serum or circulating cancer cells.

Another foreseeable development is the creation of complex biomarkers, based on multiple simple predictors, to provide information about the potential usefulness of not just different monotherapies, but also combinations of them. Technological sophistication must also address the possibility of incorporating different technologies, such as FISH and mutational analysis, into the same diagnostic test.

Lastly, if the use of predictors in clinical practice in lung cancer patient care seems likely to intensify, two further issues require special attention: firstly, the fine-tuning not just of each biomarker at each local site or referral centre, but also of the regulatory systems and quality-control regulations; and secondly, the financial implications for health systems of the greater use of biomarkers. Their use must therefore be optimised and based on consensus opinions evaluating the technology used and its potential usefulness.

Conclusions

The writing of this consensus statement, led by the scientific societies SEOM and SEAP, was guided by the importance of making the right choice of treatment for advanced NSCLC patients and the need for the biomarkers on which this choice is based to be tested according to the strictest quality controls, as quickly as possible.

This document is primarily concerned with healthcare, so recommendations are restricted to those biomarkers that currently delimit patient subgroups with different therapeutic management, validated and based on drugs already on the market. This battery is likely to be extended in the near future, but the proposals that guided the writing of this document, such as working in a multidisciplinary fashion within Tumour Steering Committees, joint decision-making and results of proven quality, will remain valid.

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References

1. Jemal A, Bray F, Center MM et al (2011) Global cancer statistics. CA Cancer J Clin 61:69–90
2. Pao W, Girard N (2011) New driver mutations in non-small-cell lung cancer. Lancet Oncol 12:175–180
3. Lynch TJ, Bell DW, Sordella R et al (2004) Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 350: 2129–2139
4. Paez JG, Janne PA, Lee JC et al (2004) EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 304:1497–1500
5. Rosell R, Moran T, Queralt C et al (2009) Screening for epidermal growth factor receptor mutations in lung cancer. N Engl J Med 361:958–967
6. Mok TS, Wu YL, Thongprasert S et al (2009) Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. N Engl J Med 361:947–957
7. Lee JS, Park K, Kim SW et al (2009) A randomized phase III study of gefitinib (IRESSA™) versus standard chemotherapy (gemcitabine plus cisplatin) as a first-line treatment for never-smokers with advanced or metastatic adenocarcinoma of the lung. J Thorac Oncol 4 [Suppl 1]:Abstract PRS 4
8. Mitsudomi T, Morita S, Yatabe Y et al (2010) Gefitinib versus cisplatin plus docetaxel in patients with non-small-cell lung cancer harbouring mutations of the epidermal growth factor receptor (WJTOG3405): an open label, randomised phase 3 trial. Lancet Oncol 11:121–128
9. Maemondo M, Inoue A, Kobayashi K et al (2010) Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. N Engl J Med 362: 2380–2388
10. Zhou C, Wu YL, Chen G et al (2010) Efficacy results from the randomized phase III OPTIMAL (CTONG 0802) study comparing first-line erlotinib versus carboplatin (CBDCA) plus gemcitabine (GEM), in Chinese advanced non-small-cell lung cancer (NSCLC) patients (PTS) with EGFR activating mutations. Ann Oncol 21:Abstract LBA13
11. Rosell R, Gervais R, Vergnenegre A et al (2011) Erlotinib versus chemotherapy (CT) in advanced non-small cell lung cancer (NSCLC) patients (p) with epidermal growth factor receptor (EGFR) mutations: Interim results of the European Erlotinib Versus Chemotherapy (EURTAC) phase III randomized trial. In: ASCO Annual Meeting, Abstract 7503. Chicago, IL, EEUU
12. Felip E, Gridelli C, Baas P et al (2011) Metastatic non-small-cell lung cancer: consensus on pathology and molecular tests, first-line, second-line, and third-line therapy: 1st ESMO Consensus Conference in Lung Cancer; Lugano 2010. Ann Oncol 22:1507–1519
13. Keedy VL, Temin S, Somerfield MR et al (2011) American Society of Clinical Oncology provisional clinical opinion: epidermal growth factor receptor (EGFR) Mutation testing for patients with advanced non-small-cell lung cancer considering first-line EGFR tyrosine kinase inhibitor therapy. J Clin Oncol 29:2121–2127
14. Trigo Perez JM, Garrido Lopez P, Felip Font E et al (2010) SEOM clinical guidelines for the treatment of non-small-cell lung cancer: an updated edition. Clin Transl Oncol 12:735–741
15. Morris SW, Kirstein MN, Valentine MB et al (1994) Fusion of a kinase gene, ALK, to a nucleolar protein gene, NPM, in non-Hodgkin's lymphoma. Science 263:1281–1284
16. Chiarle R, Voena C, Ambrogio C et al (2008) The anaplastic lymphoma kinase in the pathogenesis of cancer. Nat Rev Cancer 8:11–23
17. Rikova K, Guo A, Zeng Q et al (2007) Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. Cell 131:1190–1203
18. Soda M, Choi YL, Enomoto M et al (2007) Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. Nature 448: 561–566
19. Weinstein IB, Joe A (2008) Oncogene addiction. Cancer Res 68:3077–3080; discussion 3080
20. Sasaki T, Rodig SJ, Chirieac LR et al (2010) The biology and treatment of EML4-ALK non-small cell lung cancer. Eur J Cancer 46:1773–1780
21. Shaw AT, Yeap BY, Mino-Kenudson M et al (2009) Clinical features and outcome of patients with non-small-cell lung cancer who harbor EML4-ALK. J Clin Oncol 27:4247–4253
22. Kwak EL, Bang YJ, Camidge DR et al (2010) Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. N Engl J Med 363:1693–1703
23. Ding L, Getz G, Wheeler DA et al (2008) Somatic mutations affect key pathways in lung adenocarcinoma. Nature 455:1069–1075
24. Beau-Faller M, Ruppert AM, Voegeli AC et al (2008) MET gene copy number in non-small cell lung cancer: molecular analysis in a targeted tyrosine kinase inhibitor naive cohort. J Thorac Oncol 3:331–339
25. Eder JP, Vande Woude GF, Boerner SA et al (2009) Novel therapeutic inhibitors of the c-Met signaling pathway in cancer. Clin Cancer Res 15: 2207–2214
26. Hirsch FR, Langer CJ (2004) The role of HER2/neu expression and trastuzumab in non-small cell lung cancer. Semin Oncol 31:75–82
27. Perera SA, Li D, Shimamura T et al (2009) HER2YVMA drives rapid development of adenosquamous lung tumors in mice that are sensitive to BIBW2992 and rapamycin combination therapy. Proc Natl Acad Sci U S A 106:474–479
28. Shimamura T, Ji H, Minami Y et al (2006) Non-small-cell lung cancer and Ba/F3 transformed cells harboring the ERBB2 G776insV_G/C mutation are sensitive to the dual-specific epidermal growth factor receptor and ERBB2 inhibitor HKI-272. Cancer Res 66:6487–6491
29. Wang SE, Narasanna A, Perez-Torres M et al (2006) HER2 kinase domain mutation results in constitutive phosphorylation and activation of HER2 and EGFR and resistance to EGFR tyrosine kinase inhibitors. Cancer Cell 10:25–38
30. Paik PK, Arcila ME, Fara M et al (2011) Clinical characteristics of patients with lung adenocarcinomas harboring BRAF mutations. J Clin Oncol 29: 2046–2051
31. Okudela K, Suzuki M, Kageyama S et al (2007) PIK3CA mutation and amplification in human lung cancer. Pathol Int 57:664–671
32. Kawano O, Sasaki H, Endo K et al (2006) PIK3-CA mutation status in Japanese lung cancer patients. Lung Cancer 54:209–215
33. Angulo B, Suarez-Gauthier A, Lopez-Rios F et al (2008) Expression signatures in lung cancer reveal a profile for EGFR-mutant tumours and identify selective PIK3CA overexpression by gene amplification. J Pathol 214:347–356
34. Travis WD, Brambilla E, Müller-Hermelink HK, Harris CC (2004) Pathology and genetics of tumours of the lung, pleura, thymus and heart. WHO classification of tumours. IARC Press, Lyon
35. Salido M, Pijuan L, Martinez-Aviles L et al (2011) Increased ALK gene copy number and amplification are frequent in non-small cell lung cancer. J Thorac Oncol 6:21–27
36. Sharma SV, Haber DA, Settleman J (2010) Cell line-based platforms to evaluate the therapeutic efficacy of candidate anticancer agents. Nat Rev Cancer 10:241–253
37. SEAP-IAP (2011) Garantía de Calidad en Patología de la Sociedad Española de Anatomía Patológica y la División Española del International Academy of Pathology. http://www.seap.es/gcp/molecular/index.asp
38. Travis WD, Brambilla E, Noguchi M et al (2011) International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society international multidisciplinary classification of lung adenocarcinoma. J Thorac Oncol 6:244–285
39. Mukhopadhyay S, Katzenstein AL (2011) Subclassification of non-small cell lung carcinomas lacking morphologic differentiation on biopsy specimens: utility of an immunohistochemical panel containing TTF-1, napsin A, p63, and CK5/6. Am J Surg Pathol 35:15–25
40. Boldrini L, Gisfredi S, Ursino S et al (2007) Mutational analysis in cytological specimens of advanced lung adenocarcinoma: a sensitive method for molecular diagnosis. J Thorac Oncol 2: 1086–1090
41. Ladanyi M, Pao W (2008) Lung adenocarcinoma: guiding EGFR-targeted therapy and beyond. Mod Pathol 21[Suppl 2]:S16–22
42. Pirker R, Herth FJ, Kerr KM et al (2010) Consensus for EGFR mutation testing in non-small cell lung cancer: results from a European workshop. J Thorac Oncol 5:1706–1713
43. Angulo B, Garcia-Garcia E, Martinez R et al (2010) A commercial real-time PCR kit provides greater sensitivity than direct sequencing to detect KRAS mutations: a morphology-based approach in colorectal carcinoma. J Mol Diagn 12: 292–299
44. Brevet M, Arcila M, Ladanyi M (2010) Assessment of EGFR mutation status in lung adenocarcinoma by immunohistochemistry using antibodies specific to the two major forms of mutant EGFR. J Mol Diagn 12:169–176
45. Conde E, Angulo B, Tang M et al (2006) Molecular context of the EGFR mutations: evidence for the activation of mTOR/S6K signaling. Clin Cancer Res 12:710–717
46. Kato Y, Peled N, Wynes MW et al (2010) Novel epidermal growth factor receptor mutation-specific antibodies for non-small cell lung cancer: immunohistochemistry as a possible screening method for epidermal growth factor receptor mutations. J Thorac Oncol 5:1551–1558
47. Kawahara A, Yamamoto C, Nakashima K et al (2010) Molecular diagnosis of activating EGFR mutations in non-small cell lung cancer using mutation-specific antibodies for immunohistochemical analysis. Clin Cancer Res 16:3163–3170
48. Marchetti A, Normanno N, Pinto C et al (2010) Recommendations for mutational analysis of EGFR in lung carcinoma. Pathologica 102:119–126
49. Pan Q, Pao W, Ladanyi M (2005) Rapid polymerase chain reaction-based detection of epidermal growth factor receptor gene mutations in lung adenocarcinomas. J Mol Diagn 7:396–403
50. Pao W, Ladanyi M (2007) Epidermal growth factor receptor mutation testing in lung cancer: searching for the ideal method. Clin Cancer Res 13:4954–4955
51. Yu J, Kane S, Wu J et al (2009) Mutation-specific antibodies for the detection of EGFR mutations in non-small-cell lung cancer. Clin Cancer Res 15: 3023–3028
52. Travis WD, Rekhtman N (2011) Pathological diagnosis and classification of lung cancer in small biopsies and cytology: strategic management of tissue for molecular testing. Semin Respir Crit Care Med 32:22–31
53. Billah S, Stewart J, Staerkel G et al (2011) EGFR and KRAS mutations in lung carcinoma: molecular testing by using cytology specimens. Cancer Cytopathol 119:111–117