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REVIEW

# Targeting EGFR in non-small-cell lung cancer: Lessons, experiences, strategies

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**Summary**
Cancer is a genetic disease and this concept is now widely exploited by both scientists and clinicians to design new targeted molecules. Indeed many data have already allowed us to ameliorate not only our knowledge about cancer onset, but also about patients treatment. Correlation between mutations in cancer alleles and drug response is a key point to identify drugs that match the genetic profile of each individual tumors. On the other hand, experience derived from inhibition of tyrosine kinase receptors has pointed out that targeted treatment is really successful only in a small subset of tumors. The latter are eventually addicted to those genetic alterations which are responsible for receptors activation and for the continued expression of their signalling. Overall these observations provide a strong rationale for a molecular-based diagnosis and patients selection for targeted therapies.

This review analyses the current state of the art of molecularly-tailored pharmacological approach to lung cancer, one of the biggest killers among human solid tumors. Main relevance is addressed to genetic lesions activating the EGFR pathway transducers, focusing on their role as markers of targeted drug response.
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**Contents**



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## Introduction

Lung cancer is the leading cause of death for solid tumors worldwide with an annual mortality of over one million.[1] Lung carcinoma includes a series of different diseases which are roughly divided into two groups based on clinical and histo-pathological features: non-small-cell lung cancer (NSCLC), accounting for almost 80% of lung cancer diagnosis and small cell lung cancer (SCLC) responsible for the remaining 20%. NSCLCs were further classified as: adenocarcinoma (ADC, and its variants); squamous cell carcinoma (SCC) and large cell carcinoma (LCC) — comprising the neuroendocrine variant (LCNEC).[2] Recently the American Thoracic Society and the European Respiratory Society approved a new classification of lung adenocarcinomas [3] which eliminates the form term bronchiole-alveolar (BAC) carcinoma and introduces the new concepts of adenocarcinoma *in situ* (AIS) and minimally invasive adenocarcinoma (MIA) for small solitary adenocarcinomas with either pure lepidic growth (AIS) or predominant lepidic growth with $\leq 5$ mm invasion (MIA). Invasive adenocarcinomas are classified by predominant pattern after using comprehensive histologic subtyping with lepidic (formerly most mixed subtype tumors with non-mucinous BAC), acinar, papillary, and solid patterns; micropapillary is added as a new histologic subtype.

Despite advances in defining the molecular mechanisms involved in lung oncogenesis and the remarkable efforts made to improve screening programs for secondary cancer prevention, patients prognosis remains poor. Lung cancer is mainly diagnosed at locally-advanced/metastatic stages and if untreated, the median survival after diagnosis is of 4—5 months whereas the 1-year survival is less than 10%.[4] In those cases the platinum-based combination schedule, although more advantageous than best supportive care (BSC),[5] results in only modest increases of survival rates.[6]

More than 75% of all lung cancer histological types are related to tobacco smoking habit and the association is stronger for SCLC and SCC,[7,8] From more than fifty years chronicle exposure to carcinogens (tobacco smoke) has been recognized as a responsible for that pathological process known as 'field cancerization',[9,10] while only recently the genetic alterations responsible of the growth of a field lesion have been defined,[11—13] It is conceivable that in smokers, 'field cancerization' of the lungs might culminate in malignant transformation starting from stem-like cancer precursors grown in pre-neoplastic histological settings.[14] Indeed burgeoning evidence points out that lung cancers arising in smokers and in never smokers can be thought as separate entities, since they feature distinctions at epidemiological, clinical and bio-molecular level. Noticeably they display different mutational profiles (e.g. p53, KRAS, EGFR), which can significantly impact on both prognosis and drug responsiveness.[15] It should be noted that the proportion of lung cancer in never smokers is expected to increase in parallel with the implementation of smoking prevention and quitting programs,[16,17] Although the relevance of tobacco exposure, factors other than smoke have been suggested as lung carcinogens (environment,[18,19] hormones,[20,21] genetics,[22] viruses,[23—25]). Adenocarcinoma, which is now accounting for 35—40% of all NSCLC diagnosis, is the commonest form in never smokers.[26]

Lung cancer identifies an extremely heterogeneous group of disorders, and remains a difficult disease to treat. An extremely diverse collection of genomic alterations has been documented in NSCLC; a proportion of unknown dimension is still concealed,[27,28] However a number of tumor activating somatic genetic lesions ('driving' lesions [29]) have already been detected in a substantial fraction of patients and translated into a system from detection and determination of the disease prognosis. Alterations in several oncogenes — among which EGFR, KRAS, PIK3CA, MET, c-MYC -, tumor suppressor genes — such as p53 and LKB1- and transcription factors (e.g. TTF1) have been reported in NSCLCs, mainly in adenocarcinomas,[30,31] Dissection of such a complex scenario represents a still open challenge for both researchers and clinicians.

Although the topic of targeted therapy for the treatment of NSCLC has been covered by several reports,[32—35] this review aims to point out the unprecedented clinical value of translation of the molecular oncology findings as well as to focus on still unresolved questions emerged after the advent of the EGFR-targeted molecules. Personalized approach to NSCLC treatment started with the observation that lung cancers respond differently to EGFR inhibitors, based on their genetic status. From this initial point several successes have been reached; however much remains to be done and studied in terms of patient genotyping and stratification as well as in understanding the molecular mechanisms of primary and secondary resistance to these agents.

## Molecular profiling of tyrosine kinase genes in cancer

Cancer cells accumulate somatic DNA alterations which are responsible for oncogenic activation or tumor suppression genes silencing. Changes affecting single nucleotides (e.g. point mutations) occur in transformed cells as well as small deletions, insertions and more complex lesions involving larger portions of chromosomes such as translocations and amplifications. Today almost 300 cancer-related genes, approximately 1% of all human genes, have been identified[36]; about eighty genes are activated by somatic

mutations which arise in the only malignant clone while do not affect non transformed cells.[37]

Among cancer genes, the protein tyrosine kinase (TK) genes family plays a central role and several of these enzymes have been found to be altered in cancers by a variety of molecular mechanisms.[38] Kinases — and their inhibitors, phosphatases — are key regulators of several cellular functions such as proliferation, migration, metabolism, differentiation and survival and their appropriate activity is required for the cellular homeostasis; on the contrary their aberrant activation is crucial in driving oncogenesis. Receptor tyrosine kinases (RTKs) represent a subclass of transmembrane proteins displaying an intrinsic, ligand-controlled TK activity. In resting cells, RTKs activity is quiescent; in presence of activating lesions — as it occurs in cancer-RTKs become inappropriately phosphorylated (Fig. 1).

Mutations affecting RTKs have been demonstrated to have a causative role in many solid cancers, among which NSCLC.[39] In lung cancer, kinases tend to be altered by heterozygous missense mutations that affect residues involved in their enzymatic activity; this suggests that mutations are activating and operate by increasing the catalytic activity of the mutated protein. This evidence also points out that mutated kinase genes act as dominant oncogenes,[40,41] Besides RTKs translocation as well as increased gene copy number, have been described in NSCLC: relevant examples are the transforming ALK-EML4 fusion gene[42] on one hand and EGFR (7p12)[43] and MET (7q31.1)[44] genes amplification on the other.

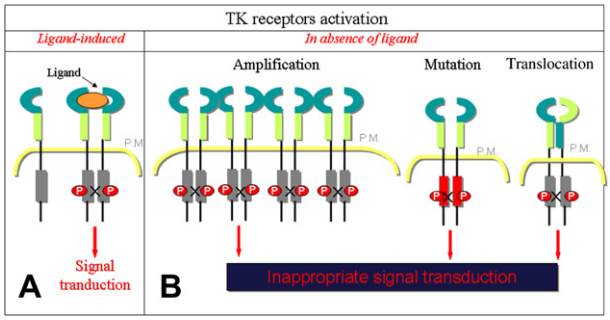



**Figure 1** Receptor tyrosine kinases (RTKs) activation. A) Ligand-induced receptor activation of transmembrane tyrosine kinase receptor. In physiological conditions, ligand binding to extracellular portion of the receptor leads to receptor dimerization and consequent trans-phosphorylation of two tyrosine residues located at the intracytoplasmic enzymatic domain. The latter activate downstream transducers involved in several biological functions of the cell. B) Inappropriate mechanisms of RTKs activation that might lead to activation of cellular proto-oncogenes. The process of gene amplification occurs through redundant replication of genomic DNA, often giving rise to karyotypic abnormalities called double-minute chromosomes (DMs) and homogeneous staining regions (HSRs). DMs are characteristic mini-chromosome structures without centromere, while HSRs are segments of chromosomes that lack the normal alternating pattern of light-and dark-staining bands. Both DMs and HSRs represent large regions of amplified genomic DNA containing up to several hundred copies of a gene. Amplification leads to the increased expression of genes, which in turn can confer a selective advantage for cell growth. Nucleotide changes such as mutations lead to structural alterations in their encoded proteins. Chromosome rearrangement lead to malignancy via two different mechanisms: the transcriptional activation of proto-oncogenes or the creation of fusion genes encoding chimeric proteins with transforming properties.

From the cloning of the first cDNA encoding an RTK — the EGFR[45] — many progresses have been reached in human cancer therapy and several tumor types have benefit from this knowledge. Importantly, the concept that mutated kinases molecularly mark 'druggable' targets has lead to intensive efforts to survey the kinome across a wide spectrum of human cancer types for mutations.[46]

Among RTKs, the epidermal growth factor (EGF) receptor family has been extensively studied in several solid cancers, mainly in colorectal and non-small-cell lung carcinomas. It consists of four members: EGFR (ErbB1, HER1), ErbB2 (HER2, neu in rodents), ErbB3 (HER3) and ErbB4 (HER4). The binding of soluble ligand to the ecto-domain of the receptor promotes homo-and hetero-dimer formation between receptors, a crucial step for activation of the intracellular TK domain and subsequent phosphorylation of the C-terminal tail.[47] Phosphotyrosine residues then activate, either directly or through adaptor proteins, downstream components of the TK signaling pathway which are involved in promoting cell proliferation, motility and invasion.

EGFR is overexpressed - when detected by immunohistochemistry (ICH) — in several cancer types[48] and in more than 60% of lung cancers; its activation correlates to poorest prognosis.[49]

Selective block of EGFR and ErbB2 has been reported to be effective as therapeutic approach in several solid cancers. In 2004 the first EGFR-mutant lung cancers were described and it was reported that most of NSCLC patients who showed clinical response to EGFR inhibitors carried EGFR mutated tumors,[50,51] Somatic changes affected sequences encoding for receptor TK domain; mutated receptors sustain a hyper-activated downstream signaling,[52,53] Notably, it was then demonstrated that cultured cell lines displaying the same EGFR genetic lesions that have been found in human tumors, undergo *in vivo* cell-cycle arrest or apoptosis in response to EGFR inhibition. This phenomenon is named 'oncogene addiction[54]' and applies to those settings in which cancer cells appear to be dependent on a single overactive oncogene for their own survival and proliferation.[55] As lung tumor cells depend on the aberrant activity of a specific mutated gene (e.g. EGFR) to survive and proliferate, it is virtually sufficient to inactivate that gene to induce growth arrest and provoke cell death (apoptosis). Therefore, switching off the oncogenic activity by specific EGFR inhibitors may trigger an 'oncogenic shock' [56] which eventually will lead tumor cells to die. This hypothesis is based on the concept that oncoproteins emanate both pro-survival and pro-apoptotic signals: oncogene elimination creates a temporal window during which apoptotic outputs persist in the absence of survival signals, resulting in cell death. As suggested by Sharma and colleagues,[57] this model has two relevant implications. The first is that co-administration of TKIs with standard chemo, due to its own effects on DNA-damage,

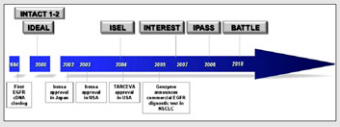
might attenuate the acute effect of growth factor signal withdrawal. The second is that the more gradual signal attenuation induced by anti-EGFR monoclonal antibodies in respect to small molecules, might explain the differential effects displayed by these two classes of EGFR inhibitors.

The paradigm of TKs genetic alterations in cancer facts highlights two crucial clinical points. The first is that targeted therapy is effective only in those patients whose tumor DNA contains the alteration which makes the tumor itself susceptible to the specific drug. Thus, before subjecting patients to targeted treatments, the presence of those genetic lesions which are predictive of potential response, must be ascertained. In addition, this diagnostic/therapeutic approach inevitably will put into question traditional medical approach to neoplastic disease: from the standpoint of translation oncology, tumor molecular profiling must be associated to the standard histopathological characterization in selecting patients who will benefit from targeted drugs. As a consequence, cancer-associated genetic lesions might — ideally — identify genetic determinants of drug response since they can display a predictive (provide information on outcome with regards to a specific therapy) and/or prognostic (provide information on outcome, regardless of which treatment is used) value.[58]

## Pharmacological targeting of the EGFR signaling pathway

Pharmacological block of EGFR represents one of the first examples of rationally designed therapeutic strategy. In 2003 and 2004 with the approval of the first small EGFR inhibitors - gefitinib and erlotinb — for advanced NSCLC treatment, a large enthusiasm aroused (Box 1). However limitations of their efficacy in unselected NSCLC became in short time evident. Indeed EGFR might be overactivated mainly in consequence to mutations or to gene amplification and its inhibition can be exploited either through anti-EGFR monoclonal antibodies (mAbs) or through small tyrosine kinase inhibitors (TKIs).

### Box 1. Timeline of EGFR small inhibitors development in NSCLC.




In 1994 AstraZeneca developed the first EGFR inhibitor and the first clinical trial with gefitinb was presented at AACR in 1997. At the beginning of the XXI century the Expanded Access Programme (EAP) started in parallel phase II/III trials: IRESSA Dose Evaluation in Advanced Lung Cancer (IDEAL[1]) and IRESSA NSCLC Trial Assessing Combination Treatment (INTACT[2,3]) which confirmed that EGFR block could be effective in pretreated NSCLC. Consequently in 2003 FDA approved gefitinb and in 2004 erlotinib as II line approach to advanced NSCLC treatment. Phase III ISEL[4] (IRESSA Survival Evaluation in Lung Cancer) study demonstrates non-significant survival advantage over placebo in overall population but subgroups show benefit: patients of Asian origin and those who have never smoked. These results where coherent with first report by Lynch and colleagues of the occurrence of EGFR somatic point mutations in responders to gefitinib. More recently the INTEREST[5] (IRESSA Non-small-cell lung cancer Trial Evaluating REsponse and Survival against Taxotere) study conducted in 24 countries demonstrated non inferiority of gefitinib vs docetaxel in 2nd line approach to advanced NSCLC while the IPASS (IRESSA Pan-Asia Study) trial showed superiority of EGFR inhibitors EGFR mutated cancers. In 2009 European Medicines Agency EMEA approved gefitinib for treatment of adults with locally advanced or metastatic NSCLC with activating mutations of EGFR TK across all lines of therapy. In april 2010 the BATTLE (Biomarker-integrated Approaches of Targeted Therapy for Lung Cancer Elimination) confirms that treatments tailored for each patient's particular type of lung cancer (e.g. erlotinib in EGFR mutated patients) may improve outcomes but unexpectedly the strategy has limited gin in survival rates.

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Tumor responsiveness to monoclonal antibodies has been associated to the copy number on the corresponding gene present in individual tumors[59]; on the other hand sensitivity to small molecules correlates with mutations affecting the catalytic domain of the receptor.[60]

Anti-EGFR mAbs bind to the extracellular domain of EGFR, occlude the ligand binding region competing with receptor ligands, inhibit the ligand-induced phosphorylation of the catalytic region and eventually block the intracellular signaling cascade. Cetuximab (Erbitux®, Merck), a chimeric immunoglobulin G1 monoclonal antibodies (mAb) and Panitumumab (Vectibix®, Amgen) a fully human antibody, are mAbs which have been developed to block EGFR (http://www.ema.europa.eu). Although two large phase III trials in chemonaive patients (FLEX and BMS090) have shown conflicting results regarding overall survival (OS), Cetuximab has been found to improve clinical outcome of untreated unresectable NSCLCs in combination with platinum-based chemotherapy [61] and chemoradiotherapy.[62] There are very few data on response to Cetuximab when administered as second and third line approach,[63–65] Nevertheless no biomarkers have been validated to predict sensitivity to treatment in NSCLC.

On the other hand, activating EGFR somatic mutations have emerged as the most relevant predictor of response to small EGFR inhibitors[54,58,66] (Fig. 2). First-generation small drugs are essentially represented by the two quinazoline-based molecules gefitinib (Iressa®, Astra Zeneca, www.astrazeneca.com) and erlotinib (Tarceva®, OSI/Roche/Genetech, www.roche.com) which behave as reversible inhibitors of the EGFR kinase.[67] EGFR activating somatic mutations mainly occur at TK domain coding sequences. Somatic changes affect four exons (18–21) which encode for the ATP binding pocket: mutations induce repositioning of critical residues at the receptor ATP binding site thus reducing receptor affinity for ATP and enhancing signal silencing after drug binding. However, this fact does not imply that mutated EGFRs are necessarily constitutively or fully active, as their degree of ligand independence might be a function of experimental context.[68,69]

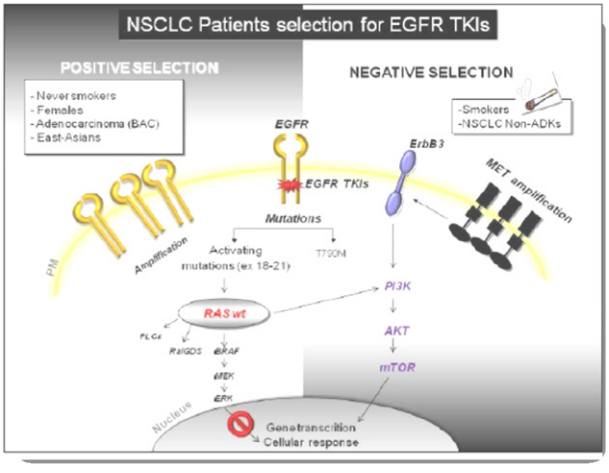


**Figure 2** NSCLC patients selection for EGFR targeting with small molecules. Careful selection of NSCLC patients is needed before starting anti-EGFR pharmacological targeting. EGFR activating mutations confer sensibility to small inhibitors in absence of KRAS changes. EGFR amplification seems to add a gain in drug response. Co-existence of MET amplification — through ErbB3 activation — leads to intracellular signaling activation even in presence of EGFR block.

Overall the incidence of EGFR mutations in NSCLCs is about 26% (according to COSMIC database, www.sanger.ac.uk): this frequency increases up to 77% among EGFR TKIs responders, while it is 7% in unsensitive cases[58]. Exon 19 mutations are mainly characterized by in-frame deletions of aminoacids 747–750 and account for 45% of mutations; the most frequent exon 21 mutation results in L858R substitution and is detected in 40–45% of mutated samples; mutations in exons 18 and 20 are found in the remaining 10% of cases. Interestingly NSCLCs harboring exon 19 deletions seems to better respond to small molecule inhibitors than L858R mutants[70,71]; fewer data are available about drug sensitization conferred by mutations in exons 18 and 20.[72] It is well documented that mutation frequency increases to over 50% in a restricted subset of NSCLC patients: East-Asians, women, not smokers, affected by ADC[73–75] Preclinical data suggest that EGFR mutations occur as early events during NSCLC onset.[76] Transgenic mice with lung specific expression of exon 19 deletion or L858R mutation, develop atypical adenomatous hyperplasia (AAH) which defines the precursor lesion of peripheral ADCs which display early distant dissemination.[69]

In the past years at least six large phase III studies comparing standard platinum-based chemotherapy versus erlotinib or gefitinib in chemonaive metastatic NSCLC demonstrated that in patients whose tumors harbor activating EGFR mutations, EGFR TKIs are superior to chemotherapy in terms of response rates progression free survival (PSF), quality of life and toxicity profile.[77] Two additional studies (SATURN[78] and ATLAS[79]) have investigated the efficacy of erlotinib as maintenance therapy in NSCLC patients with metastatic disease not progressing after standard chemo. Both studies demonstrated that patients receiving erlotinb have a significant reduction in the risk of progression, with the highest PFS in EGFR mutated cases.

In second line approach a recent metanalysis including data from 4 randomized trials (INTEREST,[80] V-15-32,[81] SIGN,[82] ISTANA[83]) confirmed that gefitinb appeared to be no different from docetaxel in unselected pre-treated NSCLC. In the INTEREST trial, EGFR mutated patients benefit more from gefitinib than docetaxel in terms of PFS, in absence of differences in survival rates. Recently the TITAN study,[84] a phase III randomized trial comparing erlotinib versus docetaxel or pemetrexed in chemorefractory NSCLC, showed no differences in PFS in both arms.

It should be noted that a small proportion (1–20% depending on trials) of patients with no detectable EGFR activating mutations, show a radiographic response when treated with EGFR TKIs. This observation might be partially explained by the fact that molecular analysis should have detection limits; however it is possible that genetic lesions other than intrinsic mutations could activate the EGFR signal cascade.

In conclusion, although detection of somatic mutations identifies the best predictor of response to anti-EGFR molecules — so that mutational screening is mandatory to define first line therapy -, new biomarkers must be

investigated to clarify the potential efficacy of EGFR blockade in EGFR wild type tumors.

EGFR amplification (detected by FISH in 20—40% of NSCLCs, according to different studies) seems to add a gain in response rates to gefitinb and erlotinib,[85—87] EGFR increased gene copy number as detected by FISH does not predict the overall survival; however Hirsh and colleagues have recently reported that EGFR-FISH positive NSCLC patients had a longer progression free survival and higher response rates to EGFR TKIs.[88]

## Dissecting the EGFR signaling to overcome the problem of resistance

Experience derived from EGFR TKIs has pointed out that lung tumors might display *de novo* resistance to TKIs therapy (primary resistance); moreover in many cases responses are not durable, more often they stabilized the disease for 6—12 months (secondary or acquired resistance [89]).

The EGFR activation triggers two main signaling pathways: KRAS-BRAF-MEK pathway, which sustains cell proliferation and in PIK3CA-AKT-mTOR axis, which is mainly involved in cell survival and motility.[90,91] Other critical activated pathways include the STAT signal cascade and ERBB mediated angiogenesis.[92] Several studies are now directed to investigate the whole EGFR-induced signaling in mediating sensitivity or resistance to EGFR inhibitors.

Both primary and acquired resistance might be related to the occurrence of EGFR activating mutations, mainly affecting the exon 20. For example small insertions or duplication in exon 20 have been described in patients harboring progressive disease in the course of anti-EGFR molecules. Besides the EGFR T790M mutation (also affecting exon 20) is often found in tumor samples from patients who did not respond to reversible anti-EGFR molecules,[93,94] The recently developed irreversible EGFR inhibitors — the pan-erbB inhibitors PF00299804[95] (Pfizer,) and neratinib (HKI-272[96]) (Wyeth) — have shown *in vitro* promising activity in inhibiting T790M-mutated NSCLC cells. These molecules irreversibly block erbB tyrosine kinase activity through binding the ATP site and inducing covalent modification of nucleophilic cysteine residues (Cys 797) in the catalytic domains of erbB family members. The covalent bond seems to permit local persistence of high drug concentrations thus allowing the inhibition of the catalytic function even in the presence of T790M mutation.[97]

Other genomic alterations can occur with EGFR mutations in inducing primary resistance to EGFR inhibitors. They include: i) mutations of PIK3CA; ii) loss of PTEN expression; iii) altered IGFR signaling[77].

On the other hand, resistance in EGFR wild type tumors is mainly related to the occurrence of genetic lesions affecting the EGFR downstream transducers.

KRAS mutations have been identified in NSCLC more than 20 years ago,[98,99] but only recently they have become clinically significant as biomarkers of anti-EGFR therapy response. KRAS is mutated in about 17% of NSCLCs, mostly in ADCs (COSMIC database, www.sanger.ac.uk). Almost 97% of KRAS mutations in NSCLC result in aminoacid substitution at codon 12 and 13. Likewise colorectal cancers (CRCs), in advanced/metastatic NSCLCs activating KRAS mutations are highly specific negative predictors to single anti-EGFR TKI agent.[100] KRAS mutations seem to arise more frequently in smoke-induced lung carcinomas: in particular G to T substitutions are associated in tobacco-related lung ADCs, while G to A changes have been recently found also in NSCLC in never smokers.[101] In addition, detection of KRAS exon 2 activating mutations is associated to resistance to conventional chemotherapy. The role of KRAS mutational status as a marker of response to standard chemo alone in NSCLC is poorly understood but it has been clearly demonstrated that KRAS mutations occurrence is associated with shortest survivals in NSCLC patients treated with carboplatin plus paclitaxel.[102] KRAS mutations occur as an early event in lung oncogenesis and mutated cells are detectable in pre-invasive lesions, such as AAH.[103] Although early studies reported the association between occurrence of KRAS mutations and poorest prognosis,[104] the role of KRAS pathway activation as prognostic marker is still debated.[105] More recent data demonstrate higher mutational frequencies of KRAS gene in lung ADCs with a dominant micropapillary growth pattern which is associated to high invasive capacity and aggressive phenotype,[106,107] At the present no direct RAS inhibitors have proven clinically effective; however several molecules are under investigation. Among them inhibitors of the enzyme farnesyl transferase (FT) — involved in RAS protein maturation and function — have been studied in lung cancer therapy: in phase II study the FT inhibitor R115777 (Zarnestra®, Johnson & Johnson) has shown only modest clinical activity as first line treatment in advanced NSCLCs.[108]

The most known and studied mediator of KRAS downstream signaling is BRAF. The BRAF gene (located on 7q34) codifies for a threonine-serine kinase. BRAF mutations rarely (2%) affect lung cancer (COSMIC database, www.sanger.ac.uk); interestingly the BRAF V600E mutation mainly affects female sex and represents a negative prognostic factor.[109] BRAF mutations are known to predict sensitivity to MEK inhibitors.[110] Few data are available, but the recent phase II study on the MEK oral inhibitor PD-0325901 did not reach its primary endpoint measured by objective response.[111]

The other key axis downstream EGFR is mediated by PIK3CA. Mutations affecting PIK3CA coding sequence rarely occur in lung cancer since mutational frequency is about 3% of NSCLCs and SCLCs (according to COSMIC database, www.sanger.ac.uk). PIK3CA oncogenic activation can be driven by increased gene copy number: the region of chromosome 3q where PIK3CA gene is located is frequently altered in lung cancer.[112]

Alterations in other components of the pathway include: loss of function of the inhibitor PTEN which is involved in sustaining an additional mechanism of EGFR-mutants resistance to TKIs [113] or — even if rarely described — activating mutations of AKT gene [46]. The PIK3CA signaling cascade is early activated during tobacco-induced lung carcinogenesis.[114] An important mediator of the PI3KCA pathway is the mammalian target of rapamycin (mTOR): it is a member of the phosphoinositide kinase-related kinase family, which also includes PIK3CA. mTOR acts as a central sensor for nutrient/energy availability, and can also be

modulated by PI3KCA dependent mechanisms.[115] Loss of PTEN[116] or AKT activation[117] have been suggested sensitizing cancers to the effects of mTOR inhibition; preliminary clinical results are available from combinatorial approach of anti-EGFR plus mTOR inhibitors.[118] Very initial data in NSCLC cell lines seem to demonstrate that mutations in either LKB1 or KRAS genes display sensitivity to the single-agent treatment with the MEK inhibitor CI-1040 or with rapamycin.[119]

PI3KCA activation might be also related to MET gene overexpression in consequence to increased gene copy number. It is well known that amplification of the MET oncogene represents a mechanism of acquired resistance to EGFR TK inhibitors.[120] Amplified MET mediates PIK3CA activation through ErbB3 activation which represents an alternative signaling pathway which induces cell proliferation even in the presence of EGFR inhibitors. Interestingly Engelmann and colleagues have recently showed that NSCLC cells carrying MET gene amplification are already detectable at tumor onset and undergo a clonal selection through anti-EGFR therapy.[121]

Taken together occurrence of the T790M mutation and MET amplification stand for 70% of causes of acquired resistance to EGFR inhibitors in NCLC. More often the two genetic lesions arise independently in different metastases of the same tumor.[122] This observation sustains a strong rationale for combinatorial anti-EGFR/anti-MET approach, at least in relapsed patients. Furthermore geldanamycin represents an interesting molecule.[123] This antibiotic induces heat shock protein 90 (Hsp90) inhibition. The Hsp 90 chaperone is required for the conformational maturation and stability of multiple oncogenic kinases (among which EGFR and MET) that drive signal transduction and proliferation of lung cancer cells. It has been recently demonstrated that also mutated EGFR is an Hsp90 client irrespective of the presence of secondary T790M mutation.[124]

Growing evidence indicates that EGFR and KRAS wild type tumors may otherwise display the EML4-AKT fusion protein[42]. This novel gene derives from fusion of echinoderm microtubule associated protein-like 4 (EML4) and the anaplastic lymphoma kinase (ALK) genes both of which are closely mapped to the same short arm of the chromosome 2.[125] The fusion induces a constitutive dimerization and a consequent activation of the ALK kinase domain. Several ALK specific inhibitors are now under investigation to specifically treat the EML4-ALK positive lung tumors and preliminary data seem to be promising. Based on the co-crystal structure of the MET inhibitor PHA665752 with the MET domain, Pfizer designed PF2341066 an orally available 2-amino-3-benzyloxy-5-arylpyridine compound that selectively targets MET and ALK. PF2341066 shows efficacy at well tolerated doses including marked citoreductive ant-tumor activity and antiangiogenic activity in several tumor models; it is currently under phase II/III trials on different solid tumors including NSCLC.[126]

## Post-genomics approach to lung cancer

As discussed above, lung cancer arises from the acquisition of somatic genetic — and epigenetic — lesions affecting gene sequence and copy number, protein structure and chromosome organization. The advent of next-generation technologies has allowed creation of catalogues of cancers somatic alterations, thus revealing a number of novel potential therapeutic targets. Over the course of the next years much more information will be accessible on somatic alterations affecting human cancers, within protein coding-genes, non-coding RNA genes and non-coding regions of the genome, as well as mithocondrial genome,[127–129] In respect to lung cancer, these technologies have been able to show a very high mutational rate in transformed versus the wild type surrounding parenchyma (genome-wide somatic mutation rate: 17.7 per megabase); as expected somatic changes mainly affect tyrosine kinase genes.[130]

Functional validations of these results are now mandatory to determine whether they have an active role in tumorigenesis, prognosis and therapy. In other words, the identification of an altered gene indicates a candidate gene rather than a causal cancer gene. As a result, great efforts are now directed to identify genetic lesions/mutations that drive oncogenesis among the several changes (most of which are passenger variations) that usually affect cancerized tissues.[131] Indeed growing evidence from the so-called 'landmark studies' on cancer genetics has pointed out that in a given tumor there are few picks of frequently mutated genes among several hills of infrequently altered genes, resulting in an extreme genetic heterogeneity. In respect to NSCLC — a still 'evolving landscape'[132] — little is known about functional characterization of mutations affecting mediators others than those involved in EGFR pathway. Besides even if EGFR is known to be activated during lung carcinogenesis, it should be kept in consideration that not all EGFR somatic mutations are functionally equivalent. Also the histological context in which a somatic alteration arises should be relevant for the interpretation of the role of the altered gene. For example both in Europe and in USA monoclonal antibodies against EGFR are recommended in colorectal cancer therapy only for those tumors which display wild type KRAS.[133] Conversely, as described above, in lung cancer EGFR is rarely activated by amplification and the KRAS status test is not required before starting TKI inhibitors.

The recently presented pioneering BATTLE (Biomarker-integrated Approaches of Targeted Therapy for Lung Cancer Elimination) trial — attempting to group patients by predominant biologic features of their cancer, including genetic changes in EGFR, KRAS, RXR/CyclinD1 or VEGF — has demonstrated that it is feasible to identify subgroups of advance/metastatic NSCLCs who are more likely to benefit from a specific drug. Importantly the study has been carried on only on fresh tissues. However the results are mixed and not fully understood. The median survival went up to nearly a year from seven months; the study confirms that EGFR mutated tumors might be more responsive to erlotinib, while it highlighted that sorafenib appeared to have good outcome for most groups of patients including those carrying KRAS mutations, which have been particularly hard to treat. Notably, most of the chosen biomarkers turned out to be less selective than expected in discriminating the groups.

## Conclusions

Over few years, the EGFR-targeted therapy has significantly modified the principles of lung cancer treatment. Many efforts are now directed to the identification of genetic markers of lung carcinogenesis in order to achieve a classification that could integrate conventional histopathological features with new parameters derived from bio-molecular knowledge. From this perspective a major and still open problem is represented by the histological and molecular heterogeneity that usually characterize lung cancer; this point is further highlighted in consideration the small size of tumor samples on which histological and molecular analyses are routinely performed. Lessons from recent 'umbrella trials' indicate a need in a predetermining few markers of which the predictive value will be strongly demonstrated. Those results point out that molecular classifiers need probably to be selected in respect to the stage of the disease and that we are only assuming — but confirmation will derive from bigger and more powerful studies — that the same genetic lesion found in a cancer will be associated to the same sensitivity to a specific inhibitor in a different patient.[134] The translational relevance of these data is not immediately evident but several of '-omics' applications will take advantage of introducing new technology over using standard care. This will ultimately lead to a better design of clinical trials that will definitely 'bridge the gap' between the worlds of personalized medicine and evidence-based medicine.[135]

## Conflict of interest statement

None declared.

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