Identifying activating mutations in the *EGFR* gene: prognostic and therapeutic implications in non-small cell lung cancer*

Identificação de mutações ativadoras no gene *EGFR*: implicações no prognóstico e no tratamento do carcinoma pulmonar de células não pequenas

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Abstract

Lung cancer is the leading cause of cancer-related deaths worldwide. Promising new therapies have recently emerged from the development of molecular targeted drugs; particularly promising are those blocking the signal transduction machinery of cancer cells. One of the most widely studied cell signaling pathways is that of EGFR, which leads to uncontrolled cell proliferation, increased cell angiogenesis, and greater cell invasiveness. Activating mutations in the *EGFR* gene (deletions in exon 19 and mutation L858R in exon 21), first described in 2004, have been detected in approximately 10% of all non-squamous non-small cell lung cancer (NSCLC) patients in Western countries and are the most important predictors of a response to EGFR tyrosine-kinase inhibitors (EGFR-TKIs). Studies of the EGFR-TKIs gefitinib, erlotinib, and afatinib, in comparison with platinum-based regimens, as first-line treatments in chemotherapy-naïve patients have shown that the EGFR-TKIs produce gains in progression-free survival and overall response rates, although only in patients whose tumors harbor activating mutations in the *EGFR* gene. Clinical trials have also shown EGFR-TKIs to be effective as second- and third-line therapies in advanced NSCLC. Here, we review the main aspects of EGFR pathway activation in NSCLC, underscore the importance of correctly identifying activating mutations in the *EGFR* gene, and discuss the main outcomes of EGFR-TKI treatment in NSCLC.

Keywords: Molecular targeted therapy; Receptor, epidermal growth factor; Lung neoplasms/drug therapy; Mutation; Oncogenes.

Introduction

Because of its high incidence and high mortality, lung cancer represents a major challenge for modern oncology. In Brazil, there were an estimated 27,330 new cases of lung cancer in 2014.[1] Recent global estimates indicate that there are 1.6 million new cases and 1.4 million lung cancer deaths each year, the majority of cases (55%) occurring in developing countries.[2,3] Historically, non-small cell lung cancer (NSCLC) presents response rates to classical cytotoxic chemotherapy in the range of 20-30%, the median overall survival typically being 8-10 months.[4] The recent development of novel therapeutic agents directed at targets that are aberrantly activated in cancer cells, particularly those within the signal transduction machinery, has opened new vistas for the treatment of NSCLC.

Among the components of the neoplastic phenotype, potential therapeutic targets include cell surface receptors, which have been the focus of intensive research because they play an important role in the processes of cell proliferation, survival, and invasiveness. Remarkable progress has been achieved with the advent of EGFR tyrosine-kinase inhibitors (EGFR-TKIs), which are able to inhibit EGFR signal transduction. Among patients with NSCLC, those with tumors that harbor activating mutations in the *EGFR* gene can benefit from treatment with an EGFR-TKI. It is therefore important that such patients are

correctly identified in clinical practice. Ten years after activating mutations in the *EGFR* gene were recognized as being the most important predictors of a response to EGFR-TKIs,[5,6] the present article will review the literature related to the EGFR signaling pathway and to activating mutations in the *EGFR* gene, as well as discussing the implications of this knowledge for daily practice.

EGFR and its signaling pathways

Cell surface receptors, which are proteins located in the plasma membrane, play a key role in cellular and tissue physiology. These receptors are activated by stimuli that originate from the external environment (ligands), generating intracellular signals that are transduced by multiple molecular cascades, in which successive phosphorylation of substrates activates the transcription of genes involved in cell proliferation, differentiation, invasion, angiogenesis, metastasis, and resistance to apoptosis. The ErbB receptor family, also known as the c-erb-B or human EGFR (HER) family, has four members: EGFR (or c-erb-B1 or HER-1), c-erb-B2 (or HER-2/neu), c-erb-B3 (or HER-3), and c-erb-B4 (or HER-4). The structure of EGFR, first described in the 1960s by Cohen,[7] comprises three domains: the extracellular domain (the N-terminal portion); the transmembrane domain; and the intracellular C-terminal domain (a hydrophobic portion with tyrosine-kinase activity). The extracellular domain confers binding specificity, ligands including EGF itself as well as TGF-α, amphiregulin, and betacellulin.[8] The intracellular domain is capable of phosphorylating tyrosine residues within the receptor itself (autophosphorylation) and within proteins involved in signal transduction.

The interaction between EGFR ligands and the extracellular domain of the receptor leads to its dimerization,[9] which promotes the activation of the tyrosine-kinase domain located in the intracellular domain of the receptor. Once active, the latter domain promotes autophosphorylation of specific sites within the C-terminal domain of EGFR.[10] Signal transduction is then continued by the interaction of those autophosphorylation sites with proteins that contain a Src homology 2 domain or a phosphotyrosine binding domain.[11] Various phosphorylation sites have been identified in the C-terminal domain of EGFR, each leading to interaction with different types of molecules and activation of various cellular pathways. Foremost among these is the Ras/Raf/mitogen-activated protein kinase (MAPK) pathway, in which the adaptor protein Grb2 binds to phosphorylated tyrosine residues of EGFR, thus activating the Son of sevenless protein.[12] This protein in turn activates the G-protein Ras, which initiates a cascade of phosphorylation of MAPKs, which are specific serine/threonine kinases. Those proteins in turn activate gene transcription related to various regulatory functions, including cell division, motility, and adhesion.[13] Another important pathway related to EGFR and activated in NSCLC is that mediated by PI3K, which is responsible for activation of serine/threonine kinase Akt. Together with the mammalian target of rapamycin, Akt participates in the regulation of many cellular processes, such as glycolytic metabolism, apoptosis, proliferation, and angiogenesis.[14]

The role that EGFR plays in carcinogenesis became clearer after the identification (in the 1980s) of the v-erb-B oncogene protein, which is related to avian erythroblastosis virus and structurally similar to EGFR.[15] The mechanisms leading to increases in proliferative activity, invasiveness, and angiogenesis, as well as in resistance to chemotherapy and radiotherapy, include paracrine and autocrine stimulation in the tumor microenvironment through increased production of ligands (mainly EGF and TGF-α), overexpression of EGFR molecules on the membrane of tumor cells, and activating mutations in the *EGFR* gene, all of which affect their signal transduction pathways.[16]

The development of EGFR-TKIs for the treatment of NSCLC

As studies have clarified the role of EGFR in carcinogenesis, interest in inhibiting the tyrosine-kinase activity of EGFR has grown. The first EGFR-TKIs were synthesized in the 1990s. The 4-anilinoquinazoline derivative gefitinib (ZD1839; AstraZeneca, London, England) was the first EGFR-TKI to obtain approval from the US Food and Drug Administration (FDA). In 2003, the FDA approved gefitinib for the treatment of advanced NSCLC after failure of conventional therapy.[17] In 2004, another EGFR-TKI, erlotinib (OSI-774; Genentech, Roche Group, South San Francisco, CA, USA) was approved by the FDA for the treatment of NSCLC after failure of cytotoxic chemotherapy.[18] More recently (in 2014), the irreversible ErbB family blocker afatinib (BIBW-

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2992; Boehringer Ingelheim Pharmaceuticals, Ingelheim am Rhein, Germany) was also approved by the FDA for clinical use in chemotherapy-naïve patients whose tumors harbor activating mutations in the *EGFR* gene.[19]

Mechanisms of action

Inhibition of the tyrosine-kinase activity of EGFR, whether reversibly (by gefitinib and erlotinib) or irreversibly (by afatinib), is due to the competition of these drugs with ATP molecules for binding sites in the C-terminal domain (catalytic sites) of the receptor. Blocking the phosphorylation of those sites prevents signal transduction through downstream components of the pathway by blocking activation of, for example, the MAPK and PI3K/Akt/mammalian target of rapamycin pathways.[20] As a result, these TKIs interfere with important aspects of tumor viability, leading to reduced proliferation, survival, and angiogenesis of cancer cells, as well as promoting their apoptosis by increasing their sensitivity to the toxic effects of chemotherapy and radiotherapy.[21]

Results from clinical trials in unselected populations

Before activating mutations in the *EGFR* gene had been identified as predictors of a response to EGFR-TKIs,[5,6] clinical trials were conducted in unselected populations of patients with advanced NSCLC. Two sequential phase II trials evaluating the activity of gefitinib in patients with NSCLC previously treated with cytotoxic chemotherapy showed response rates of up to 19%, median overall survival of approximately 8 months, and one-year survival of up to 35%.[22,23] However, in two sequential phase III trials, the combination of gefitinib and platinum-based conventional chemotherapy, as a first-line treatment, was not found to be superior to placebo plus chemotherapy, with no significant differences being found in terms of response rates or overall survival.[24,25] Likewise, two separate phase III trials showed that the combination of erlotinib and cytotoxic chemotherapy did not improve response rates or overall survival in comparison with chemotherapy alone.[26,27]

An international, randomized, placebo-controlled trial of treatment with erlotinib after the failure of standard chemotherapy for NSCLC, involving 731 patients with advanced NSCLC that had previously been treated with first- or second-line platinum-based chemotherapy, was conducted between August of 2001 and January of 2003.[28] Among those patients (some of whom were treated in Brazil), erlotinib led to improvements in overall survival (median of 6.7 months in the erlotinib-treated group vs. 4.7 months in the placebo group; hazard ratio [HR] = 0.70; p < 0.001) and in one-year survival (31% vs. 22%). The response rate was also higher in the erlotinib group (8.9% vs. < 1%; p < 0.001). It is of note that the authors of that study identified specific subgroups of patients in which treatment with erlotinib provided greater benefit: those with adenocarcinoma; those who were female; those of Asian descent; and those with no smoking history. Those results corroborated the emerging body of literature regarding which patients are most likely to derive clinical benefit from the use of EGFR-TKIs.[22,23,29] A study using a similar design compared gefitinib with placebo in 1,692 patients with advanced refractory NSCLC and demonstrated that the median progression-free survival was significantly longer in the gefitinib-treated group (2.2 months vs. 1.8 months; HR = 0.61; p < 0.001).[30] However, that was considered a negative study, because there was no significant difference in the primary endpoint, i.e., overall survival. Another phase III trial involving an unselected population compared gefitinib with docetaxel in 1,443 patients with advanced NSCLC after failure of platinum-based, first-line chemotherapy.[31] In that study, gefitinib was not found to be inferior to docetaxel in terms of median overall survival (7.6 months vs. 8.0 months; HR = 1.02). It is noteworthy that pemetrexed has also been shown to be comparable to docetaxel in this setting.[32]

Activating mutations in the *EGFR* gene and response to EGFR-TKIs

Independent work by two groups led to the seminal discovery that tumors responding to an EGFR-TKI typically harbor activating mutations, most often located in exon 19 (del19) or exon 21 (L858R) of the *EGFR* gene.[5,6] These mutations cause structural alterations in the ATP-binding site of the intracellular domain of EGFR, thus increasing the affinity for TKIs and leading to clinical responses. Although the first four exons encoding the tyrosine-kinase domain of EGFR

(exons 18 through 21) have been identified as major sites for activating mutations, small deletions in exon 19 and point mutations in exon 21 account for over 90% of such mutations.[33] Other, less common mutations have also been identified, including specific nucleotide substitutions at codon 719 of exon 18 (G719S) and insertions at codon 20.[34] Exon 19 deletions and the L858R mutation lead to a constitutively activated receptor state, as well as to a greater response upon ligand stimulation.[35] It has also been shown that these mutations lead to constitutive activation of Akt, which translates to greater survival.[36] Activating mutations in the *EGFR* gene have been observed in 8-15% of all NSCLC patients worldwide and in 25-30% of those in Brazil.[37,38]

Some patients who show an initial response to first-generation TKIs (erlotinib and gefitinib) experience disease progression, and many of those patients display secondary EGFR mutations or MET amplification. Approximately 50% of such patients have tumors that harbor the T790M mutation, and another ≈20% have tumors with MET amplifications.[39,40] Specific inhibitors of the T790M mutation (AZD9291 and CO1696) are under study, as are potential MET inhibitors (onartuzumab and tivantinib).[40] However, none of those have proven to be clinically effective in this scenario.[41] The second-generation EGFR-TKI afatinib is an irreversible ErbB family blocker that is effective in patients with the more common mutations and seems to be effective even in patients with the less common mutations, including the T790M mutation in exon 20, which is one of the main mechanisms of resistance to first-generation EGFR-TKIs.[39]

Clinical studies in mutation-rich populations

The findings described above paved the way for a novel generation of clinical trials that aimed to evaluate the performance of EGFR-TKIs in populations selected for activating mutations in the *EGFR* gene. The results from the main studies are summarized in Table 1.

A phase III clinical trial of the EGFR-TKI gefitinib, in comparison with the carboplatin-paclitaxel combination, in patients with advanced lung adenocarcinoma (designated the IPASS study, conducted in Asia) included patients with clinical features known to be associated with a higher rate of response to TKIs.[42] The patients, all of whom were nonsmokers or former light smokers, were randomized to first-line treatment with gefitinib or with carboplatin plus paclitaxel. The one-year progression-free survival rate was higher in the gefitinib arm than in the carboplatin-paclitaxel arm (24.9% vs. 6.7%). In addition to achieving its primary objective of demonstrating the non-inferiority of gefitinib as a first-line treatment for advanced lung adenocarcinoma in clinically selected patients, the IPASS study also demonstrated the superiority of gefitinib in this setting. In addition, retrospective evaluation of the EGFR mutation status in tumor samples demonstrated that even within the clinically selected IPASS population, a response to gefitinib correlated strongly with the presence of activating mutations in the *EGFR* gene, corroborating their predictive role. Among the mutation-positive patients, the objective response rate to gefitinib was 71.2%, compared with 47.3% for the carboplatin-paclitaxel combination, whereas the inverse was observed among the patients with no mutations, who showed objective response rates to gefitinib and to the carboplatin-paclitaxel combination of 1.1% and 23.5%, respectively. The IPASS data also demonstrate that tumors harboring activating mutations in the *EGFR* gene are more chemosensitive, showing higher response rates than do wild-type tumors. Maemondo et al.[43] also evaluated gefitinib, in comparison with the carboplatin-paclitaxel combination, as first-line therapy in patients whose tumors harbored activating mutations in the *EGFR* gene. The authors found that the median progression-free survival among the patients treated with gefitinib was 10.8 months, double the 5.4 months observed among the patients treated with the carboplatin-paclitaxel combination. In addition, the one- and two-year progression-free survival rates were 42.1% and 3.2%, respectively, in the gefitinib group, compared with 8.4% and 0%, respectively, in the carboplatin-paclitaxel group. Finally, the objective response rate was significantly higher in the gefitinib group than in the carboplatin-paclitaxel group (73.7% vs. 30.7%).[43] In a similar study, Mitsudomi et al.[44] compared gefitinib with cisplatin plus docetaxel as the first-line treatment of patients with mutations. As in the other studies cited, the median progression-free survival and objective response rate were better in the gefitinib group (9.2 months vs. 6.3 months and 62.1% vs. 32.2%, respectively).

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Table 1 – Randomized trials of EGFR-TKIs in selected populations rich in activating mutations in the *EGFR*
gene.

Reference	N	Prevalence of EGFR mutations	TKI	Response rate*			Progression-free survival*			
				(%)			(median in months)			
		(%)		TKI	CT	p	TKI	CT	HR	p
Mok et al.[42]	1,217	60	G	71	47	< 0.001	10.0	6.0	0.48	< 0.001
Maemondo et al.[43]	230	100	G	74	31	< 0.001	10.8	5.4	0.36	< 0.001
Mitsudomi et al.[44]	177	100	G	62	32	< 0.0001	9.2	6.3	0.49	< 0.0001
Zhou et al.[45]	165	100	E	83	36	< 0.0001	13.0	4.6	0.16	< 0.0001
Rosell et al.[46]	174	100	E	58	15	< 0.0001	9.7	5.2	0.37	< 0.0001
Sequist et al.[47]	345	100	A	56	23	0.001	11.1	6.9	0.58	0.001
Wu et al.[48]	364	100	A	67	23	< 0.001	11.0	5.6	0.28	< 0.0001

A: afatinib; CT: chemotherapy; E: erlotinib; G: gefitinib; HR: hazard ratio; and TKI: tyrosine-kinase inhibitor. *Among
patients with EGFR mutations.

A phase III trial comparing the EGFR-TKI erlotinib with the gemcitabine-carboplatin combination showed that the former provided significant gains in progression-free survival (median, 13.1 months vs. 4.6 months) and in the objective response rate (83% vs. 36%).[45] Another phase III trial of erlotinib, designated the EURTAC study,[46] was the first to compare it with platinum-based chemotherapy as a first-line therapy in Caucasian patients with activating mutations in the *EGFR* gene. The authors of that study also reported that, in comparison with the cytotoxic chemotherapy, treatment with erlotinib provided significant gains in progression-free survival (median, 9.7 months vs. 5.2 months) and in the objective response rate (58% vs. 15%).

In a phase III trial designated the LUX-Lung 3 study,[47] afatinib was compared with the current standard of cisplatin plus pemetrexed in the first-line treatment of Asian and non-Asian patients with adenocarcinoma. The authors found that progression-free survival was significantly better in the afatinib group (median, 11.1 months vs. 6.9 months). In that study, the superiority of afatinib over the cisplatin-pemetrexed combination was found to be even greater in patients with common mutations, such as del19 and L858R. It is noteworthy that the LUX-Lung 3 study used the cisplatin-pemetrexed combination, which is considered more effective, as a reference treatment. In a subsequent phase III trial, designated the LUX-Lung 6 trial,[48] afatinib was compared with gemcitabine plus cisplatin in Asian patients with tumors harboring EGFR mutations. Median progression-free survival and the response rate were better in the afatinib group than in the gemcitabine-cisplatin group (11 months vs.

5.6 months and 67% vs. 23%, respectively).[48] Cross-trial comparisons indicate that afatinib provides the longest progression-free survival. A recent combined analysis of the LUX-Lung 3 and LUX-Lung 6 studies (the largest of such trials) suggested an overall survival gain for afatinib over chemotherapy, mainly in patients whose tumors harbor exon 19 deletions.[49]

Despite the markedly better activity of first- and second-generation EGFR-TKIs, when compared with traditional chemotherapy, no difference in overall survival has been found among mutation-rich populations. In the majority of patients with EGFR mutations, second-line treatment with an EGFR-TKI was used in those who had disease progression after chemotherapy, thus obscuring the treatment effect of these agents on overall survival, something that has been demonstrated for the IPASS and EURTAC trials.[46,50] Consequently, when EGFR genotype results are not available, we suggest starting conventional cytotoxic platinum-based chemotherapy in patients with tumor-related symptoms and introducing an EGFR-TKI only after a mutation has been detected. An EGFR-TKI could also be used as maintenance therapy, or even as a second-line treatment, given that EGFR-TKIs have been shown to have no negative impact on survival.[51]

One of the common adverse effects of EGFR-TKIs is papulopustular (acneiform) rash, which is in fact a favorable prognostic and predictive factor of a response to such drugs. Other reported adverse effects include gastrointestinal symptoms (such as hyperbilirubinemia, diarrhea, nausea, and anorexia), dyspnea, fatigue, and edema, although all of these effects are generally well tolerated and manageable.[52]

All EGFR-TKIs have some penetration across the blood-brain barrier and are therefore effective modes of therapy for patients with central nervous system metastases, as well as being well tolerated by such patients.[53] Treatment with an EGFR-TKI is particularly helpful when such metastases are small, because it can (in some cases) allow radiation therapy or surgery to be postponed.[53] A recent phase II study confirmed that it is feasible to continue treatment with an EGFR-TKI (erlotinib) in patients with asymptomatic progressive disease—as determined on the basis of the response evaluation criteria in solid tumors—and showed that such treatment has no impact on overall survival.[54] However, antacids that modify gastric pH can affect absorption and thus reduce the efficacy of EGFR-TKIs.[55]

Diagnosing EGFR mutations

Given the role of activating mutations as predictors of a benefit from EGFR-TKIs, there is a clear need to accurately genotype tumor samples obtained from patients with NSCLC. In some studies, large-scale screening for such mutations has proven feasible. The most widely studied activating mutations (EGFR mutations, Kirsten rat sarcoma viral oncogene homolog mutations, and echinoderm microtubule-associated protein-like 4-anaplastic lymphoma kinase rearrangement) are usually mutually exclusive. Therefore, in day-to-day practice, physicians can discontinue the molecular investigation when one such mutation is identified. It is of note that, because these types of mutations occur in only 5% of patients with squamous cell carcinoma, such patients are not routinely screened for them. In addition, even if an activating mutation is identified, there is no clear evidence that treatment with EGFR-TKIs provides a benefit in cases of squamous cell carcinoma.[56]

General recommendations

At present, there are guidelines recommending screening for EGFR mutations in patients with advanced pulmonary adenocarcinoma who are candidates for first-line therapy with erlotinib, gefitinib, or afatinib, regardless of performance status or smoking history. However, in the adjuvant therapy scenario, it is not recommended to incorporate such screening into the clinical routine, because of the scarcity of data regarding the use of EGFR-TKIs in patients with localized or locally advanced disease.[57] It is recommended that greater attention be paid to patients with adenocarcinoma and to those who are never-smokers. Ideally, sample collection for molecular testing should be done at the time of the histological classification of the tumor, the ideal turnaround time being ≤ 7 days. It should be borne in mind that mutation screening in blood samples is still considered experimental.[58] Careful consideration should be given to the type of biopsy, taking into account the number of malignant cells likely to be present in the sample, because some mutation-detection techniques require large fractions of tumor cells in the sample. Tissue for molecular testing can be obtained by bronchoscopy, mediastinoscopy, thoracoscopy, pleural biopsy (for malignant pleural effusion), or CT-guided percutaneous biopsy. Endobronchial ultrasound with transbronchial biopsy can also be helpful in select cases. Obtaining bone samples is feasible, although they must be processed only by laboratories with experience, in order to avoid losses. However, other biopsy sites are preferred, if available.

Techniques for identifying EGFR mutations

Direct DNA sequencing

First described by Sanger in 1977,[59] direct DNA sequencing has contributed greatly to the development of biotechnology, culminating in the sequencing of a large part of the human genome. The method relies on the so-called dideoxy reaction, in which dideoxynucleotides (ddNTPs) are used in order to interrupt the replication of the genetic material, thus generating segments of different sizes. Fluorescently labeled ddNTPs can reveal the sequence of DNA bases in the sample through the analysis of the various bands thus generated. Although well established and reliable, direct sequencing requires samples containing a large fraction of tumor cells, usually more than 30% of the sample, a proportion not easily obtained, given that non-neoplastic tissue often comprises most of the biopsy material. Newer sequencing methods show great potential for future application; among them, pyrosequencing deserves mention, because it can detect mutations in samples containing only 0.2% tumor cells. This extremely sensitive technique can be used in order to detect EGFR mutations in pleural

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effusion samples containing only 10% of neoplastic cells. However, the method is still not widely available and requires sophisticated, expensive equipment.[60]

Methods based on PCR

The PCR technique can be used not only to amplify genetic material but also to detect mutations of interest. One of the PCR methods most commonly used for the latter purpose is the amplification refractory mutation system (ARMS), which is based on the differential activity of the enzyme Taq DNA polymerase during amplification of sequences that have mismatch points at 3′. The primers used in the ARMS reaction, when pairing with mutated sequences, generate mismatch points, allowing detection of mutations through the identification of differences in the band patterns generated. Kimura et al.[61] reported interesting results from the use of the ARMS technique in samples comprising less than 1% mutant EGFR-containing material. According to the College of American Pathologists, laboratories should use EGFR tests that are able to identify mutations in specimens with at least 50% cancer cells, although it has recently encouraged the use of more sensitive tests that can detect mutations in specimens with less than 10% cancer cells.[62] The technique known as TaqMan PCR uses probes that are specific for the wild-type and mutant sequences of EGFR. The presence of mutated sequences is indicated by the fluorescence peaks generated. Jian et al.[63] were able to identify mutations in samples comprising at least 10% mutant EGFR-containing material.[63] This approach facilitates testing by using a single step, no post-PCR processing being required. Variations of the PCR technique include other sensitive methods that apply selective amplification of mutated sequences. Such variations, which display high sensitivity in samples with low proportions of mutant EGFR-containing material, include mutant-enriched PCR assay, peptide nucleic acid-locked nucleic acid PCR clamping, and the smart-amplification process. Using mutant-enriched PCR assay, Asano et al.[64] detected mutations that were present in only 0.05% of the tumor samples evaluated. These techniques provide new possibilities for developing diagnostic tests that will be capable of detecting mutations in a less invasive manner, often using small samples.

RFLP

The RFLP technique is based on the use of restriction enzymes, which cleave sequences of genetic material at specific sites. As a result, DNA segments of different sizes are generated according to the presence of mutations. Those segments in turn present different patterns of electrophoretic mobility, which allows the detection of mutations through the analysis of the band patterns observed. Using this method, Pan et al.[65] detected mutations that were present in proportions as low as 6.25% for exon 19 deletions and 3.25% for L858R.

Other techniques

Several other methods for the detection of EGFR mutations have been described. Probes designed specifically for the detection of mutated alleles, such as the cycleave probe, emit a fluorescence peak in the presence of the mutation and have shown good results with samples in which at least 5% of the cells harbor mutations.[66] Other methods include the so-called loop-hybrid mobility shift assay, the single-strand conformation polymorphism technique, and HPLC. The latter technique, which is a means of comprehensively characterizing the DNA sequence under study and not only previously known mutations, has shown good sensitivity in samples with only 1% mutated material.[67] Finally, if labeled antibodies are directed against mutant EGFR proteins resulting from the transcription of *EGFR* genes with known activating mutations, immunohistochemistry can be used to detect mutations of interest. The validation of mutation-specific immunohistochemistry in clinical practice is eagerly awaited, because this technique could greatly facilitate the identification of the patients most likely to benefit from treatment with EGFR-TKIs.[68]

Final considerations and perspectives

The latest guidelines for the classification of lung adenocarcinoma recommend including EGFR genotyping in the diagnostic algorithm.[69] The appropriate selection of patients who are potential candidates for EGFR-TKI therapy becomes even more critical when one considers other genetic alterations observed in lung adenocarcinomas, such

as RAS mutations, anaplastic lymphoma kinase translocations, and HER-2 amplification. Some of these alterations seem to be mutually exclusive and can also be targeted by specific therapies under development or already in clinical use.[70] Genome-wide projects evaluating multiple genetic changes in patient samples have shown promising results and might lead to the identification of relevant targets for future intervention.[71] Data in the Cancer Genome Atlas suggest that pulmonary adenocarcinoma could be classified by molecular subtype based on next-generation sequencing reads, the new nomenclature for the transcriptional subtypes being terminal respiratory unit (formerly bronchioid), proximal-inflammatory (formerly squamoid), and proximal-proliferative (formerly magnoid). Adenocarcinomas that present activating mutations in the *EGFR* gene are clustered in the terminal respiratory unit subtype. The other two subtypes (proximal-inflammatory and proximal-proliferative) do not seem to be associated with EGFR-mutated tumors.[72]

In advanced NSCLC patients with EGFR-wild type tumors, the use of EGFR-TKIs cannot be considered a valid second-line treatment option after failure of a platinum-based regimen.[73] In patients with known activating mutations, a TKI can be used as a first-line treatment and should be maintained until there is clinically documented disease progression.[74,75] This "personalized medicine" approach represents a new frontier in modern oncology, in which the treatment of each cancer patient will be targeted according to genetic alterations present in the tumors—treating the right patient with the right drug, at the right dose, and at the right time.

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