EGFR Mutation Analysis

Background

Cancers of the lung are aggressive diseases, accounting for approximately 30% of all cancer deaths in the U.S. in 2009, with the overall survival rate of patients with metastatic disease less than 15%.[1] Different histological subtypes exist, including small-cell lung cancer (SCLC) that accounts for approximately 20% of lung cancer cases, and non-small-cell lung cancer (NSCLC) that represent the majority of lung cancer cases. NSCLC includes adenocarcinoma, squamous cell carcinoma and large-cell carcinoma. NSCLC tumors are also categorized according to molecular criteria.

Lung cancers harboring mutations in the epidermal growth factor receptor (*EGFR*) have demonstrated some success in responding to EGFR tyrosine kinase inhibitors. *EGFR*-mutant NSCLC is defined as a distinct, clinically relevant subset of lung cancer. *EGFR*-mutant tumors are histologically similar to adenocarcinomas, and are often associated with better prognosis than *EGFR* wild-type tumors, due primarily to their increased sensitivity to tyrosine kinase inhibitors (TKIs) such as erlotinib (Tarceva from Genentech and OSI Pharmaceuticals) or gefitinib (Iressa from AstraZeneca). These two drugs have demonstrated an increase in progression-free and overall survival in patients who receive *EGFR*-TKI therapy as a first-line therapy for the treatment of NSCLC. However, not all mutations in the *EGFR* gene confer sensitivity to TKIs, and primary resistance is still observed. Therefore, the mutation status of *EGFR* can be a useful marker by which patients are selected for *EGFR*-targeted therapy.

EGFR is a transmembrane receptor belonging to the ERBB family of receptor tyrosine kinases, which also includes HER2, HER3 and HER4. After ligand binding, the EGFR receptor forms a dimer that activates receptor autophosphorylation through tyrosine kinase activity, which triggers a series of intracellular pathways that may result in cancer-cell proliferation, blocking apoptosis, activating invasion and metastasis, and stimulating tumor-induced neovascularization.[2,6] Small-molecule EGFR TKIs inhibit *EGFR* autophosphorylation and downstream signaling by reversibly competing with ATP to bind to the intracellular catalytic domain of EGFR tyrosine kinase.[2]

Clinical Indications

Patients with advanced, non-treated NCSLC should have their tumor tested for *EGFR* mutations to guide first-line therapy, such as treatment with EGFR TKIs or chemotherapy. Activating mutations in *EGFR* occur in exons 18 to 21, which encode the kinase domain. These mutations are usually heterozygous, and amplification of the mutant allele can also occur.[1] The most common activating mutations found in *EGFR* are deletions in exon 19 centered around four amino acids (LREA) at positions 747–750, and a missense mutation (L858R) arising as a result of the leucine to arginine amino acid substitution at position 858 within exon 21. Together, these account for approximately 90% of TKI-sensitive mutations within the *EGFR*-mutant tumors.[1,7] The L861Q mutation in exon 21 and the substitution of glycine at position 719 in exon 18 with alanine (G719A), serine (G719S) or cysteine (G719C) also confer increased sensitivity to EGFR TKIs,[7,8] although these are much less common. Together, the activating mutations are associated with response rates of approximately 70% when treated with erlotinib or gefitinib.[9] The S768I mutation in exon 20 is a rare mutation, which has also been reported to confer sensitivity to TKIs, although at a lower level than the L858R mutation or exon 19 deletions.[7] Analysis of the crystal structures of L858R and G719S EGFR mutant proteins demonstrated that the kinase is activated through disruption of autoinhibitory interactions, resulting in receptors with 50-fold more activity compared to wild-type EGFR receptors.[1] Recent studies have shown that patients with tumors harboring exon 19 deletions are associated with longer time to progression (TTP) and overall survival (OS) when compared with L858R point mutations,[9] presumably due to greater sensitivity to TKI treatments.

Mutations in exons 18 to 21 can also be associated with decreased sensitivity or primary resistance to TKIs. Small insertions or duplications in exon 20 (including D770_N771insG) have been shown to be less sensitive to TKIs than exon 19 deletion and L858R mutants. In fact, most patients with these mutations show progressive disease while undergoing TKI therapy.[1]

Acquired resistance can also arise, and despite an initial response to EGFR TKI therapy, patients with *EGFR* mutations rarely achieve a complete radiographic or pathologic response.

Prolonged TKI treatment provides a selective pressure for the development of tumor cells with acquired resistance to gefitinib or erlotinib.[8] The T790M mutation that arises as a result of the substitution of threonine to methionine at position 790 in exon 20 has been found in 50% of patients with *EGFR*-mutant tumors who develop acquired resistance to TKIs.[1,3,4,8] This phenomenon is also seen in chronic myelogenous leukemia (CML) cells harboring *ABL* translocations and gastrointestinal stromal tumor (GIST) cells harboring activating KIT mutations, whereby the threonine gatekeeper residues are substituted with bulkier isoleucine residues that alter drug binding in both *ABL* (T315I) and *KIT* (T670I).[1] The T790M mutation is rare, occurring in <5% of untreated *EGFR*-mutant tumors, but is detected as a second site mutation in half of patients who develop acquired resistance to EGFR TKI therapy.[1] Evidence exists to show that acquired resistance can be lost after a period without TKI therapy,[1,5] however the mechanisms involved in this are not yet fully understood, and a recommendation regarding discontinuation of EGFR TKI therapy after developing acquired resistance has not yet been made. Collectively, these results deepen our understanding of resistance to *EGFR* inhibitors and underscore the importance of repeatedly assessing cancers throughout the course of the disease.

Numerous studies and trials have emphasized the importance of using genomic assessments of *EGFR* rather than using clinical characteristics, as the former is more accurate in selecting a group of patients with increased chance of sensitivity to EGFR-TKI therapy.[9]

Methodology

The *EGFR* Mutation Analysis assay is an allele specific polymerase chain reaction (ASPCR) assay performed on the Rotor-Gene® Q 5plex HRM® instrument (Qiagen) using two

Table 1: NSCLC Predictive Testing — *Mutations detected by the EGFR Mutation Analysis Assay*

Exon	Mutation	EGFR TKI Therapy Response Associated with Mutation	References	COSMIC ID	Base Change (CDS Mutation)	Amino Acid Change
18	G719A G719S G719C	Sensitive to EGFR TKI treatment	1, 7, 8	6239 6252 6253	c.2156G>C c.2155G>A c.2155G>T	p.G719A p.G719S p.G719C
19	Deletions in exon 19	Sensitive to EGFR TKI treatment	1, 7, 9	6223 13551 12728 12678 12367 12384 6225 6220 12422 12419 6218 6254 6255 12382 12387 6210 12370 12369 12383	c.2235_2249del15 c.2235_2252>AAT (complex) c.2236_2253del18 c.2237_2251del15 c.2237_2254del18 c.2237_2255>T (complex) c.2236_2250del15 c.2238_2255del18 c.2238_2248>GC (complex) c.2238_2252>GCA (complex) c.2239_2247del9 c.2239_2253del15 c.2239_2256del18 c.2239_2248TTAAGAGAAG>C (complex) c.2239_2258>CA (complex) c.2240_2251del12 c.2240_2257del18 c.2240_2254del15 c.2239_2251>C (complex)	p.E746_A750del p.E746_T751>I p.E746_T751del p.E746_T751>A p.E746_S752>A p.E746_S752>V p.E746_A750del p.E746_S752>D p.L747_A750>P p.L747_T751>Q p.L747_E749del p.L747_T751del p.L747_S752del p.L747_A750>P p.L747_P753>Q p.L747_T751>S p.L747_P753>S p.L747_T751del p.L747_T751>P
20	Insertions in exon 20	Lack of responsiveness to EGFR TKIs	1	12376 12377 12378	c.2307_2308ins9 c.2319_2320insCAC c.2310_2311insGGT	p.V769_D770insASV p.H773_V774insH p.D770_N771insG
20	T790M	Resistance to EGFR TKIs	1, 3, 4, 5, 8	6240	c.2369C>T	p.T790M
20	S768I	Low level sensitivity to EGFR TKI treatment	7	6241	c.2303G>T	p.S768I
21	L858R	Sensitive to EGFR TKI treatment	1, 7	6224	c.2573T>G	p.L858R
21	L861Q	Sensitive to EGFR TKI treatment	7, 8	6213	c.2582T>A	p.L861Q

technologies – Scorpions and ARMS® – to qualitatively detect the following 29 somatic mutations (*full details in Table 1*) against a background of wild-type genomic DNA:

- 19 deletions in exon 19 (detects the presence of any of the 19 deletions, but does not distinguish between them).
- T790M.
- L858R.
- L861Q.
- G719X (detects the presence of any of G719S, G719A or G719C, but does not distinguish between them).
- S768I.
- 3 insertions in exon 20 (detects the presence of any of 3 insertions but does not distinguish between them).

The ARMS® (Amplification Refractory Mutation System) technology achieves allele- or mutation-specific amplification. Taq DNA polymerase is effective at distinguishing between a match and a mismatch at the 3' end of a PCR primer. Specific mutated sequences are selectively amplified, even in samples where the majority of the sequences do not carry the mutation. When the primer is fully matched, the amplification proceeds with full efficiency. When the 3' base is mismatched, only low level background amplification occurs.[10]

Scorpions, or bi-functional molecules containing a PCR primer covalently linked to a probe, are used to detect amplification. The fluorophore in this probe interacts with a quencher, also incorporated into the probe, which reduces fluorescence. During PCR, when the probe binds to the amplicon, the fluorophore and quencher become separated. This leads to an increase in fluorescence from the reaction tube.[10] A total of seven mutation reactions, each labeled with FAM are performed. Each mutation reaction contains Scorpion probes plus primers for discrimination between the wild-type DNA and mutant DNA detected by real time PCR.

A control reaction (a region of exon 2 for which the primers and probe have been designed to avoid any known *EGFR* polymorphisms), also labeled in FAM, is performed to assess the amount of amplifiable DNA in the sample and to calculate the difference in cycle threshold (ΔC_T) between the mutation reaction and the control reaction from the same sample.

Interpretation of Results

A sample is considered mutation positive if the ΔC_T is less than the cut-off ΔC_T for that mutation reaction. Above this value, the sample may either contain less than the percentage of mutant DNA detectable by the assay, or the sample does not contain that specific mutation. The assay is capable of detecting mutations in samples containing at least 10% mutant allele. This is representative of 20% tumor within a heterogeneous sample containing tumor and non-tumor cells.

When using ARMS primers, some inefficient priming may occur, giving a very late background C_T from DNA not containing a mutation. All C_T values calculated from background amplification will be greater than the cut-off ΔC_T values and the samples will be classed as mutation negative.[10] This is particularly noticeable in the T790M mutation reaction, due to the fact that the T790M mutation is found in a very GC rich region. With primer design, GC content is critical for PCR efficiency. The sensitivity of the T790M assay is affected due to an increased limit of blank (LOB), which only allows an increased % of mutant to be discriminately detected. In the assay, this reflects itself in a lower ΔC_T cut-off, and the potential for 'breakthrough' (non-specific) amplification late in the reaction.

Specimen Preparation

Up to 10 unstained slides cut at a thickness of 7μm on unbaked microscope slides plus the pre- and post-H&E stained sections with the best tumor area circled by a pathologist are needed to perform the *EGFR* mutation analysis assay. The number of slides required will vary, depending on the size of the tumor area.

Alternatively, cellular material obtained from the patient's lungs by fine needle aspiration and preserved in CytoLyt solution can be used. Such material should be stored at 4°C for up to 2 weeks prior to analysis. The specimen submitted for analysis should contain >20% tumor cells, as determined by microscopic examination of ThinPrep slides, H&E stained slide or other slides.

Limitations of the Assay

A validation panel consisting of 100 specimens was used to assess the clinical performance of the *EGFR* Mutation Analysis assay. This consisted of samples for which *EGFR* mutation status had been determined by analysis at external CLIA-certified laboratories.

Sensitivity of the assay greatly depends on the extent of fragmentation and quality of the isolated DNA.

References

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2. Ciardiello & Tortora (2008) *EGFR* Antagonists in Cancer Treatment. *N Engl J Med* 358:1160-74.

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4. Kobayashi *et al.* (2005) EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. *N Engl J Med* 352(8):786-92.
5. Sequist *et al*. (2011) Genotypic and histological evolution of lung cancers acquiring resistance to *EGFR* inhibitors. *Sci Transl Med*. 2011 Mar 23;3(75):75ra26.
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7. Masago *et al.* (2010) Good Clinical Response to Gefitinib in a Non-small Cell Lung Cancer Patient Harboring a Rare Somatic Epidermal Growth Factor Gene Point Mutation; Codon 768 AGC > ATC in Exon 20 (S768I). *Jpn. J. Clin. Oncol*. (2010) 40 (11):1105-1109.
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9. Jackman *et al.* (2009) Impact of Epidermal Growth Factor Receptor and KRAS Mutations on Clinical Outcomes in Previously Untreated Non-Small Cell Lung Cancer Patients: Results of an Online Tumor Registry of Clinical Trials. *Clin Cancer Res* 2009.15:5267-5273.
10. *EGFR* RGQ PCR Kit Handbook, Qiagen, July 2010.

Test Overview

Test Name	*EGFR* Mutational Analysis
Ordering Mnemonics	EGFRCP (cell pellet); EGFRTI (tissue)
Methodology	Qiagen *EGFR* RGQ PCR Assay
Specimen Requirements	Acceptable specimens are cellular material obtained from the patient's lungs by fine needle aspiration, formalin fixed paraffin-embedded (FFPE) tissue block or cytopathology cell blocks. Submit specimen in one of the following: **Aspirate, Fine Needle** - Container: CytoLyt solution or PreservCyt solution. - Transport specimen at ambient temperature. **Formalin-Fixed, Paraffin-Embedded Block - Formalin Fixation Only:** - Tissue should be well-fixed in formalin. - The specimen submitted for analysis should contain >20% tumor cells. Please include Thin Prep slide and Cytology report. - Transport specimen at ambient temperature. **Unstained Slides:** - 8-10 unstained slides from formalin fixed block cut in 7 micron sections. - The specimen submitted for analysis should contain >20% tumor cells. Please include H&E slide and a copy of pathology report. - Transport specimen at ambient temperature.
CPT Codes	81235; 88381
Billing Codes	88871 (EGFRCP); 88877 (EGFRTI)

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