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MOLECULAR ANALYSES

# A comparative study of EGFR mutation screening methods in non-small cell carcinoma of lung

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**Abstract:** Epidermal growth factor receptor (EGFR) is a transmembrane receptor tyrosine kinase of the ERBB2 family that has important roles in the proliferation and metastasis of tumor cells. It is frequently overexpressed in common solid tumors and has become a favored target for orally administered small-molecule tyrosine kinase inhibitors (TKI) and monoclonal antibody-based therapy. Gain-of-function somatic mutations of the *EGFR* tyrosine kinase domain have been associated with the response of some patients with non-small-cell lung carcinoma to TKIs. We evaluated three methods of *EGFR* mutation analysis to identify an optimal assay for clinical testing based on comparison of diagnostic sensitivity, technical difficulty, and cost *(Tab. 1, Fig. 1, Ref. 12)*. Full Text in free PDF *www.bmj.sk.*
Key words: lung cancer, EGFR, molecular analysis.

Epidermal growth factor receptor (EGFR), a transmembrane receptor tyrosine kinase of the ERBB2 family, plays an important role in proliferation and metastasis of tumor cells. It is frequently overexpressed in common solid tumors and has become a favored target for orally administered small-molecule inhibitor (TKI) and antibody-based therapy (1, 2, 3).

Somatic mutations in the tyrosine kinase domain of the *EGFR* gene are present in non-small cell lung carcinomas (NSCLC) that respond to TKI, such as gefitinib and erlotinib (4). Mutations are more frequent in never-smokers (51 % vs 9 %), females (38 % vs 13 %), adenocarcinomas (31 % vs 2 %) and patients of East Asian origin (29 % vs 8 %) (4). Approximately 10 % of lung adenocarcinomas from American or European patients who are unselected for response to EGFR inhibitors contain *EGFR* mutations (4).

Two "hot spot" mutations account for ~90 % of *EGFR* mutations reported in NSCLCs (5). The most common mutation type, seen in ~45 % of cases with *EGFR* mutations, is a short in-frame deletion of 9, 12, 15, 18, or 24 nucleotides in exon 19. The second most common mutation, seen in another ~45 % of cases with *EGFR* mutations, is a point mutation (CTG to CGG) in exon 21 at nucleotide 2573, that results in substitution of leucine by arginine at codon 858 (NM_005228.3: c.2573T>G, p.Leu858Arg). Other, much less common mutations have been described in exons 18, 20, and 21 (5). Combining data from several studies, it appears that ~80 % of tumors that respond to TKIs contain missense mutations or in-frame deletions in *EGFR*, compared to none of drug-refractory tumors (6, 7, 8). These studies show that these *EGFR* mutations correlate strongly with sensitivity to specific EGFR inhibitors and that their detection could be used to predict which patients will respond to these novel drugs. The vast majority of these mutations were found in moderately to well differentiated adenocarcinomas, in particular those with partial or complete bronchioloalveolar features (4). The same histological features had been shown to correlate with response to EGFR inhibitors before the discovery of these mutations (1). Therefore, it is important to correlate the clinical and histopathologic features before testing for mutations.

Recently, a new EGFR mutation has been described in patients who initially responded to TKI treatment but subsequently experienced disease progression while still on treatment. Part of this "acquired resistance" is attributable to a secondary point mutation resulting in threonine to methionine at codon 790 (T790M) of *EGFR* (9). Clinical molecular testing for *EGFR* mutations in patients with NSCLC may aid in selecting therapy in adjuvant, neoadjuvant and advanced/metastatic settings.

Our goal was to compare the diagnostic sensitivity, technical aspects and cost of some of the three most common testing methods in clinical practice to identify the optimal assay for clinical testing.

## Materials and methods

*Tissue Processing*

The tumor samples were received as formalin-fixed paraffin-embedded tissue blocks or frozen tissues. Five 10-micron thick sections of tissue were macrodissected manually with a clean scalpel blade and prepared for DNA extraction using

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QIAamp® DNA Mini Kit (Hilden, Germany). Frozen specimens were cut with a cryostat, with a single 5 micron section taken first for H & E staining, followed by twenty 30 micron sections for DNA preparation, followed by another section for hematoxilin and eosin staining. The percentage of tumor cells as compared to normal cells was estimated from the two H & E stained slides (first and last sections) in both frozen and paraffin embedded tissue samples. Only samples in which the tumor represented more than 70 % of the tissue before macrodissection were processed further.

*Sequencing analysis*

*EGFR* exons 19 and 21 were amplified from genomic DNA obtained from the paraffin and frozen samples by polymerase chain reaction (PCR) with the following primers: EGFRex19Forward 5'GCAATATCAGCCTTAGGTGCGGCTC3', EGFRex19 Reverse 5'CATAGAAAGTGAACATTTAGGATGTG3' and EGFRex21Forward 5'CTAACGTTCGCCAGCCATAAGTCC3', EGFRex21Reverse 5'GCTGCGAGCTCACCCAGAATGTCTGG3' (10). PCR was performed using the following amplification conditions: initial denaturation at 95 °C for 15 min followed by 35 cycles of the following steps: denaturation at 95 °C for 30s, annealing at 56.4 °C for 30 s, and elongation at 72 °C for 30 s. After the last cycle, a final extension at 70 °C for 10 minutes was performed. The amplified products were electrophoresed on a 1.2 % gel at 110V for 1.5 hours and the EGFR exon 19 and exon 21 bands (~205 and ~235bp) were cut using sterile blade and purified using Qiagen Gel Extraction Kit (Hilden, Germany). The samples were analyzed on an ABI PRISM 3130 Sequence Analyzer (Applied Biosystems, Foster City, CA, USA). Mutations were to be confirmed by reamplification by polymerase chain reaction and by sequencing from both 5' and 3' ends.

*PCR analysis*

A 207-bp genomic fragment including all of exon 19 was amplified using primers EGFR-Ex19-forward1 (GCACCATC TCACAATTGCCAGTTA) and EGFR-Ex19-reverse1 (Fam-AA AAGGTGGGCCTGAGGTTCA) (10). The PCR reaction mix was made up as follows: HotStarTaq DNA polymerase and 10 x buffer (Qiagen, Valencia, CA), EGFR-Ex19-forward1 and EGFR-Ex19-reverse1 primers, genomic DNA template, dNTPs, *N*-glycosylase, $MgCl_2$ and sterile distilled water. The PCR was performed as follows: 95 °C x 15 minutes (to inactivate *N*-glycosylase and activate *Taq*DNA polymerase), followed by 40 cycles of 95 °C x 0.5 minutes, 60 °C x 1 minute, 72 °C x 1 minute, and a final extension of 72 °C x 10 minutes. 1 in 50 dilution of the product was made of which 1 µl was added into 20 µl of formamide plus 1 µl of Genescan 400 HD size standard (Applied Biosystems). The samples were denatured and subjected to capillary electrophoresis on an ABI PRISM 3130 Sequence Analyzer (Applied Biosystems).

A 222-bp genomic fragment including all of exon 21 was amplified using primers EGFR-Ex21-FWD1 (CCTCACAGCAG GGTCTTCTCTGT) and EGFR-Ex21-REV1 (Fam-TCAGGAA AATGCTGGCTGACCTA ) (10). The PCR reaction mix was made up as above, but with EGFR-Ex21-FWD1 and EGFR-Ex21-REV1 primers. The PCR was performed as follows: 95 °C x 15 minutes (to inactivate *N*-glycosylase and activate *Taq*DNA polymerase), followed by 95 °C x 0.5 minutes, 60 °C x 1 minute, 72 °C x 1 minute, and a final extension of 72 °C x 10 minutes for 40 cycles. The PCR products were then purified using PCR Kleen Spin column (Bio-Rad, Hercules, CA) at 735 x *g* for 2 minutes. To identify a new restriction site introduced by the mutation enzymatic digestion of the amplicons was performed. *Sau*96I digestion reaction was performed at 37 °C for 2 hours and consisted of the following: PCR product, 10x NEBuffer 4 (New England Biolabs, Beverly, MA), *Sau*96I restriction enzyme (New England Biolabs), and sterile distilled water. After digestion, 1 µl of undiluted *Sau*96I-digested PCR product was added to 20 µl of formamide and 1 µl of Genescan 400HD size standard. The samples were denatured and subjected to capillary electrophoresis as described above.

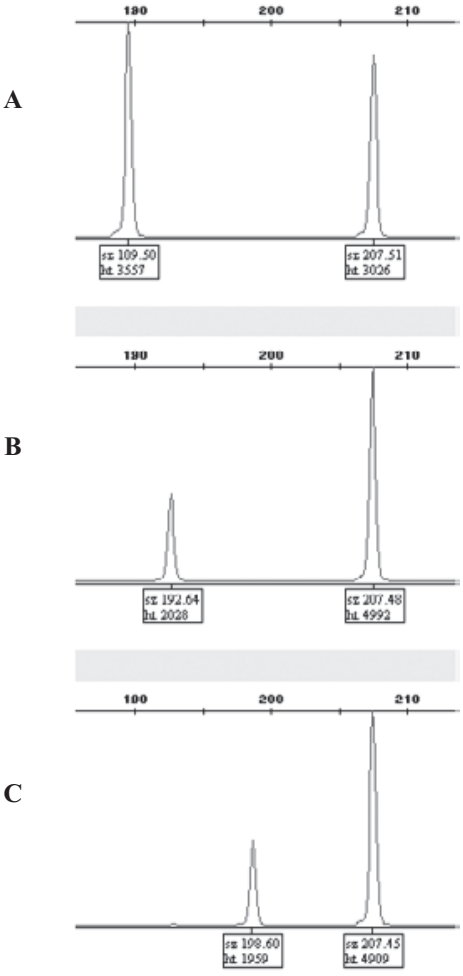


**Fig. 1. Exon 19 mutations. Length analysis of fluorescently labeled PCR products (left peak mutant, right peak wild type): 18 bp deletion (A), 15 bp deletion (B) and 9 bp deletion (C)**

*EGFR Real Time Assay*

The real time PCR assay (DxS; Manchester, UK)(11) enables the specific detection of the EGFR Exon 19 in frame dele-

**Tab. 1. Summary of results.**

| SAMPLES | CLINICAL DATA | SEQUENCING | RT-PCR | PCR-CE |
|---|---|---|---|---|
| S05-29845 | M, NS | WT | WT | WT |
| S05-28418 | F, NS | WT | WT | WT |
| S05-28067 | M, NS | WT | WT | WT |
| S05-25660 | M, NS | E746_S752del7insV | WT | Del18(189-207) |
| S05-26455 | F, A, NS | Inconclusive | Del15 | Del15(192-207) |
| S05-23686 | F, NS | Inconclusive | WT | Del9(198-207) |
| S05-26331 | F, NS | WT | WT | WT |
| S05-25559 | M, NS | WT | WT | WT |
| S04-8377 | F, NS | WT | WT | WT |
| S05-8357 | F, NS | WT | Del15 | Del15(192-207) |

F – female, M – male, NS – non-smoker, A – Asian origin, WT: wild type

tion of codons 746-750 and the amino acid substitution L858R in Exon 21. The assay detects mutant alleles in the background of excess wild type DNA based upon ARMS™ and DxS Scorpion™ (Scorpions Amplified Refractory Mutation System; DxS; Manchester, UK) technology. The use of ARMS technology allows for allele specific amplification based upon the ability of Taq DNA Polymerase to distinguish between a match and mismatch at the 3'-end of the PCR primer. Efficient amplification only occurs when the primer is fully matched allowing for mutated sequences to be selectively amplified. Scorpions are molecules containing a PCR primer covalently linked to a fluorescent probe, which interacts with a quencher. Upon amplification the fluorophore and quencher are separated leading to increased fluorescence in the reaction. These two technologies have been combined to provide an assay with a high level of sensitivity and specificity for detecting EGFR mutations. The reaction mix was composed of one of following primer mixes: Primer Mix del 15 WT, Primer Mix del 15 MUT, Primer Mix L858R WT and Primer Mix L858R MUT; additionally Taq Polymerase and sterile distilled water was added to each of four reaction mixes, according the manufacturer protocol. Thermal cycling conditions were as follows: Stage 1: 95 °C – 10 minutes – 1cycle and Stage 2: 95 °C – 30 seconds, 62 °C – 30 seconds – 45 cycles.

## Results

Using PCR with capillary electrophoresis we found four exon 19 mutations (deletions of 9, 15, 15, and 18 nucleotides (Fig. 1) and no exon 21 mutation in total of 10 cases tested (Tab. 1). Only two exon 19 mutations were found by real time assay (not shown); the other two mutations (deletions) were different than 15 in base pair length, therefore not detectable by the kit we used. No exon 21 mutations were found by real time assay. Dye terminator sequencing analysis identified only one exon 19 mutation (not shown) and failed to identify another one detected by both PCR with capillary electrophoresis and real time assay. Two inconclusive results were most likely attributable to low sample DNA concentration. Dye terminator sequencing analysis detected no exon 21 mutation.

PCR with capillary electrophoresis and dye terminator sequencing analysis appeared to be equally cost effective in comparison with the real time PCR kit. Sequencing analysis required the most hands-on time (2–3 days) followed by the PCR with capillary electrophoresis and the real time PCR kit, both of which yielded results within 24 hours.

Our results were not intended to have any influence on patient management.

## Discussion

EGFR expression as determined by immunohistochemical methods was the first biomarker investigated as potential predictor of response. However, most studies have failed to show any relationship between EGFR expression and the clinical activity of anti-EGFR drugs (12). The discovery of mutations in the tyrosine kinase domain of the *EGFR* gene in non-small cell lung cancer accelerated the research of molecular targeted therapy by EGFR-tyrosine kinase inhibitors (1). About 90 % of EGFR mutations were found to be clustered in exons 19 (deletion) and 21 (point mutation at codon 858) and patients with these mutations had great response to EGFR-TKIs (1, 2).

In this comparative study we performed molecular analysis of 10 lung adenocarcinoma samples with regards to *EGFR* mutational status targeting the previously reported hot spot mutations. Our goal was to identify the most optimal assay considering several factors, including the diagnostic sensitivity. We found that PCR with capillary electrophoresis was able to detect the largest number of mutations in our samples (four mutant samples found, one mutation in each) followed by the real time PCR kit (two mutant samples) and sequencing analysis (one mutant sample). This was not surprising, since the real time assay we used was designed to detect only two specific mutations, i.e. exon 19 in frame deletion of codons 746–750 and the amino acid substitution L858R in exon 21, and different length deletions were missed by this assay.

Although real-time detection involved less hands-on technical time, the high cost of reagents and inferior diagnostic sensitivity in comparison to the PCR with capillary electrophoresis

made this method less than optimal. The latter method performed well compared to DNA sequencing in this study and may also have the benefit of great analytic sensitivity as previously reported (10). Similarly, the real time detection kit we used also has the advantage of high analytical sensitivity when compared with sequencing analysis (11). Currently newer generation real time research kits are available with increased diagnostic repertoire including detection of more than 20 most frequent mutations.

Sequencing analysis required several days to be completed and we found that it is perhaps the least practical method considering the potential need of specimen send out if the clinical laboratory does not perform routine sequencing analysis. Thus, we found that PCR with capillary electrophoresis detection for hot spot mutations of EGFR is best suited to our clinical laboratory setting.

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