PCR-based Testing for Therapy-related EGFR Mutations in Patients with Non-small Cell Lung Cancer

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Abstract. Background: In patients with non-small cell lung cancer (NSCLC), mutations in the epidermal growth factor receptor gene (EGFR) have been associated with improved response to tyrosine kinase inhibitors. Two hotspot mutations located in exon 19 and exon 21 account for about 90% of all EGFR mutations. Materials and Methods: We designed a Bi-PASA (bidirectional PCR amplification of specific alleles) assay to detect the most common exon 19 deletion (codons 746-750) and an allele-specific PCR for the L858R mutation in exon 21. To validate the assays for use in clinical diagnostics, 35 lung adenocarcinoma samples were analyzed. Results: Both assays provided the predicted amplification pattern for normal and mutant genotypes with high specificity and sensitivity. In serial dilution experiments, the mutant alleles were detectable in mixed samples with an at least 6-fold excess of normal DNA. Three exon 19 deletions were identified in the tumor samples. Conclusion: Both assays are fast and easy to perform in any routine PCR laboratory with no special equipment other than thermocyclers. They provide sensitive and cost-effective initial EGFR testing for identifying lung cancer patients who might clinically benefit from tyrosine kinase inhibitors.

Non-small cell lung cancer (NSCLC) is one of the most common types of cancer worldwide. Relapses are frequent after therapy and often evolve into lethal metastatic disease. Recent studies (1-3) have identified a subset of NSCLC patients with rapid and durable clinical responses to EGFR tyrosine kinase inhibitors (i.e. gefitinib, erlotinib) and found an underlying association between mutations in the EGFR tyrosine kinase domain and drug-responsive NSCLC. These heterozygous EGFR tyrosine kinase mutations, clustered within the ATP-binding pocket (4), cause ligand-independent activation of EGFR, while simultaneously increasing sensitivity to tyrosine kinase inhibitors. Two mutations account for approximately 90% of all EGFR mutations reported to date in lung adenocarcinoma (5-6). An in-frame deletion of 9-24 nucleotides in exon 19 centered around codons 746-750 makes up about 50% of mutations. The second most common mutation, found in up to 45% of cases with EGFR mutations, is a point mutation at nucleotide 2573 (CTG to CGG) in exon 21 that results in substitution of leucine by arginine at codon 858 (L858R). For current clinical practice in the care of lung cancer patients, simple screening assays for molecular predictors of response to EGFR tyrosine kinase inhibitors are needed. The gold standard for mutation detection is the genomic sequencing of DNA amplified from tumor tissues, however, this method is labor-intensive and of suboptimal sensitivity because clinical tumor specimens may contain a high percentage of nonneoplastic cells. Over the past three years, many methods have been proposed for the improved detection of EGFR mutations [reviewed in (7)]. Most of them involve complex assays or require expensive equipment or reagents and cannot be easily adapted for use in routine diagnostics. The aim of this study was to develop two simple and sensitive assays for the initial screening of the two most common therapy-related EGFR mutations in clinical tumor samples based on allele-specific PCR.

Materials and Methods

Samples and DNA isolation. Genomic DNA was extracted and pooled from oral mucosa samples of five healthy individuals. This pooled DNA was used as a normal DNA control for the development of the PCR assays. Heterozygous mutant control DNA was extracted from lung cancer cell lines NCI-H-1650 and NCI-H-1975 (American Type Culture Collection, LGC Promochem, Wesel, Germany) which contain the heterozygous exon 19 deletion (H-1650) and the exon 21 L858R mutation (H-1975) (4).

Formalin-fixed tumor samples were obtained from 35 patients treated at the HELIOS Clinical Center Erfurt, Germany, with the
histopathological diagnosis of lung adenocarcinoma. The study cohort consisted of 19 males and 16 females with a median age at diagnosis of 68.8 years. Informed consent was obtained in writing from all patients. This study was conducted in accordance with the principles of the Declaration of Helsinki, as adopted by the 29th World Medical Assembly, Helsinki, Finland, and revised at the 48th World Medical Assembly in Somerset West, Republic of South Africa, 1996. DNA was isolated using the QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s instructions.

**Exon 19 E746_A750del deletion assay.** For detecting the exon 19 deletion, a so-called bidirectional PCR amplification of specific alleles procedure (8) was designed. Bi-PASA contains four primers: i) two allele-specific primers complementary to the sequence variation in a bi-directional orientation such that both primers terminate at the variation site and ii) two so-called outer primers that anneal at different distances from the sequence variation to differentiate the downstream and upstream reactions on an agarose gel. In this way, the assay has an inherent control, since at least one allele specific fragment is amplified per reaction. The two outer primers (P and Q) were placed on the opposing strands at pre-defined bases, resulting in an amplicon of 444 bp as internal standard. The 3’ end of allele-specific inner primer A matches a part of the deletion sequence, i.e. the AQ fragment is only amplified in the case of the wild-type sequence. The inner primer B matches the mutant DNA sequence containing the deletion, i.e. PB can be amplified only in homozygous mutant DNA. A heterozygous genotype (N/M) is characterized by both a 325 bp AQ- and a 134 bp PB amplification fragment (Figure 1). The allele-specific primers are designed to detect both possible nucleotide deletions (c.2235_2249del15 and c.2236_2250del15) that give rise to an E746_A750del protein change. Parameters important for designing the four Bi-PASA primers were the melting temperature (with Tm values of A and B lower than that of the PQ segment) and the annealing temperature (relatively high to avoid hybridization between the multiple PCR products).

**Exon 21 L 858R point mutation assay.** The basis for discrimination using allele-specific PCR is that a PCR primer mismatched at its 3’ end with the DNA template will react less efficiently than one that is entirely complementary. Allele-specific primers, A and B, were designed with mismatches at their 3’ end – A complements the normal allele (2573T) while B complements the mutant allele (2573G). At the allele-specific primer’s 3’ end, one (21A) or two (21B) further mismatches were added in order to weaken hydrogen bonding between primer and template and therefore increase the likelihood of discrimination. A consensus primer, P, was designed in the sense direction within exon 21 overlapping the intron 20-21/exon 21 boundary. The exon 21 PCR assay involves two reactions: i) the ‘T-reaction’, containing the 5’ consensus primer P, and the primer A complementary to the normal EGF allele; ii) the ‘G-reaction’ containing P, and the primer B complementary to the mutant EGF allele (Figure 2). For testing and proving the specificity of the allele-specific PCR primers A and B, a homozygous mutant control sample was artificially created: 2 primers, 21F (5’-TGATGATCT GTCCCTCACAGCA-3’) and 21R (5’-ACCAGCATTGTGGCG CGC-3’) were designed with 21R complementary to the mutant allele (2573 G) at its 3’ end. DNA of the cell line NCI-H-1975 was amplified with these primers, resulting in a 160 bp PCR fragment containing the 2573G point mutation. All PCR primers were designed with the aid of OLIGO software (Software IDT SciTools, Integrated DNA Technologies, Coralville, USA). Primer sequences and PCR parameters are listed in Table I.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequences (5’-3’)</th>
<th>Optimal primer concentration (μmol/l)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bi-PASA exon 19</td>
<td>19 P GTAACATCCACCCAGATCAGT 0.25</td>
<td>PA: 137</td>
<td></td>
</tr>
<tr>
<td></td>
<td>19Q GTTGTCAAGAAACTAGTGTGTGG 0.25</td>
<td>AQ: 325</td>
<td></td>
</tr>
<tr>
<td></td>
<td>19A CCCGTGCTAACAAGGAATAA 0.25</td>
<td>PB: 134</td>
<td></td>
</tr>
<tr>
<td></td>
<td>19B GGTGGCCTTTCGAGAGTTTGTAG 0.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allele-specific PCR exon 21</td>
<td>21P AGGTTCTTCTCTGTTTG 0.1</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>21A TTCCGACCCACGATTTGCTA 0.1</td>
<td>PA: 137</td>
<td></td>
</tr>
<tr>
<td></td>
<td>21B CGC ACC CAG CAG TTT GGTTC</td>
<td>0.1</td>
<td>PB: 134</td>
</tr>
</tbody>
</table>

PCR. PCR reactions were run at a final volume of 25 μl. Reactions consisted of: 80-100 ng genomic DNA; 200 μmol/l dNTP; 0.1-0.25 μmol/l of primers; 1.5 mmol/l MgCl2; 0.5 U Taq polymerase (Roche Diagnostics, Penzberg, Germany). Cycling conditions were as follows: 5 min of denaturing at 94°C and 30 cycles of 94°C for 0.30 min, annealing 58°C (exon 19) or 60°C (exon 21) for 45 s and 72°C for 1 min. A volume of 10 μl of the PCR products was electrophoresed on a standard 2% agarose gel stained with SYBR-Green I for visualization under UV light.

**Sequencing.** The EGF mutations were analyzed by DNA sequencing of the relevant regions of exon 19 and 21. PCR products were purified with the MinElute PCR Purification Kit (Qiagen GmbH). Genomic sequencing (MWG Biotech, Penzberg, Germany) was performed using the appropriate downstream PCR primer.

**Results**

Identification of the exon 19 E746_A750del deletion and the exon 21 nt2573 point mutation in control samples. Before testing clinical samples, both allele-specific PCR assays were validated in known genomic samples (normal wild-type control DNA, heterozygous mutant control DNA from cell lines NCI-H-1650 and NCI-H-1975 and an nt2573 homozygous mutant PCR fragment). The predicted amplification pattern for normal and mutant genotypes was
obtained with high specificity i.e. detection of only the normal or only the mutant allele, high sensitivity i.e. no spurious PCR fragments and an acceptable yield (Figure 1b and 2b, lanes 1-3).

Sensitivity of the PCR-based assays in mixed cell populations. Because tumor specimens may contain a high percentage of nonneoplastic cells, screening assays must be able to detect mutations in heterogenous genomic samples.
In order to provide defined heterogenous samples, serial dilutions were established by mixing DNA from the heterozygous mutant cell line NCI-H-1650 and NCI-H-1975 with wild-type normal DNA. The exon 19 Bi-PASA assay was able to detect the mutant allele in an 8-fold excess of normal DNA. The mutant exon 21 2573T allele was detectable in a mixed sample with a 4-fold excess of normal DNA (Figure 3).

**Screening of patients samples.** DNA was isolated from 35 formalin-fixed paraffin-embedded lung tumor samples (obtained by biopsy or surgical resection) and screened for the both most common EGFR mutations. The PQ fragment in the exon 19 assay was always amplified, indicating the integrity of the isolated DNA from clinical tissue samples. Three mutations were found of which all were deletions in exon 19. Genomic sequencing confirmed the exon 19 deletion to be the variant c.2235_2249del15 in all three samples. Both wild-type and mutant amplification curves were found in all three cases indicating that the samples were most likely heterozygous. Case 1 was a 79-year-old female patient with a subpleural lung adenocarcinoma metastasis (previously diagnosed with a moderately differentiated lung adenocarcinoma in 2003). Case 2 was a 71-year-old female patient with a moderately differentiated primary lung adenocarcinoma of the right middle lobe after having tuberculosis decades earlier. Case 3 was a 74-year-old female patient with a primary moderately differentiated lung adenocarcinoma.

**Discussion**

A major effort to improve the clinical management of lung cancer patients entails the use of molecular markers to provide accurate predictions of the outcome after cancer treatment. EGFR mutation testing in lung adenocarcinoma is already used to help guide treatment decisions and/or to enroll patients on specific arms of clinical trials (7). For clinical routine, mutation screening will need to rely on fast, inexpensive, simple and sensitive assays. Because of its universal availability as a standard methodology in molecular medicine, we designed EGFR mutation screening assays based on PCR. Our assays are able to detect the two most common EGFR mutations in lung adenocarcinoma simply by PCR amplification and discrimination of the allele-specific PCR fragments by agarose gel electrophoresis. Allele-specific PCR, also known as amplification refractory mutation system (ARMS) is a well-established method for discriminating between different alleles at specific loci resulting from single base mutations (9, 10). Compared to other proposed methods for the detection of EGFR mutations in lung cancer, such as SSCP (11), TaqMan PCR (12), MALDI-TOF (13), dHPLC (14-16), single molecule sequencing (17), or SMAP (18), our allele-specific PCR assays do not need special equipment other than thermocyclers, reaction steps are simple and allow troubleshooting, reagents are inexpensive and the methodology can be easily implemented at a clinical laboratory.

For assay design, we had to solve the problem of having no homozygous mutant control DNA because EGFR mutations have been only found in a heterozygous status, both in patient samples and in the lung cancer cell lines NCI-H-1650 and NCI-H-1975. Consequently, these 'mutant' controls contain one wild-type allele and are not suitable to entirely control the allele-specificity of the primers, especially in the case of a point mutation in which it is demanded that one single base pair change is distinguished. There are several possible experimental approaches for creating artificially constructed mutation samples, including sample spiking, transfection, and genetic engineering, through such techniques as homologous recombination and site-directed mutagenesis (19). However, these techniques are time-consuming, labor-intensive and require some expertise with recombinant DNA techniques. Therefore, we created a homozygous mutant control fragment for the L858R point mutation artificially by PCR.

![Figure 3. Serial dilution experiment. a) Exon 19 assay. Serial dilution of the heterozygous mutant cell line DNA NCI-H-1650 with normal control DNA (lane 2: H-1650 undiluted; lane 3: 1:2 dilution; lane 4: 1:4; lane 5: 1:8). Lane 1: molecular weight marker. The PB fragment (arrow) is detectable in mixed samples in an up to 8-fold excess of normal DNA. b) Exon 21 assay. Serial dilution of the heterozygous mutant cell-line DNA NCI-H-1975 with normal control DNA (lane 2: H-1975 undiluted; lane 3: 1:2 dilution; lane 4: 1:4; lane 5: 1:8). Lane 1: molecular weight marker. The 'mutant' amplification fragment in the G-reaction (arrow) is detectable in mixed samples in a 4-fold excess of normal DNA.](image)
and were able to validate the primer specificity and to establish appropriate PCR conditions. Creating a similar control fragment simply by PCR was technologically not possible in the case of the 15 bp deletion in exon 19. But our assay design as a Bi-PASA with two outer and two inner primers ensures the discrimination between the normal and the mutant allele because the allele-specific inner primers involve the deletion. In practice, in screening patients’ samples, our exon 19 Bi-PASA was able to specifically detect the heterozygous \( \text{EGFR} \) mutation status in three patients. The PQ fragment that is always amplified serves not only as an internal control within the assay but also confirms the integrity of the isolated DNA from clinical tissue samples.

Approximately 10\% of lung adenocarcinomas contain \( \text{EGFR} \) mutations (5), as also found in our study cohort. Of these, 90\% are either nucleotide deletions around codons 746-750 in exon 19, or the exon 21 nt2573 point mutation (6). Both hot-spot mutations are detectable using the two PCR assays described herein. Among exon 19 mutations, few 9-, 12-, 18- and 24-bp deletions have been detected, and by far the most common deletion size was 15 bp (5). We decided to focus our assay design for lung cancer screening in clinical diagnostics on just the most common \( \text{EGFR} \) mutations related to chemotherapy sensitivity. Both PCR protocols should be appropriate for initial \( \text{EGFR} \) mutation analysis in patients with lung adenocarcinoma and provide a rapid, sensitive, and cost-effective screening method. Three other \( \text{EGFR} \) mutations, exon 19 D761Y, exon 20 T790M and exon 20 D770_N771insNPG, represent only about 10\% of kinase domain mutations associated with drug resistance and should be analysed after an initial negative PCR test.

**References**


6. \( \text{EGFR} \) mutation database: http://www.cityofhope.org/cmdl/egfr%5Fdb/


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