A Mutation-Sensitive Switch Assay to Detect Five Clinically Significant Epidermal Growth Factor Receptor Mutations

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Epidermal growth factor receptor (EGFR) mutations can affect the therapeutic efficacy of drugs used to treat nonsmall-cell lung cancer (NSCLC). We aimed to develop methods to detect five common EGFR somatic mutations in tumor tissues from NSCLC patients by using a nanoscale mutation-sensitive switch consisting of a high-fidelity polymerase and phosphorothioate-modified allele-specific primers. The five clinically significant EGFR mutations examined here are S768I, T790M, L858R, and 15- and 18-bp deletion mutations in exon 19. Our assays showed sensitivities of 100 copies and specificities of more than three log scales for matched templates relative to mismatched templates by routine polymerase chain reaction (PCR), real-time PCR, and multiplex PCR. This assay would be superior to DNA sequencing in situations where mutant DNA is not abundant.

Introduction

MANY EXPERIMENTAL AND CLINICAL studies have shown that epidermal growth factor receptor (EGFR) mutations play an important role in the development of nonsmall-cell lung cancer (NSCLC). At the same time, the emergence of tyrosine kinase inhibitors gefitinib and erlotinib that target EGFR has brought new hope for the treatment of lung cancer, especially NSCLC (Mitsudomi *et al.*, 2005).

However, the long-term use of gefitinib in NSCLC treatment can eventually result in drug resistance that is often closely associated with a specific type of EGFR gene mutation (Huang *et al.*, 2004; Lynch *et al.*, 2004; Han *et al.*, 2005; Mu *et al.*, 2005; Qin *et al.*, 2005). For example, gefitinib treatment of NSCLC patients carrying nucleotide deletions in exon 19 is more successful than that for patients with an L858R mutation or other EGFR mutations (Kosaka *et al.*, 2004; Paez *et al.*, 2004; Green, 2006; Jänne and Johnson, 2006; Jänne *et al.*, 2006; Mitsudomi and Yatabe, 2007; Ansari *et al.*, 2009; Igawa *et al.*, 2014; Ko *et al.*, 2014; Wang *et al.*, 2014).

Therefore, a better and more efficient identification of which EGFR mutations are carried by patients who enroll in clinical trials for potential NSCLC therapeutics is needed. Currently developed assays have mainly focused on five clinically significant EGFR mutations as detection targets: L858R, S768I, and T790M, as well as a 15-bp deletion (E746–A750) and an 18-bp deletion (L747–S752) in exon 19 (Hoshi *et al.*, 2004; Pao *et al.*, 2004; Shigematsu *et al.*, 2005;

Chen *et al.*, 2014). Conventional gene sequencing is complex, time-consuming, and can have a low sensitivity for rare somatic mutations. In situations where normal and cancerous tissues are mixed, successful detection of mutant sequences requires that mutated sequences account for > 10–30% of the total genetic content (Fan *et al.*, 2001; Tokumo *et al.*, 2005).

We previously applied a molecular on/off switch combining 3' phosphorothioate-modified allele-specific primers with an *exo+* polymerase to detect gene mutations. This mutation-specific molecular switch showed sensitive “on” and “off” effects toward mutant and wild-type DNA templates, respectively (Zhang *et al.*, 2004; Li *et al.*, 2005; Zhang *et al.*, 2005; Yung *et al.*, 2009; Wang *et al.*, 2011; Xiao *et al.*, 2011; Guo *et al.*, 2012). Here, we used this newly developed assay to detect the five EGFR-specific mutations described above. This assay could be useful for monitoring gefitinib-resistant mutations during NSCLC therapy and allows the selection of the most appropriate treatment according to the patient's individual genetic situation.

Materials and Methods

Preparation of mutant and wild-type templates

Blood samples from normal controls and tissue samples from lung cancer patients were obtained from the Second Affiliated Hospital of Soochow University. Mutant and wild-type templates were obtained from either regular polymerase

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TABLE 1. PRIMERS USED TO CONSTRUCT WILD-TYPE AND MUTANT TEMPLATES

Primers	Sequence
Primer 1	5'-CCAAGCTTATGTGCCCTCCTTCTGGCCACCATG-3'
Primer 2	5'-CGGTCGACGGGAAGCAATATTCCTTCTCCACTAC-3'
Primer 3	5'-GGTGAGGCAGATGCCAGCAGGCGGCACACGTGGGGGTTGTCCACGATGGCCA-3'
Primer 4	5'-CCTGCTGGGCATCTGCCTCACCTCCACCGTGCAGCTCATCATGCAGCTCA-3'
Primer 5	5'-GCGTCGACGGTCTTCTCTGTTTCAGGGCATGAACTA-3'
Primer 6	5'-CCGGATCCGCACCCCTTAGTGCTGTGAGGGTCCCT-3'
Primer 7	5'-GCGTCGACGCAGCATGTCAAGATCACAGATTTTGGGCGGGCCAAA-3'
Primer 8	5'-TGTGATTTCGTGGAGCCCAACA-3'
Primer 9	5'-AGGCCAGTGCTGTCTCTAAG-3'
Primer 10	5'-GGCTTTCGGAGATGTTTGTATAGCGACGGGAATTTTAACTTTCTC-3'
Primer 11	5'-GCTATCAAAACATCTCCGAAAGCCAACAAGGAAATCCTCGAT-3'
Primer 12	5'-CTTGTTGGCTTTCGATTCTTGTATAGCGACGGGAATTTTAAAC-3'
Primer 13	5'-ATCAAGGAATCGAAAGCCAACAAGGAAATCCTCGAT-3'

Primers 1 and 2 were used to construct wild-type templates S768I and T790M.

Primers 2 to 4 were used to construct mutant templates S768I and T790M.

Primers 5 and 6 were used to construct the wild-type template L858R.

Primers 6 and 7 were used to construct the mutant template L858R.

Primers 8 and 9 were designed for the construction of wild-type recombinant plasmid.

Primers 8 to 11 were designed for the construction of EGFR 15-bp deletion mutant recombinant plasmid.

Primers 8, 9, 12, and 13 were designed for the construction of EGFR 18-bp deletion mutant recombinant plasmid.

EGFR, epidermal growth factor receptor.

chain reaction (PCR) using Taq DNA polymerase or overlap extension PCR with the primers, as shown in Table 1. Wild-type templates of S768I, T790M, and L858R, as well as a 15-bp deletion and an 18-bp deletion in exon 19 of our assays were all obtained by regular PCR using Taq DNA polymerase. Genomic DNA was obtained from the blood samples of healthy controls. The PCR products were purified using a universal DNA purification kit (Tiangen, Beijing, China) and then subcloned into the PMD19-T vector. The mutant templates of S768I and T790M were obtained by the overlap extension PCR technology with two rounds of primer extension (Wang *et al.*, 2012).

In the first round, primers 1 and 3 were used to amplify the upstream fragment, and primers 2 and 4 were used to amplify the downstream fragment from the synthesized mutant template for S768I and T790M. Primers 3 and 4 have a 22-nucleotide overlap. The mixed upstream and downstream fragments were then used as the template for a second round of PCR with primers 1 and 2. The final PCR products were gel purified and subcloned into the PMD19-T vector. Cloning products harboring both normal and mutant EGFR fragments were confirmed by direct sequencing. The mutant templates

L858R, 15-bp deletion, and 18-bp deletion were obtained by regular PCR using Taq DNA polymerase.

On/off switch assays of five EGFR mutations

Five pairs of primers were used to detect the five mutations listed in Table 2. These primers were designed according to the reference sequences of EGFR protease and reverse transcriptase genes in GenBank (NC_000007.14) and perfectly matched the four mutant templates. The forward primers had a 3' terminal phosphorothioate modification, while the common reverse primer had no specific modification. All primers were synthesized by Genewiz (Suzhou, China). A nanoscale proofreading polymerase-mediated on/off switch was employed in these five loci to detect the S768I, T790M, and L858R point mutations, as well as the 15-bp (E746–A750) and 18-bp (L747–S752) deletion mutants in exon 19.

The molarity of the wild-type and mutant templates was adjusted to 1:1 and then diluted to 10^7 , 10^6 , 10^5 , 10^4 , 10^3 , 10^2 , and 10^1 copies/ μ L to test the sensitivity of the phosphorothioate modification and high-fidelity enzyme.

TABLE 2. PHOSPHOROTHIOATE-MODIFIED PRIMERS DESIGNED FOR DETECTING EPIDERMAL GROWTH FACTOR RECEPTOR MUTATIONS

Gene	Phosphorothioate forward	Common reverse	Fragment
S768I	5'-CTACGTGATGGCCATAGTA-3'	5'-TGATTGATGAGAGTTTCCACATGCA-3'	395
T790M	5'-CGTGTCAGCTCATCATACAA-3'		329
L858R	5'-CACAGATTTTGGGCGAGCG-3'	5'-AGAAACCGAGCCAGTGAAGGGAGAGAAG-3	529
15-bp deletion	5'-AAATTCCCCTCGCTATCAAA <u>ACCG</u> -3'	5'-TAGGATGTGGAGATGAGCAGGGTC-3'	162
18-bp deletion	5'-CCCGTCGCTATCAAGGAAT <u>CGATT</u> -3'		154

The underlined nucleotides are phosphorothioate modified. These primers are designed for detecting EGFR mutations.

FIG. 1. Sequencing results for S768I, T790M, and L858R. Sequencing chromatography illustrates that the wild-type and mutant template sequences prepared *in vitro* are correct. The *rectangular box base* is the mutant site.

FIG. 2. Sequencing results for the 15- and 18-bp deletions in exon 19. Sequencing chromatography illustrates that the wild-type and mutant template sequences prepared *in vitro* are correct. The *rectangular box base* is the mutant site.

The following formula was used to calculate the number of copies:

$$\text{Copies}/\mu\text{L} = (6.02 \times \text{OD} \times 10^{12}) / (\text{DNA length} \times 660),$$

where OD represents the value measured by the Thermo Scientific NanoDrop 2000 device.

PCR was performed in a volume of 25 μL containing 2.5 μL of 10 $\times$ Pfu buffer with MgSO_4 , 10 μM allele-specific primer, 10 μM of the relevant antisense primer, 0.75 U Pfu, 0.4 μL dNTP, and 1 μL 10^1 – 10^7 copies of plasmid DNA. After denaturation at 95°C for 5 min, primer extension was performed for 30 cycles with denaturation at 95°C for 30 s, annealing at 58°C for 1 min, and extension at 72°C for 30 s. A final extension step at 72°C for 10 min was added to minimize premature products. The optimal annealing temperatures for each mutation were obtained through gradient PCR ranging

from 50°C to 60°C. The optimized annealing temperatures are crucial for the nanoscale on/off switch to achieve relatively high sensitivity and specificity in detecting the selected drug-resistant mutation.

Real-time PCR was performed in a volume of 25 μL containing 10^1 – 10^7 copies of plasmid DNA, 0.5 μL 20 $\times$ EvaGreen fluorochrome, 0.4 μL dNTP, 0.75 U Pfu DNA polymerase, 2.5 μL 10 $\times$ PCR-balanced solution, 10 μM allele-specific primer, and 10 μM of the relevant antisense primer. After denaturation at 95°C for 5 min, primer extension was performed for 50 cycles with touchdown PCR, where the first 10 cycles of the first stage had denaturation at 95°C for 30 s and annealing at 68°C for 40 s with each subsequent cycle decreasing by 1°C. The second stage of 40 cycles had a denaturation step at 95°C for 30 s and annealing at 59°C for 1 min. Fluorescence signals were collected during the extension phase.

FIG. 3. On/off switch sensitivity and specificity. Representative results showing the relatively high sensitivity and specificity of the on/off switch for detecting five different EGFR mutations. (1) AGC/ATC codon of S768I; (2) ACG/ATG codon of T790M; (3) CTG/CGG codon of L858R; (4) 15-bp deletion of E746–A750; and (5) 18-bp deletion of L747–S752. The *upper section* of each panel shows results from the matched amplicons, while the *lower sections* show results from the mismatched amplicons where there are no amplified products until the template level is $\geq 10^6$ copies. The first lane is a bp marker. EGFR, epidermal growth factor receptor.

Multiplex PCR was carried out in a total volume of 25 μ L with 2.5 μ L of 10 $\times$ Pfu buffer with MgSO₄, 10 μ M allele-specific primers for S768I, T790M, L858R, or 15-bp deletion, 0.75 U Pfu, 0.4 μ L dNTP mix, and 2 μ L 10¹–10⁷ copies of plasmid DNA. The reaction conditions were similar to the stage 2 PCR mentioned above and the annealing temperature was 58°C.

Results and Discussion

Identification of wild-type and mutant recombinant plasmids

As shown in Figure 1, the wild-type fragments for S768I, T790M, L858R, and exon 19 were successfully inserted into the recombinant plasmids, and the mutant templates for S768I, T790M, L858R, 15-bp deletion (E746–A750), and 18-bp deletion (L747–S752) were also successfully prepared (Figs. 1 and 2).

Specific and sensitive on/off switch assays of the five EGFR mutations

The major components of these specific assays include high-fidelity polymerases with an intrinsic proofreading activity and nuclease-resistant-specific primers targeting the wild-type sequence and mutant plasmids harboring S768I, T790M, L858R, and the 15- and 18-bp deletions. Template concentrations ranging from 10¹ to 10⁷ copies were used to test the sensitivity and specificity of the five phosphorothioate-modified specific primer pairs. After determination of optimal annealing temperatures using gradient PCR for each amplicon, five assays were successfully developed for each of the selected mutations.

The allele-specific primers for the S768I, T790M, L858R, and 15- and 18-bp deletion mutations could detect the perfectly matched template at a sensitivity of about 100 copies

FIG. 4. Amplification curves for S768I mutation detection. The x-axis shows the amplification curves, in which the matched amplicons amplified well from 10² to 10⁷ template copies (in dark gray), whereas the mismatched amplicons amplified only when the templates were present at $\geq 10^3$ copies (in light gray).

FIG. 5. Multiplex PCR detection of four mutations. Multiplex PCR detection of four EGFR mutations in plasmid samples. PCR, polymerase chain reaction.

(Fig. 3). The same assay conditions yielded no primer extension products until template levels met or exceeded 10⁶ copies of the mismatched amplicon.

The assays to detect the S768I, T790M, L858R, and 15- and 18-bp deletion mutations are highly sensitive and can detect as few as 10–100 template copies. These assays are also sufficiently specific so that mismatched primers are not extended until the number of template copies exceeds 10⁶.

Real-time PCR quantitative evaluation of the on/off assay

The S768I, T790M, L858R, and 15- and 18-bp deletion mutations were used to test the compatibility of the newly developed assays with the real-time PCR platform. The accuracy and sensitivity of fluorescence quantitative PCR is higher than ordinary PCR, as is shown in Figure 4, where real-time PCR had weak amplification with 10⁵ copies for the wild-type template, which is similar to the gel electrophoresis results.

Multiplex PCR

Forward primers targeting the S768F, S790F, S858F, and exon 19 deletion mutations were mixed together with the common reverse primer to detect the specificity and sensitivity of multiplex PCR of these mutations. The assay developed here has a sensitivity that allows the detection of 100 copies of the mutant template and is sufficiently specific so that mismatched primers are not extended until the wild-type template levels reach 10⁵ or more copies. For single PCR of the five EGFR mutations, we obtained five bands, but for multiplex PCR, only four bands with distinct sizes according to each mutant fragment can be detected (Fig. 5). The 15- and 18-bp deletions in

FIG. 6. Multiplex PCR detection of EGFR L858R in tumor samples. No product was obtained from samples from a healthy subject (Lane 1). Lanes 2 and 3 show results from lung tissue samples from patients carrying the L858R mutation in EGFR.

wild type

mutant type1

mutant type2

FIG. 7. Sequencing chromatography illustrated the L858R mutations in tumor samples. Sequencing chromatography illustrated the correctness of two L858R mutations in tumor samples. The rectangular box base is the mutant site.

exon 19 only differ by 8 bp, which makes their bands difficult to distinguish. As such, we first detected the 15-bp deletion by multiplex PCR. Following amplification, 25 μ L of the multiplex PCR was run alongside a 100-bp ladder in 3.5% agarose. Similar results were obtained for multiplex PCR for S768F, S790F, S858F, and the 5-/18-bp deletions in exon 19 (Fig. 5).

Detection of five EGFR mutations in tumor samples using the new assay

We collected and analyzed 10 NSCLC tumor samples (provided by the Second Affiliated Hospital of Soochow University) from patients using a mutation-sensitive switch assay and sequencing analysis. We also used multiplex PCR to detect the S768F, S790F, and S858F point mutations, as well as the 15- and 18-bp deletions in exon 19 in tumor samples. In screening DNA samples from these 10 NSCLC cases, two cases with S858F mutations were identified (Fig. 6). The results from a sequencing analysis were consistent with these experimental results (Fig. 7).

Conclusions

In this study, the high-fidelity DNA polymerase-mediated mutation-sensitive switch was applied to analyze five clinically significant EGFR mutations. The results showed that the mutation-sensitive switch is sufficiently sensitive and specific to detect these mutations in both plasmid samples

and DNA from tumor samples. This new assay is easy to perform, economical, and can also be combined with other platforms such as real-time PCR and multiplex PCR for detecting mutations. The on/off switch assay-based methods showed reliable, accurate, and reproducible results in the detection of these five EGFR mutations. Multiplex PCR was successfully developed for synchronous detection of these mutations with detection of around 1000 copies and no mismatch primer extension below 10^5 copies of the wild-type template.

These high specificities of the on/off switch could be very useful for the early detection of EGFR mutants that are related to drug resistance. As shown in Figure 8, our

FIG. 8. PCR detection of the 15-bp deletion EGFR in exon 19 in plasmid samples. PCR detection of the 15-bp deletion in EGFR exon 19 in plasmid samples. Lane 1: No product was present at 10^4 copies of wild-type template. Lanes 2 and 3: 10^4 copies of wild-type template were mixed with 10^1 copies of mutant template. Lane 4 is a negative control.

assay can even detect the 0.1% difference between the 15- and 18-bp deletion mutations in plasmid samples. As compared to conventional sequencing analysis, we can detect mutations even when they are present as the minor population. The assays in the present study are simple and reliable with relatively high sensitivity and specificity. Thus, they may have a potential use for monitoring EGFR-targeted therapies.

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Author Disclosure Statement

No competing financial interests exist.

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