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Simple polymerase chain reaction for the detection of mutations and deletions in the epidermal growth factor receptor gene: Applications of this method for the diagnosis of non-small-cell lung cancer

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ABSTRACT

Background: Somatic mutations in the epidermal growth factor receptor (*EGFR*) gene are associated with the responses to the tyrosine kinase inhibitors gefitinib and erlotinib in patients with non-small-cell lung cancer (NSCLC). Although various methods for detecting *EGFR* gene mutations have been developed, they have several disadvantages. We attempted to establish a new method for the detection of *EGFR* gene mutations with the use of paraffin-embedded samples.
Methods: The detections of T790M mutations in exon 20 and L858R mutations in exon 21 are based on the principle of allele-specific oligonucleotide polymerase chain reaction (PCR). We also designed PCR primers that enable to detect all types of deletions in exon 19. We assessed the basic performance efficiency of this method, and to confirm its clinical applicability, we performed PCR using DNA extracted from 66 tissue sections that were obtained from patients with NSCLC and embedded in paraffin.
Results: The sensitivity of this method for the detection of deletions or mutations was as low as 0.5%. In the 66 subjects whose samples were analyzed, we detected the following deletions and mutations in the *EGFR* gene: 11 deletions in exon 19, 8 L858R mutations, and 1 double mutation of L858R and T790M.
Conclusion: The present method is sensitive and specific for the detection of deletions and mutations in the *EGFR* gene and is thus suitable for use in laboratory tests.

1. Introduction

Detection of mutations in the epidermal growth factor receptor (EGFR) gene is critical for predicting responses to tyrosine kinase inhibitors (TKIs, e.g.; gefitinib and erlotinib) therapy. Further, these mutations affect the response rate and provide a rationale for prolonging TKI therapy in patients with non-small-cell lung cancer (NSCLC) [1,2].

The detection of the epidermal growth factor receptor (EGFR) gene mutations is critical to predict the response to the TKIs therapy [1–3]. The sensitivity and efficacy of TKI therapy in patients with NSCLC depend on whether the patients carry mutations in the *EGFR* gene [1–4].

Thus far, various methods have been used to detect *EGFR* mutations, such as direct DNA sequencing, restriction fragment length polymorphism analysis [5,6], mutant-enriched PCR [7], allele-specific oligonucleotide PCR (ASO-PCR) [8], and melting curve analysis [9,10]. Of these, the direct DNA sequencing method is well established and is the most common and conventional method used for the detection and identification of mutations in tumor cells. However, contamination of normal cells, which yields a high-intensity background signal of wild-type *EGFR* genes, reduces the detection sensitivity limit of this method [8,11,12]. The peptide nucleic acid-locked nucleic acid PCR clamp method [11] and real-time PCR [12,13] have been developed to overcome the above-mentioned problems associated with the direct DNA sequencing method. Although the sensitivity of these methods is superior to that of other methods, these methods are rarely used in routine clinical assays because they require special equipments and expensive reagents.

Genetic analyses of paraffin-embedded sections are often conducted during clinical examinations for the histopathological diagnosis of diseases. Paraffin-embedded sections offer the advantage of prolonged stability even at room temperature, such that the paraffin-embedded sections can be used for fluorescence in situ hybridization (FISH) analyses and sufficient DNA can be extracted from these sections for use in PCR analyses [14,15]. Thus, these sections yield DNA samples that can be used not only for diagnosis but also for retrospective studies.

Abbreviations: EGFR, epidermal growth factor receptor; NSCLC, non-small cell carcinoma; ASO-PCR, allele-specific oligonucleotide PCR; PAGE, polyacrylamide gel electrophoresis; AGE, agarose gel electrophoresis.

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Table 1
Primers for PCR

Exon		Forward	Reverse
19		5′-agggactctggatcccagaaggtg-3′	5′-cccacacagcaaagcagaaactcac-3′
20	Wild-type	5′-gaagccacactgacgtgcct-3′	5′-gccgaagggcatgagctg**Tg**-3′
	T790M	5′-accatgcgaagccacactgacg-3′	5′-gccgaagggcatgagctg**Ga**-3′
21	Wild-type	5′-gcttggtgcaccgcgacctg-3′	5′-cgcacccagcagtttggc**Ga**-3′
	L858R	5′-gcttggtgcaccgcgacctg-3′	5′-cgcacccagcagtttggc**Gc**-3′

Wild or mutation specific bases depicted in lowercase italics, bold and underlined; while, −1 mismatch is bold uppercase.

In this study, we established a simple and sensitive PCR method involving the use of mutation-specific primers, including those with a mismatched base pair to detect *EGFR* mutations and primers recognizing all types of deletions, and confirmed its specificity by direct sequencing analysis. Further, we investigated the clinical applicability of this method by using paraffin-embedded sections as samples.

2. Materials and methods

2.1. Cell lines

The human lung cancer cell lines NCI-H1975 and PC-14 were from the American Type Culture Collection (ATCC; Rockville, MD) and the Riken Bioresource Center (Tsukuba, Japan), respectively. Both cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum at 37 °C in air containing 5% CO_2.

2.2. Clinical samples

Tumor samples were excised from 66 patients with NSCLC, who had been hospitalized at Shinshu University Hospital from 2006 to 2007. The samples were fixed with formalin and embedded in paraffin. The histopathologic diagnoses of these tumors were as follows: adenocarcinoma (n=44), squamous cell carcinoma (n=18), large cell carcinoma (n=2), adenosquamous carcinoma (n=1), and other carcinomas (n=1). This study was approved by the medical ethical committee of the Shinshu University School of Medicine, Japan.

2.3. DNA extraction

We extracted DNA from the human lung cancer cells (NCI-H1975 and PC-14) and from whole peripheral blood samples obtained from healthy volunteers, by using a QIAamp DNA blood mini kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. We also extracted DNA from the formalin-fixed paraffin-embedded tissue sections by the following procedure. First, 10-μm-thick sections were cut from the paraffin-embedded tissue samples and cleared of paraffin by treatment with xylene, followed by rinsing with 100% ethanol. The samples were then dried at 50 °C for evaporation of the residual ethanol. After the complete removal of paraffin, the samples were treated with 20 mg/ml proteinase K (Promega, Madison, WI) in lysis buffer (50 mmol/l Tris–HCl pH 8.5, 100 mmol/l NaCl, 1 mmol/l EDTA, 0.5% Tween-20, 0.5% NP40, 20 mmol/l DTT) at 50 °C overnight. We then inactivated the proteinase K by heating the solution at 95 °C for 5 min and used this treated tissue lysate for PCR analysis.

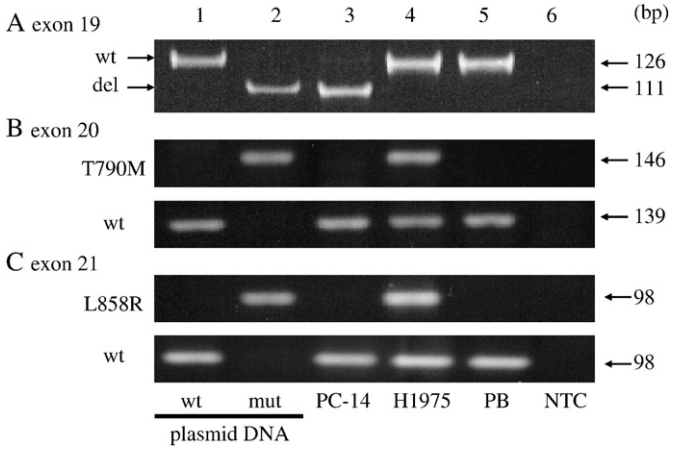

Fig. 1. Specificity of PCR for the detection of *EGFR* mutations. PCR was carried out for exons 19 (A), 20 (B), and 21 (C) by using plasmid DNA, lung cancer cell lines (PC-14 and NCI-H1975), and normal peripheral blood, respectively. The PCR products were subjected to polyacrylamide gel electrophoresis (for the detection of deletions) or agarose gel electrophoresis (for the detection of mutations). (A) A 111-bp deletion-specific band and a 126-bp wild-type band derived from exon 19 were detected. (B) A 146-bp T790M mutation-specific band and a 139-bp wild-type band were detected. (C) An L858R mutant and 98-bp wild-type products were detected. wt, wild type; mut, mutant; del, deletion; PB, peripheral blood; NTC, no template control.

2.4. Construction of plasmids carrying wild-type and mutant EGFR

To construct plasmids carrying wild-type *EGFR*, PCR products obtained by amplification of DNA samples procured from healthy volunteers were cloned into pCR2.1 vectors by using the TA cloning kit (Invitrogen, Paisley, UK). Plasmids carrying the mutant *EGFR* gene were then synthesized from the wild-type plasmids by the oligonucleotide-directed mutagenesis method performed using the site-directed mutagenesis kit (Stratagene, La Jolla, CA). The nucleotide sequences of both the wild-type and mutant plasmids were confirmed by using an automated DNA sequencer (ABI Prism 3100 Genetic Analyzer, Applied Biosystems, Foster City, CA).

2.5. Preparation of specific primers

The *EGFR* gene exons were identically amplified by PCR. The primers used in this study are listed in Table 1. The primers used for recognizing the deletions in exon 19 were designed to encompass nucleotides 2185–2283 nucleotides, which cover all types of deletions reported thus far (e.g., E746-A750del, L747-A750del, and L747-S752del; 1, 5, 7, 12). Further, we designed 2 specific primer sets for the wild-type sequence and the T790M mutation in exon 20 by introducing a wild-type or mutated nucleotide at the 3′ terminal end and a mismatch in the penultimate nucleotide at the 3′ end. Similarly, we designed 2 specific primer sets for the wild-type sequence and the L858R mutation in exon 21.

2.6. PCR amplification of the EGFR gene

For PCR, 5 μl of the extracted DNA was added to 15 μl of a reaction mixture comprising PCR buffer containing 1.5 mmol/l $MgCl_2$ (1.0 mmol/l for exon 19 and 1.25 mmol/l for L858R in exon 21), 200 μmol/l dNTPs, 2.5 μmol/l of each primer, and 1.0 U of Ampli Taq Gold DNA polymerase (Applied Biosytems). PCR was performed on a Gene Amp PCR system 9700 (Applied Biosystems) under the following conditions: 95 °C for 10 min, followed by 35 cycles of treatment at 94 °C for 30 s, 56 °C for 30 s (60 °C for exon 19 and L858R in exon 21), 72 °C for 30 s; and final extension at 72 °C for 5 min. The PCR products were then subjected to 3.0% agarose gel electrophoresis for detection of the mutations and to 10.0% polyacrylamide gel electrophoresis for detection of the deletions.

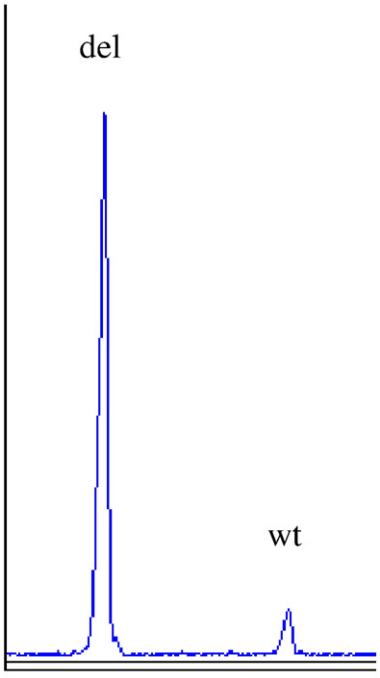

Fig. 2. Fragment analysis of *EGFR* exon 19 in the PC-14 cell line. The percentage of the peak obtained for the wild-type allele (wt) against that obtained for the deletion-type allele (del) was less than 7%.