ORIGINAL ARTICLE

The PCR-invader method (structure-specific 5' nuclease-based method), a sensitive method for detecting EGFR gene mutations in lung cancer specimens; comparison with direct sequencing

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Received: 23 March 2010/Accepted: 7 January 2011/Published online: 11 February 2011 © Japan Society of Clinical Oncology 2011

Abstract

Background Several sensitive assays, including the PCRinvader method (structure-specific 5' nuclease-based method), have been used to detect EGFR mutations in nonsmall-cell lung cancer (NSCLC). However, validation has not been reported. We assessed the detection rate of EGFR mutation by the PCR-invader method and direct sequencing using same clinical specimens.

Patients and methods EGFR mutations were analyzed with the PCR-invader method and compared with direct sequencing using paraffin tissues and pleural and pericardial effusions from NSCLC patients. The relationships between the treatment responses and mutations were evaluated retrospectively.

Results Fifty-four samples from 42 NSCLC patients were studied. EGFR mutations were identified in 52% of the patients and 52% of the samples with the PCR-invader method, but only in 43% of the patients and in 35% of the samples by direct sequencing. In the samples obtained from the same patients at different sites and different times, EGFR mutations were coincident in nine out of ten patients

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H. Okamoto · N. Hida · K. Watanabe Department of Pulmonary Medicine, Yokohama Municipal Citizen's Hospital, Yokohama, Japan by the PCR-invader method but in six out of ten patients by direct sequencing. Seventeen patients with EGFR mutations were treated with gefitinib; the response rate (RR) and disease control rate (DCR) were 41 and 94%, and median treatment duration was more than 6 months. Seven EGFR mutation-negative patients were treated with gefitinib; the RR and DCR were 0 and 14%, and median treatment duration was 1 month.

Conclusion The PCR-invader method was useful for detecting EGFR mutations in clinical lung cancer specimens and is more sensitive than direct sequencing.

Keywords EGFR mutation \cdot Non-small-cell lung cancer \cdot PCR-invader method \cdot Direct sequencing \cdot Validation \cdot Gefitinib

Introduction

Lung cancer is the leading cause of cancer death in the world today. Recent efforts, including large-scale DNA sequencing, indicate that activating mutations in EGFR, BRAF, PI3K, and K-ras genes are generally non-overlapping and identifiable in approximately 40% of non-small-cell lung cancer (NSCLC) [1–4]. EGFR mutation has been reported as a very important factor in decision-making for treatment of NSCLC [3–6]. A recent prospective randomized phase III study (IPASS study) revealed the superiority of gefitinib (an EGFR inhibitor) to standard platinum-based treatment for first line treatment of lung adenocarcinoma, and documented a significantly higher response rate (RR) of 71.2% in EGFR mutation-positive patients but an extremely low RR of 1.1% in mutation-negative patients [5].

Several different EGFR mutation-detection methods are used in daily practical settings and laboratories [7-11], but

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standardization and validation of these methods are needed [12]. The PCR-invader assay (serial invasive signal amplification reaction with structure-specific 5' nuclease using PCR product) is a sensitive method with which to detect gene mutations such as SNPs [13–17]. To compare the detection rate of EGFR mutation with the PCR-invader method and direct sequencing, which is the current standard [12], we analyzed clinical samples from NSCLC patients.

Patients and methods

Patients with NSCLC whose specimens were available for DNA extraction were eligible. From May 2007 to August 2008, 42 patients provided written informed consent at Yokohama Municipal Citizen's Hospital. Approval for the study was obtained from the institutional review board. Specimens (archived paraffin-embedded tissues, pleural effusions, and pericardial effusions) were obtained by surgery, transbronchial lung biopsy (TBLB), lymph node biopsy, or effusion drainage. DNA was extracted using a QIAamp DNA Mini Kit (Qiagen, California, USA). In cases with paraffin-embedded specimens, DNA was extracted with gross dissection with confirmation of adjacent slices having enough (at least 70% of surface area) cancer cells. DNA extraction, the PCR-invader method, and direct sequencing were performed by BML (Tokyo, Japan).

EGFR exons 18, 19, 20, and 21 were amplified by polymerase chain reaction (PCR) using specific primers (Table 1) with Ex Taq polymerase (Takara Bio, Shiga, Japan). PCR reaction was performed with the cycles: 94°C 2 min, 96°C 10 s, 65°C 30 s for 50 cycles, 72°C 7 min, 95°C 5 min, 4°C: hold. EGFR mutations were analyzed using the PCR-invader method (Fig. 1) [13–17] and the results were compared with those of direct sequencing. Briefly, in the initial reaction of the PCR-invader method, the target nucleic acid, invader-oligo, and signal probe form a threedimensional invader structure. A highly specific enzyme (cleavase) recognizes the structure and cleaves the flap portion. In the second reaction, the released flap hybridizes with FRET-probe to make a three-dimensional structure as in the first reaction, and cleavase cleaves it to produce a fluorophore whose signal can be measured [13-17]. The PCR-invader method can only detect known mutations (exon 18: G719A/C/S, exon 19: deletion, exon 20: S768I, exon 21: L858R·L861Q, and known resistant mutation exon 20: T790M) by using probes specific to those mutations (Table 1). The "exon 19 deletion" mutations are in fact several types. The invader assay probes (Table 1) used here for the PCR-invader method are specially constructed to detect three types of "exon 19 deletion" mutations: E746-A750del type1 (DEL1; 2235-2249del GGAATTAAG AGAAGC), E746-A750del type2 (DEL2; 2236-2250del GAATTAAGAGAAGCA), and L747-P753del insS (INS-S). There are other mutations with the "exon 19 deletion", although the frequency is not high. To ensure detection of other types of "exon 19 deletion" mutations, the PCR-invader method included electrophoresis of exon 19 PCR products in the clinical setting. If there were several bands suggesting "exon 19 deletion" but the invader assay probes did not detect the three types of "exon 19 deletion", sequencing followed to elucidate the exact deletion.

Direct sequencing was performed using purified PCR products with a BigDye terminator sequencing kit (Ver. 1.1; Applied Biosystems, California, USA). Sequencing was carried out with the primers as indicated in Table 1. Sequencing was confirmed with forward and reverse reactions. An ABI Prism 3130xl genetic analyzer (Applied Biosystems) was used for analysis.

In patients treated with an EGFR-tyrosine kinase inhibitor (EGFR-TKI), the relationships between the responses (according to RECIST criteria [18]) and EGFR gene mutations were evaluated retrospectively.

To show the sensitivity of the PCR-invader method, sensitivity assay was done using cell lines with known EGFR mutation status, H1650 (exon 19 deletion: DEL1), H1975 (L858R and T790 M), and SK-MES-1 (wild type) [8, 19]. These cell lines were purchased from ATCC. Cell lines were mixed with different ratio, H1650 and SK-MES-1: 0:1, 0.5:0.5, 0.1:0.9, 0.01:0.99, 0.001:0.999, and 1:0; H1975 and SK-MES-1: 0:1, 0.5:0.5, 0.1:0.9, 0.01:0.99, 0.001:0.999, and 1:0. These cell mixtures were sent to BML (Tokyo, Japan) for the analysis of EGFR mutation by PCR-invader method. BML were not aware of the composition of the cell mixtures.

Results

Characteristics of patients and samples

The median age of the 42 patients was 65 years (range 33–82) (Table 2). All of the patients were Japanese. The histology was adenocarcinoma in 90% of patients, squamous cell carcinoma in 7% of patients. The samples used were archived paraffin-embedded tissues (n = 49), pleural effusion specimens (n = 4), and pericardial effusion specimens (n = 1).

EGFR mutation by the PCR-invader method

EGFR mutations were detected in 22 patients (n = 22/42: 52%) and in 28 samples (n = 28/54: 52%) by use of the PCR-invader method. Eleven patients (50%) had an exon

Table 1 Primers

PCR primers Exon 18 EGFRex18-F GAGAAGCTCCCAACCAAGCTC ECORRECT8-F GAGAAGCTCCCAACCAAGCTC EXon 19 EGFRex18-R CAGGGACCTTACCTTATACACCG Exon 19 EGFRex19-F GCCTCTCCCTCGAGACGCACCACCACG Exon 20 EGFRex20-F GCCTCTCCCTCCAGGAAGG EGFRex20-R CCGGACATAGTCCAGGAGGACGA Exon 21 EGFRex21-R CCACCTCGCTCAGGAAGG GGTRex21-R CCACCTCGCTTACCTTGGTCTT 3' Animation GGTPA SGT19A_ST ACGGACGCCGAGGCCCAGCACTTTGATCTT 3' Animation GGTPA SGT19A_ST ACGGACGCGGAGGCCAGCACTTTGATCTT 3' Animation GGTPA SGT19C_ST ACGGACGCGGAGGCCAGCACCTTGATCTT 3' Animation GGTPC SGT19C_ST ACGGACGCGGAGGCCAGGCCAGGACTTGATCTTT 3' Animation GGT19C_N GGT19C_ST ACGGACGCGGAGGCCAGGCCTGGGTG 3' Animation GGT19S_N CCTGCGGAGGCCCAGGCCCAGGCCGGAGGCCAGATTGATCTTTT 3' Animation GGT19S_STACGGACGCGGAGGCCCAGGCCCGGGGGCTCGGGTG 3' Animation GGT19C_N GGT19C_ST ACGGACGCGGAGGCCCGGGGGCTCCGGTGCG 3' Animation GGT19S_N CCTGCAGGACGCCCGGGGGGCTCGGGTGG 3' Animation GGT19S_N CCTGCAGGACGCCCGGGGGGCTCGGTGGG 3' Animation GGT19S_N CCTGCAGGACGCCGGGGGGCTCGGTGGG 3' Animation GGT19S_N CCTGCAGGACGCCGGGGGGCTCGGTGGG 3' Animation SG464LJ_S1 CGCCGCGGAGGCTCGGTGGG 3' Animation SG464LJ_S1 CGCCGCGGGGGGCTCGGTGGG 3' Animation SG464LJ_S2 ACGGACGCGGGGGTGTGTGTGGGGGGGG 3' Animation SG464LJ_S2 ACGGACGCGGGGGGCTGCGGTGGG 3' Animation SG464LJ_S1 CGCCGCGGGGGGTGTGTGGGGGGGGGGGGGGGGGGGGG	Lesion	Name	Sequence	
Exon 18 EGREx18-F GAGAAGCTCCAACCAGCTC EGREx19-F GTCATAGGAACTCGATCCA EGREx19-R CAGGAAGCTGGAAACCAGGAACTCGA Fkron 20 EGREx19-R CAGGAAGCTGGAAAGCAGAAACTCGGGA Ekon 21 EGREx21-F CAGGCAGGAGGTCCGGGA Ekon 21 EGREx21-R CCAGCAGGAGGTCCCGGAGGAC Ekon 21 EGREx21-R CCAGCCAGGAGGCA Ekon 21 EGREx21-R CCAGCCAGGAGGCCAGCACTTGATCTT 3' Amination s6719A_S2' ACGGACGCGGGGCCAGCACTTGATCTT 3' Amination s6719A_S2' ACGGACGCGGAGGCCAGCACTTGATCTT 3' Amination s6719A_S2' ACGGACGCGGAGGCCAGCACTTGATCTT 3' Amination s6719C_S2' ACGGACGCGGAGGCACGCACGCAGTGCGTTTT 3' Amination s6719C_S1' CGCCCGAAGCCACGCACGCACGCACTTGATCTTT 3' Amination s6719C_S1' CGCCCGAAGCCACGCACGCACTTGATCTTTT 3' Amination s6719C_S1' CGCCCGAAGCCACGCACGCACTTGATCTTTTT 3' Amination s6719C_S1' CGCCCGAAGCCACGCACGCACGTGG 3' Amination s6719C_S1' CGCCCGAAGCCACGCACGTGGG 3' Amination s6719C_S2' ACGGACGCGGAGACACGCACTTGATCTTTTTG 3' Amination s6719S_S1' CGTGCCGAAGGCCCGGGGACTGCGGAAGCGAAGT "E746-A750del type1" s746-dell_S1I CGCCCCGAGGGCGCTCTGGGAAGCGGAGACGGAGGCG s746-dell_S1I CGCCCCGGAGGTGTCTGAAGGAAGCGGGGG 3' Amination s674-dells_S1I CGCCCCGGAGGTGTCTGAGGGGCCTCTGGGGGG 3' Amination s746-dell_S1I CGCCCCGGAGGTGTCTGAAGGGCGGGGGGCTTGAAGGGGGGGG	PCR primers			
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Exon 19 EGFRex19-F GTCATAGGACTCTGGATCCCA EGRex19-F CAGCAAAGCAGAAACTCACATCG Exon 20 EGFRex19-R CCGGACCTAGGTCCAGGAGGCA Exon 21 EGFRex12-F CAGCCAGGAACCTAGGTGG Exon 21 EGFRex12-R CCACCTCCTTACTTGGCTC Invader assay probes (primer mixture) CCACCTCGGACGCCACCACTTTGATCTT 3' Amination G719A sG719A_S1F CGCCCCGAGGCCCAGCACTTTGATCTT 3' Amination G719A sG719A_S1F CGCCCCGAGGCCCAGCACTTTGATCTT 3' Amination G719C sG719C_S1F CGCCCCGAGGCCCAGCACTTTGATCTTT 3' Amination G719C sG719C_S1F CGCCCCGAGGCCCGCACCCGGAGCT 3' Amination G719S asG719S_S1F CGCGCCGAGGCCCGGGTGCGG 3' Amination aG719C saG719S_S1F CGCGCCGAGGCCCGGGTGCGG 3' Amination aG719S asG719S_S1F CGCGCCGAGGCCCGGGTGGGGGGAGGAGCGGGGGGGGAGGAGCGG 3' Amination s719C sG719S_S1F CGCGCCGAGGGCTCGGTGCGGGGGGGGGGGGGGGGGGGG		EGFRex18-R	CAGGGACCTTACCTTATACACCG	
EGFRex19-R Exon 20 CAGCAAAGCAGAAACTCACATCG GCTETCCTTCCTCCAGGAGG Exon 21 EGFRex20-F EGFRex21-F EGFRex21-F EGFRex21-F EGFRex21-R CCACCTCCTTCCTTCCTCCC Lesion Name Invader assay probes (primer mixure) CGGCCCGAGGACCCCAGCACTTTGATCTT 3' Amination G719A_S1F CGGCCCGAGGCCCAGCACCTTGATCTT 3' Amination G719A_S2: ACGGACGCCGAGGCCACCCGGAGT G719 G719A_S1F CGCCCCGAGGCCACCCGGAGT G719C G719C_S1F CGCCCCGAGGCCACCCGGAGT G719C G719C_S1F CGCCCCGAGGCCACCCGGAGT G719S G719C_S2r ACGGACCGCGGAGACCCCGGAGCT G719S G719S_S1F CGCCCCCGAGGCCCCCGGTGCG G719S asG719S_S1F CGCCCCCGAGGGCCTCCGTGCG G719S asG719S_S1F CGCCCCGAGGGCCTCCGTGCG asG719S_S1F CGCCCCGAGGGCCTCCGTGCG 3' Amination asG719S_Lav CTTGAGGATCTTGATGCTCGGAGACTGCGGGGA 3' Amination asG719S_Lav AGGACCCGGAGGTGCTCCTTGTTGATAGCACCGGGA 3' Amination asG719S_Lav AF4-dell_S2r ACGGACCCGGAGTGCTTGTTGATAGCGACGGG AF4-dell_S2r ACGGACCCGGAGTTCTGTTGTTGGCTGCGGAGTGCT "E746-A750del type?" s746-dell_S1r af4d-dellmS_S1r CGCCCCGAGGTGCTTGTCTGGAGAT	Exon 19	EGFRex19-F	GTCATAGGGACTCTGGATCCCA	
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EGFRex20-R Even 21 CCGGACATAGTCCAGGAGGCA EGFRex21-F EGFRex21-R CCAGCCAGGACCTACTGCTG CCACCTCCTTACTTGCCTCC Lesion Name Sequence Modification Invader assay probes (primer mixture) GG19A_S1F CGCCCCGAGGCCCAGCACTTTGATCTT 3' Amination GG719A_S2r G719A sG719A_S1F CGCCCCGAGGCCAGCACCTTTGATCTT 3' Amination GG719C_S1PA_Linv ACGCACCGCGAGGCCAGCCACCTTTGATCTTT 3' Amination GG719C_S1PA_Linv G719C sG719C_S1F CGCCCCGAGGCCAGCCGGGAGCCACCTTGATCTTTTG 3' Amination GG719C_G3C19C_S1r CGCCCCCGAGGCCCGGGGCC G719S asG719S_S1F CGCCCCCGAGGCCCCGGGTGCG 3' Amination GG719S_S2r ACGGACGCGGAGAGCCCCGGTGCG 3' Amination asG719S_D1vv "E746-A750del type1" s746-del _S1F CGCCCCGAGGTCTTGATGAGAATTCCAAAA AGATCAAAGTTCTGT 3' Amination asG719S_D1vv CATCGAGGGGAGTTTGATAGCGACGGGA 3' Amination asG719S_D1vv "E746-A750del type1" s746-del _S1F CGCCCCGAGGTTTGCTCTTGATTCCTTGAT 3' Amination asG719-S2P ACGGACCGGGAGATTTCCTTGTTGGGAGTCC 3' Amination asG719-Linv "E746-A750del type2" s746-del _S1F CGCCCCGAGGTTGCATAGGCACACTTCC 3' Amination asG719-Linv GCCCGAGGTGGCAACTTCCCTGAT 3' Amination asG719-Linv GCCCCGAGGTGGCCAACTTCCC	Exon 20	EGFRex20-F	GCCTCTCCCTCCAGGAAG	
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G719A sG719A_S1f CGCGCCGAGGCCCAGCACTTTGATCTT 3' Amination sG719A_Jav ACCGACCCGAGGCCCAGCACTTTGATCTTT 3' Amination sG719C sG719C_S1f CGCGCCGAGGCCAGCACTTTGATCTTTTT 3' Amination sG719C sG719C_S1r CGCGCCGAGGCCAGCACTTTGATCTTTTTG 3' Amination sG719C_Inv CGTGCCGAAGCACCGGGAGCA 3' Amination sG719S_S1f CGCGCCGAGGCCAGCAGCACCTTGATCTTTTTG 3' Amination asG719S_S2r ACGGACGCGGAGGCTCCGGTGCG 3' Amination asG719S_S2r ACGGACGCGAGGGCCTCCGGTGCG 3' Amination asG719S_Nr CTTGAGGATCTTGAAGGGAACTGAATTCAAAA AGATCAAAGTCGAT asG719S_Inv CTTGAGGATTTCCTTGTTGATAGCGACGGG 3' Amination s746-dell_S1f CGCGCCGAGGTGCCTTCTTAATTCCTTGTA 3' Amination s746-dell_S1r CGCGCCGAGGTTCCTTGTTGATAGCGACGGG 3' Amination s746-dell_S2r ACGGACGCGGAGTTCCTTGTTGAGGATGTC 3' Amination s746-dell_S1r CGCGCCGAGGTTAGCTGTGTGTGCGGGAGTGC 3' Amination s746-dell_S1r CGCGCCGAGGTGAAGCCAACAGG 3' Amination s746-dell_S1r CGCGCCGAGGTTAGGAAGCAACTTGCC 3' Amination s746-dell_S1r CGCGCCGAGGTTAGGAAGCAACTTGCGGGG 3' Amination s747-delinsS_N1r CGCGCCGAGGTGAAGCCAACAGGG 3' Amination s747-delin	Invader assay probes (primer	mixture)		
s0719A_S2r ACGGACGCGAGAGCCAGCACTTTGATCTT 3' Amination G719C s0719A_Inv ACCGTGCCGAAGCCACCGGAGT s0719C_S1f CGCCCCGAGCCCAGCACTTTGATCTTTT 3' Amination s0719C_Inv CGTGCCGAAGCCACCGCAGCACTTTGATCTTTTG 3' Amination s0719C_S2r ACGGACGCGGAGCAGCACTTGATCTTTTGA 3' Amination s0719S_S1f CGCGCCGAGGGCTCCGGGGCG 3' Amination s6719S_S1r ACGGACCGCGAAGCTCCGGTGCG 3' Amination s6719S_S1r ACGGACGCGGAGAGCTCTCGATAGAGAACTGAAATTCAAAA AGATCAAAGTGCTGT "E746-A750del type1" s746-dell_S1f CGCGCCGAGGGTCTTCTTAATCCCTTGAT 3' Amination s746-dell_S1r CGCGCCGGAGGTTTCTGATAGCGAGGGGA 3' Amination s746-dell_S1r CGCGCCGGGGGGGTCTTGATAGCGAGGGGG 3' Amination s746-dell_S1r CGCGCCGGGGGGTTTGGTAGCGCAGGGG 3' Amination s746-dell_S1r CGCGCCGGGGGGTTAGGAGAGCACATCTCC 3' Amination s746-dell_S1r CGCGCCGGGGGGGCCAACAGGGG 3' Amination s746-dell_S1r CGCGCCGGGGGGGCCAACAGGG 3' Amination s746-dell_S1r CGCGCCGGGGGGGCCAACAGCGG 3' Amination s746-dell_S1r CGCGCCGGGGGGCCAACAGGGGGGGGGGGGGGGGGGGG	G719A	sG719A_S1f	CGCGCCGAGGCCCAGCACTTTGATCTT	3' Amination
sq719A_Inv ACCGTGCCGAACGCACCGGAGT G719C sq719C_S1f CGCGCCGAGGCAGCACGCACTTTGATCTTTTT 3' Amination sq719C_Inv CGTGCCGAACGCACGTGGGGCCT 3' Amination sq719S_Inv CGTGCCGAACGCACCGGGGCTCCGGTGCG 3' Amination asq719S_S1f CGCGCCGAGGGCTCCGGTGCG 3' Amination asq719S_S1rv CTTGAGGATCTTGAAGGAACTGAATTCAAAA AGGACCAGGAGAGTCTTGATAGCGACGGGA 3' Amination asq719S_S1rv CTTGAGGATCTTGAAGGAACTGAATTCAAAA AGGACCAGGAGGTTCTCTTAATTCCTTGAT 3' Amination sq746-dell_S1r CGCGCCGAGGTGTCTCTTTAATCGCACGGGA 3' Amination sq746-dell_S1r CGCGCCGAGGTTTCGTTGATAGCGACGGGA 3' Amination sq746-dell_S1r CGCGCCGAGGTTTGCTTGATAGCGACGGGA 3' Amination sq746-dell_S1rv CATCGAGGATTTCCTTGATAGCGACGGGA 3' Amination sq746-dell_Inv CATCGAGGATTTCCTTGTTGGCTTTCCTGA 3' Amination sq746-dell_S1rv CGCGCCGAGGTTGAAACCAACACAACGG 3' Amination sq746-dell_S1rv CGCGCCCGAGGTTGGAAACCAACACACAGG 3' Amination sq747-delinsS_S1r CGCGCCGAGGTGCGAAACCACACAGGG 3' Amination sq747-delinsS_S1rv CGCGCCGGAGTGCCAACACGGGG 3' Amination sq746 <td></td> <td>sG719A_S2r</td> <td>ACGGACGCGGAGGCCAGCACTTTGATCTT</td> <td>3' Amination</td>		sG719A_S2r	ACGGACGCGGAGGCCAGCACTTTGATCTT	3' Amination
G719C sG719C_S1f CGCGCCGAGGCCAGCACTTTGATCTTTT 3' Amination sG719C_Inv CGTGCCGAAGGCCACCTTGATCTTTTG 3' Amination sG719S asG719S_S1f CGCGCCGAGGGCCCGGGTGCG 3' Amination asG719S_S1r CGCGCCGAGGGCCCCGGTGCG 3' Amination asG719S_S2r ACGGACGCGGAGGCTCCGGTGCG 3' Amination asG719S_Inv CTTGAGGATCTTGAAGGAAACTGCAAA 3' Amination s746-dell_S1F CGCGCCGAGGTGCTTCTCTAATCCGAGGGAAGTGC 3' Amination s746-dell_S2r ACGGACGCGGAGTTGCTTGTATAGCGACGGGA 3' Amination s746-dell_S2r ACGGACGCGGAGTTGCTTCTCTTAATTCCTTGA 3' Amination s746-dell_S1f CGCGCCGAGGTTGCTCTCTTAATTCCTTGGAGTGC 3' Amination s746-dell_S1f CGCGCCGAGGTTAAGGAAACCAGCAGGG 3' Amination s746-dell_S1f CGCGCCGAGGTTAAGAGAAACCAACATCTCC 3' Amination s746-dell_S1f CGCGCCGAGGTTAAGAGAAACCAACACCGG 3' Amination s746-dell_S1f CGCGCCGAGGTTAAAGAACAACACCACCGGG 3' Amination s746-dell_S1f CGCGCCGAGGTTAAAGAACAACCAACACCG 3' Amination s747-delinsS_S1f CGCGCCGAGGTGCCAACACACGGGG 3' Amination s747-delinsS_S2r ACGGACCGGGAGATGACCACGT		sG719A_Inv	ACCGTGCCGAACGCACCGGAGT	
sG719C_S2r ACGGACGCGGAGACAGCACTTGATCTTTTG 3' Amination sG719S asG719S_If CGGCCGAAGGCACCGGAGGC 3' Amination asG719S_S1F CGGCCGAGGGCTCCGGTGCG 3' Amination asG719S_S1r ACGGACGCGGAGAGCTCCGGTGCG 3' Amination asG719S_S1r CCGGCCGAGGGCTCCGGTGCG 3' Amination asG719S_Inv CTTGAGGATCTTGAAGGAAACTGAATTCAAAA AGATCAAAGTGCTGT "E746-A750del type1" \$746-del1_S1f CGCGCCGAGGTGCTTCTTTAATCCTTGAT 3' Amination s746-del1_S2r ACGGACGCGGAGTTTGATAGCGACGGGG 3' Amination s746-del2_S1f CGCGCCGAGGTTTGATAGCGACGGG 3' Amination s746-del2_S1r ACGGACGCGGAGTTTGATAGCGACACATCTC 3' Amination s746-del2_S1r ACGGACGCGGAGTTTAAGAGAAACAACATCTCC 3' Amination s746-del2_S1r ACGGACGCGGAGTTAAGAGAAACAACATCTCC 3' Amination as747-delinsS_S1r ACGGACGCGGAGATGAAAAGTCAAAAGC 3' Amination s747-delinsS_Liv CACGACGGGAGTGGCCATCACGTG 3' Amination s7681 s57681 S57681 S67681_S1r CGCGCCGAGGGGGCCATCACGTAG s790M s790M_S1f CGCGCCGAGGGGGGCATCACGGGAG 3' Amination s790M_S2r ACGGACGGGAGATGAGGCCAAACATCTCC 3' Amination s790M_S1f CGCGCCGAGGGGGCATGAGGGCCACGCGGGGGGGCCACGGTG 3' Aminati	G719C	sG719C_S1f	CGCGCCGAGGCCAGCACTTTGATCTTTT	3' Amination
s6719C_Inv CGTGCCGAACGGACCGGAGGT G719S asG719S_S1f CCGCCCGAGGGGCTCCGGTGCG 3' Amination asG719S_Inv CTTGAGGATCTTGAAGGAAACTGAATTCAAAA AGATCAAAGTGCTGT AAGATCAAAGTGCTGT 3' Amination *"E746-A750del type1" \$746-dell_S1f CGCGCCGAGGTGCTTCTCTTGATAGCGACGGGA 3' Amination \$746-dell_Inv CATCGAGGATTTCCTTGATAGCGACGGGA 3' Amination \$746-dell_Inv CATCGAGGATTTCCTTGATAGCGACGGGA 3' Amination \$746-dell_Inv CATCGAGGATTTCCTTGATAGCGACGGG 3' Amination \$746-dell_Inv CATCGAGGATTTCCTTGATAGCGACGGG 3' Amination \$746-dell_Inv CATCGAGGATTTCCTTGATAGCGACGGG 3' Amination \$746-dell_S1F CGCGCCGAGGTTGAAAGCAACACACACGG 3' Amination \$746-dell_S1rv ACATCGAGGATGCAAAAGCCAACACACGG 3' Amination \$746-dell_S2r ACGGACCCGGAGGTGAAAGCCAACACACGG 3' Amination \$746-dellinsS_S1r CGCGCCGAGGTGAAAGCCAACACACGGG 3' Amination \$747-delinsS_Linv CAGCACGGGGGGGCCAACCGGAGG 3' Amination \$768L \$5768L_S2r ACGGACCCGGAGGTGGCCAACGGAGG 3' Amination \$790M_MS2r ACGGACCCGGAGGTGGCCAAACTGGCG 3' Amination		sG719C_S2r	ACGGACGCGGAGACAGCACTTTGATCTTTTG	3' Amination
G719S asG7195_S1F CGCGCCGAGGGCTCCGGTGCG 3' Amination asG719S_Lav ACGGACCCCGAGAGCTCCGGTGCG 3' Amination asG719S_Inv CTTGAGGATCTTGAAGGAACTGAAATTCAAAA AGATCAAACTGCGT "E746-A750del type1" s746-dell_S1f CGCGCCGAGGTGCTTGCTTGATGCACGGGA 3' Amination s746-dell_S2r ACGGACCGGAGTTTGCTTGGTGGCAGGGGA 3' Amination s746-dell_S2r ACGGACCGGAGGTTCTCTTTATTCCTTGAT 3' Amination s746-dell_S1F CGCGCCGAGGTTTCCTTGTTATCCTTGAGAGTGC 3' Amination "E746-A750del type2" s746-dell_S1F CGCGCCGAGGTTAGCGAGGAGC 3' Amination s746-dell_S2r ACGGACCGGAGTTAGCGAGGAGGC 3' Amination s746-dell_S1F CGCGCCGAGGTGAAGAGAACACACTCTCC 3' Amination s747-delinsS_S1F CGCGCCGAGGTGAAGAGAACACACACACGG 3' Amination s7681 s57681_S1f CGCGCCGAGGTGGCCATCACGTAG 3' Amination s57681 s57681_S1f CGCGCCGAGGGGGGTGGTCGCACGTG 3' Amination s790M_S1f CGCGCCGAGGGGGGTGGTCACCGTG 3' Amination s1790M_S1r CCGCCCGAGGTGGCCAAACTGCTG 3' Amination s4588_Linv CCCCCCAGGTGGCCAAACTGCTG 3' Amination		sG719C_Inv	CGTGCCGAACGCACCGGAGCT	
asG7195_S2r ACGGACGCGGAGAGCTCCGGTGGG 3' Amination asG7195_Inv CTTGAGGATCTTGAAGGAAACTGAATTCAAAA "E746-A750del type1" s746-del1_S1f CGCGCCGAGGTGTCTCTTTAATCCTTGAT 3' Amination s746-del1_S2r ACGGACGCGAGGTTTGATAGCGACGGGA 3' Amination s746-del1_S1f CGCGCCGAGGTTCTCTTGATAGCGACGGGA 3' Amination s746-del2_S1f CGCGCCGAGGTTCTCTTGATAGCGACGGG 3' Amination s746-del2_S1r ACGGACCGGAGTCTTGTTGGTAGCGCGGGGG 3' Amination s746-del2_S2r ACGGACCCGGAGTCTTGATAGCGACGGG 3' Amination s746-del2_S1r CGCGCCGAGGTTAAGAGAAGCAACATCTCC 3' Amination s746-del2_S1r CGCGCCGAGGTGAAGCCAACAAGG 3' Amination s746-del2_S1r CCGGCCCGAGGTGAAGCCAACAAGG 3' Amination s747-delinsS_S1r CGCGCCCAGGTGGCAACACGTGG 3' Amination as747-delinsS_Inv CAGAACGTGGCCATCACGTAGG 3' Amination s768I s5768I_Inv GCGCCCAGGGGTGCTCACCGTAGG 3' Amination s790M_S2r ACGGACCGCGAGGTGGCCATCACGTAG 3' Amination s790M_Inv GCACCGGAGGGCGCAAACTGCTGCT 3' Amination s1790M_Inv GCACCGGAGGGGCCAAACTGCTGCT 3' Amination </td <td>G719S</td> <td>asG719S_S1f</td> <td>CGCGCCGAGGGGCTCCGGTGCG</td> <td>3' Amination</td>	G719S	asG719S_S1f	CGCGCCGAGGGGCTCCGGTGCG	3' Amination
asG719S_Inv CTTGAGGATCTTGAAGGAAACTGAATTCAAAA AGATCAAAGTGCTGT "E746-A750del type1" \$746-del1_S1f CGCGCCGAGGTTGATAGCGACGGGA 3' Amination s746-del1_S2r "E746-A750del type2" \$746-del2_S1f CGCGCCGAGGTTGCTTCTTAATTCCTTGAA 3' Amination s746-del2_S1F "E746-A750del type2" \$746-del2_S1f CGCGCCGAGGTTGCTTCTTAATTCCTTGAA 3' Amination s746-del2_Inv "T746-del2_Inv ACCGACCCGGAGTTTGCTAGCGACGGG 3' Amination afraf-del2_Inv ACATCGAGAGTTCCTTGTTGGCTTTCGGAGATGC "L747-P753del insS" as746-del2_S1f CGCGCCGAGGTTAAGAAAGCAACATCTCC 3' Amination as746-del2_S1f "L747-P753del insS" as746-del2_S1f CGCGCCGAGGTTAAGAAAGCAACATCTCC 3' Amination as746-del2_S1f S7681 s3768L_S1f CGCGCCGAGGTGAGAAAGTTAAAATTCCCGTCG CTATCAAGGAAC 3' Amination as746-del2_S1f S7681 s5768L_S1f CGCGCCGAGGCTGGCCATCACGTAG CGCGCCGAGGGTGTGTCCACGT 3' Amination asf786L_Inv S7681 s5768L_S1f CGCGCCGAGGGGGGGGGAAGGGGCCAACGTGGGGGGGG 3' Amination asf780M_S1f CGCGCCGAGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG		asG719S_S2r	ACGGACGCGGAGAGCTCCGGTGCG	3' Amination
"E746-A750del type1" s746-del1_S1f CGCGCCGAGGTGCTTCTCTTAATTCCTTGAT 3' Amination s746-del1_S2r ACGGACGCGAGGTTTGATAGCGACGGGA 3' Amination "E746-A750del type2" s746-del2_S1f CGCGCCGAGGTTGCTTCTGTAATTCCTTGA 3' Amination s746-del2_S1f CGCGCCGAGGTTGCTTCTGATAGCGACGGG 3' Amination s746-del2_S1r ACGGACGCGAGGTTTGGTTGGCTTTCGGAGATGC 3' Amination s746-del2_S1r ACGGACGCGAGGTTAAGAGAAGCAACATCTCC 3' Amination s746-del2_Inv ACATCAGAGGATTTCCTTGTTGGCTTTCGGAGATGC 3' Amination s746-del2_S1r ACGGACGCGGAGTTAAGAGAAAGCAACATCTCC 3' Amination s747-delinsS_S1f CGCGCCGAGGTGAAGCCAACAAGG 3' Amination as747-delinsS_Inv CAGAAGGTGAAAGTTAAAATTCCCGTCG CTATCAAGGAAC S768I sS768I_S1r CGCGCCGAGGGGGGGAGATGGCCATCACGTAGG 3' Amination s5768I sS768I_S1r CGCGCCGAGGGGGGGAGATGAGGGCCATCACGTAGG 3' Amination s5768I sS768L_Inv GCCACACGTGGGGGTGATGAGGTGCACGGTG 3' Amination s5780M_Inv GCAACGCGAGGGGGCAAACTGCTGC 3' Amination s4588R_Inv CGGACCGCGAGGGGCCAAACTGCTG 3' Amination s4861Q_S1r CGGCGCAGGAGGGGCCAAACTGCT		asG719S_Inv	CTTGAGGATCTTGAAGGAAACTGAATTCAAAA AGATCAAAGTGCTGT	
 s746-dell_S2r s746-dell_Inv CATCGAGGATTTGATAGCGACGGGA 3' Amination s746-dell_Inv CATCGAGGATTTCCTTGTTGGCTTTCGGAGATGTC "E746-A750del type?" s746-del2_S1f CGCGCCGAGGTTGCTTCTCTTAATTCCTTGA 3' Amination s746-del2_Inv ACCGACGCGGAGTTTCCTTGTTGGATAGCGACGGG 3' Amination as747-delinsS_S1f CGCGCCGAGGTTAAGAGAAGCAACATCTCC 3' Amination as747-delinsS_S1f CGCGCCGAGGTGAAGCCAACAAGG 3' Amination as747-delinsS_S1r CAGGACGCGGAGTCGAAAGCCAACAAGG 3' Amination as747-delinsS_S1r CAGGACGCGAGGTGACAAAGCCAACAAGG 3' Amination as747-delinsS_S1r CAGGACGCGAGCTGGCCATCACGTAG 3' Amination as7681_S1r CGCGCCGAGGCGGGGGTGATGACGAAGGTGACGAGG 3' Amination s57681_s52r ACGGACGCGGAGGTGGCCATCACGTAG 3' Amination s57681_s2r ACGGACGCGGAGGTGGCCATCACGTAG s790M_S1f CGCGCCGAGGGGGGTGATGAGGTGCACGGTG Amination s1790M_S2r ACGGACGCGAAGGGCCAAACTGCTG 3' Amination s1790M_Inv GCAGCCGAAGGGCCAAACTGCTG 3' Amination s1858R_S1f CGCGCCGAGGAGTGTCCCAGCC s1861Q_S1f CGCGCCGAGGAGTTTGGCCAACTGCG s1861Q_S1r CGCGCCGAGGAGTTTGGCCAACCGC s1861Q_S1r CGCGCCGAGGAGTTTGGCCAGCC s1861Q_S2r ACGGACGCGGAGTGTTTGGCCAGCC s1861Q_S2r ACGGACGCGGAGTTTGGCCAGCC s1861Q_S2r ACGGACGCGGAGTTTGGCCAGCC s1861Q_S2r ACGGACGCGGAGTTTGGCCAGCC s1861Q_S2r ACGGACGCGGAGTTTGGCCAGCC s1861Q_S2r ACGGACGCG	"E746-A750del type1"	s746-del1_S1f	CGCGCCGAGGTGCTTCTCTTAATTCCTTGAT	3' Amination
"E746-A750del type2" \$746-dell_Inv CATCGAGGATTTCCTTGTTGGCTTTCGGAGATGTC "E746-A750del type2" \$746-del2_S1f CGCGCCGAGGTTGCTTCTGTAATCCCTTGA 3' Amination \$746-del2_S2r ACGGACGCGGAGTTCTTGTAACGACGGG 3' Amination \$746-del2_lnv ACATCGAGGATTTCCTTGTGGCTTTCGGAGATGC "L747-P753del insS" as747-delinsS_S1f CGCGCCGAGGTTAAGAGAAGCCAACATCTCC 3' Amination as747-delinsS_S1r CAGAAGGTGAGAAAGCAAAGCCAACAAGG 3' Amination as747-delinsS_S1r CAGGACGCGAGAGTGGCCATCACGTAG 3' Amination s7681 \$57681_S1r CGCGCCGAGGCTGGCCATCACGTAG 3' Amination \$57681_s2r ACGGACGCGGAGATGACGCAGCAGGTG 3' Amination \$57681_Nv GGCACACGTGGGGGTTGTCCACGT 3' Amination \$790M_S1r CGCGCCGAGGGGGAGTGACGGGCACGGTG 3' Amination \$57681_Nv GCGCCGAGGGGAGTGATGAGGTGCACGGTG 3' Amination \$57681_S2r ACGGACCGCGAGGGGCATGAGGTGCACGGTG 3' Amination \$57681_Nv GCGCCCCAGGGGGCATGAGGTGCACGGTG 3' Amination \$57681_Nv GCGCCCGAGGGGCATGAGGCGCCC 3' Amination \$57681_Nv GCGCCCGAGGGGCATGAGGCGCCC 3' Amination \$610_Inv GCGCGCCGAGG		s746-del1_S2r	ACGGACGCGGAGTTTGATAGCGACGGGA	3' Amination
"E746-A750del type2" \$746-del2_S1f CGCGCCGAGGTTGCTTCTCTTAATTCCTTGA 3' Amination s746-del2_S2r ACGGACGCGGAGTCTTGATAGCGACGGG 3' Amination s746-del2_Inv ACATCGAGGATTTCCTTGTGGCTTTCGGAGATGC "L747-P753del insS" as747-delinsS_S1f CGCGCCGAGGTTAAGAGAAGCAACATCTCC 3' Amination as747-delinsS_S1r CCGCGCCGAGGTTAAGAGAAGCAACAACG 3' Amination as747-delinsS_S1r ACGGACCCGGAGTGAAGCCAACAAGG 3' Amination as747-delinsS_Inv CAGGAAGGTGAAAGCAACATCTCC 3' Amination s7681 \$S7681_S1r CGCGCCGAGGCTGGCCATCACGTAG 3' Amination s7681 \$S7681_S2r ACGGACGCGGAGATGACCACGTAGG 3' Amination s7790M_S1f CGCGCCGAGGGGTGTGCCACGGTG 3' Amination s7790M_S2r ACGGACCGGAGAGGGCCAAACTGCTG 3' Amination s7790M_Inv GCAGCCGAGGGGCCAAACTGCTG 3' Amination s4588_S1f CGCGCCCGAGGGGCCAAACTGCTG 3' Amination s4588_S2r ACGGACCGGAGAGGGCCAAACTGCTG 3' Amination s4588_S1f CGCGCCGAGGGGCCAAACTGCTG 3' Amination s4588_S1sf CGCGCCGAGGAGGGCCAAACTGCTG 3' Amination s4581Q \$LS61Q_S1f C		s746-del1_Inv	CATCGAGGATTTCCTTGTTGGCTTTCGGAGATGTC	
s746-del2_S2r ACGGACGCGGAGTCTTGATAGCGACGGG 3' Amination s746-del2_Inv ACATCGAGGATTCCTTGTTGGCTTCGGAGATGC "L747-P753del insS" as747-delinsS_S1f CGCGCCGAGGTTAAAGAGAAGCAACATCTCC 3' Amination as747-delinsS_S2r ACGGACCGCGAGTCGAAAGCCAACAAGG 3' Amination as747-delinsS_Inv CAGAAGGTGAGAAAGCTAAAAGTTAAAATTCCCGTCG CTATCAAGGAAC S768I sS768L_S1f CGCGCCGAGGCTGGCCATCACGTAG 3' Amination sS768L_S2r ACGGACGCGGAGATGGCCATCACGTAG 3' Amination sS768L_Inv GGCACACGTGGGGGTTGTCCACGT T790M sT790M_S1f CGCGCCGAGGCTGACAGGGTGCACGGTG 3' Amination sT790M_S2r ACGGACGCGGAGATGAGGGGCCAACGGGG 3' Amination sT790M_S2r ACGGACGCGGAGATGAGGGGCCACGGTG 3' Amination sT790M_S2r ACGGACGCGGAGATGAGGGGCCACGGTG 3' Amination sT790M_S2r ACGGACGCGGAGATGAGGGGCCAACGGTG 3' Amination sT790M_S2r ACGGACGCGGAGATGAGGGGCCAACGGTG 3' Amination sT790M_S2r ACGGACGCGGAGGTGGCCAACTGCTG 3' Amination sT80M_S2r ACGGACGCGAGGTGGCCAAACTGCTG 3' Amination sT80M_S2r ACGGACGCGAGGGGCCAAACTGCTG 3' Amination sT80M_S2r ACGGACGCGGAGGGGCCAAACTGCTG 3' Amination sL858R_Inv CCGCACGCGAGGGGCCAAACTGCTG 3' Amination sL858R_Inv CCGCACGCGGAGGGGCCAAACTGCTG 3' Amination sL861Q_S2r ACGGACGCGGAGGTGTTTGGCCAGCC 3' Amination sL861Q_S2r ACGGACGCGGAGGTGTTTGGCCAGCC 3' Amination sL861Q_S2r ACGGACGCGGAGGTGTTTGGCCAGCC 3' Amination sL861Q_S2r ACGGACGCGGAGGTGTTTGGCCAGCC 3' Amination sL861Q_Inv GCATGGTATTCTTTCTCTCTCCGCACCCAGCC Lesion Name Sequence Sequencing primers Exon 18 EGFR18F CATGCCGTGGCTGGTGCC EGFR18R AGTAGATGATGAAATATACAGCTTGCA	"E746-A750del type2"	s746-del2_S1f	CGCGCCGAGGTTGCTTCTCTTAATTCCTTGA	3' Amination
s746-del2_Inv ACATCGAGGATTTCCTTGTTGGCTTTCGGAGATGC "L747-P753del insS" as747-delinsS_S1f CGCGCCGAGGTTAAGAGAAGCAACATCTCC 3' Amination as747-delinsS_S2r ACGGACGCGGAGTCGAAAGCCAACAAGG 3' Amination as747-delinsS_Inv CAGAAGGTGAGAAAGTTAAAATTCCCGTCG CTATCAAGGAAC S768I sS768I_S1f CGCGCCGAGGCTGGCCATCACGTAG 3' Amination sS768I_Nv GGCACACGTGGCGAGATGGCCATCACGTAGG 3' Amination sS768I_Inv GGCACACGTGGGGGTGTGTCCACGT T T790M sT790M_S1f CGCGCCGAGGGGGGGTGAGACGGGGGGCACGGTGG 3' Amination sT790M_S2r ACGGACGCGGAGAGGGCCAAACTGCTG T I L858R asL858R_S1f CGCGCCGAGGGGGCCAAACTGCTG 3' Amination L861Q sL861Q_S2r ACGGACGCGGAGGGGCCAAACTGCTG 3' Amination L861Q sL861Q_S1f CGCGCCGAGGAGGGCCAAACTGCTG 3' Amination L861Q sL861Q_S1f CGCGCCGAGGAGGGCCAAACTGCTG 3' Amination L861Q sL861Q_S1f CGCGCCGAGGAGGTGTTGGCCAGCC 3' Amination L861Q sL861Q_S1f CGCGCCGAGGAGTGTTTGGCCAGCC 3' Amination L861Q sL861Q_S2r ACGGACCCGGAGGTGTTTGGCCA	21	s746-del2 S2r	ACGGACGCGGAGTCTTGATAGCGACGGG	3' Amination
"L747-P753del insS" as747-delinsS_S1f CGCGCCGAGGTTAAGAGAAGCAACATCTCC 3' Amination as747-delinsS_S2r ACGGACGCGGAGTCGAAAGCCAACAAGG 3' Amination as747-delinsS_Inv CAGAAGGTGAGAAAGTTAAAATTCCCGTCG 3' Amination as747-delinsS_Inv CAGAAGGTGAGAAAGTTAAAATTCCCGTCG 3' Amination as747-delinsS_Inv CAGAAGGTGGCATCACGTAG 3' Amination s768I sS768I_S2r ACGGACGCGGAGATGGCCATCACGTAG 3' Amination s5768L_Inv GGCACACGTGGGGGTGATGAGGGGCACGGTG 3' Amination s7790M_S1f CGCGCCGAGGGTGATGAGGGGCACGGTG 3' Amination s1790M_Lnv GCAGCCGAGGGGGGGCCAAACTGCTG 3' Amination L858R asL858R_S1f CGCGCCGAGGTGGCCAAACTGCTG 3' Amination L861Q sL861Q_S1f CGCGCCGAGGGGGGCCAAACTGCTG 3' Amination sL861Q_S1f CGCGCCGAGGAGTTGTCGCCAGCC 3' Amination sL861Q_S2r ACGGACCGCGGAGTGTTTGGCCAGCC 3' Amination sL861Q_S2r ACGGACGCGGAGTGTTTGGCCAGCC 3' Amination sL861Q_S1f CGCGCCCGAGGAGTGTTTGGCCAGCC 3' Amination sL861Q_S2r ACGGACGCGGAGGTGTTTGGCCAGCC 3' Amination sL861Q_Inv GCATG		s746-del2 Inv	ACATCGAGGATTTCCTTGTTGGCTTTCGGAGATGC	
as747-delinsS_S2rACGGACGCGGAGTCGAAAGCCAACAAGG3' Aminationas747-delinsS_InvCAGAAGGTGAGAAAGTTAAAATTCCCGTCG CTATCAAGGAAC3' Amination\$768I\$\$768I_S1fCGCGCCGAGGCTGGCCATCACGTAG3' Amination\$\$768I\$\$768I_S2rACGGACGCGGAGATGGCCATCACGTAGG3' Amination\$\$768I\$\$768I_Nv\$\$GCACACGTGGGGGTTGTCCACGT3' Amination\$\$768I_Inv\$\$GCACACGTGGGGGTGATGAGGTGCACGGTG3' Amination\$\$7790M_S1fCGCGCCCAGGGGGGATGAGGGGCCACGGTG3' Amination\$\$7790M_S2rACGGACGCGGAGGGGCCAAACTGCTG3' Amination\$\$7790M_Inv\$\$CAGCCGAAGGGCATGAGGTGCACGGTG3' Amination\$\$1790M_Inv\$\$CAGCCGAGGGGGCCAAACTGCTG3' Amination\$\$1790M_Inv\$\$CGCGCCGAGGGGGCCAAACTGCTG3' Amination\$\$1790M_Inv\$\$CGCGCGAGGGGGCCAAACTGCTG3' Amination\$\$1790M_Inv\$\$CGCGCGAGGGGGCCAAACTGCTG3' Amination\$\$1790M_S2r\$\$CGCGCGGGGGGGCCAAACTGCTG3' Amination\$\$1888_S8R_S1f\$\$CGCCCGAGGAGGGGCCAAACTGCTG3' Amination\$\$1861Q_S1f\$\$CGCCCGAGGAGGGGCCAAACTGCCGC3' Amination\$\$1861Q_S2r\$\$CGGCCGAGGGGGGGCCAGCC3' Amination\$\$2841Q_Inv\$\$CATGGTATTCTTTCTCTTCCGCACCCAGCC3' Amination\$\$29uencing primers\$\$\$\$GPR18F\$\$CATGCCGTGGCTGCTGGTCC\$\$C9FR18R\$\$GT8F18F\$\$CATGCCGTGGCTGCTGGCC\$\$\$\$C9FR18R\$\$GTAGATGATGATGAATATACAGCTTGCA\$\$	"L747-P753del insS"	as747-delinsS S1f	CGCGCCGAGGTTAAGAGAAGCAACATCTCC	3' Amination
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S768I \$\$768I_\$1f CGCGCCGAGGCTGGCCATCACGTAG 3' Amination \$\$768I_\$2r ACGGACGCGGAGATGGCCATCACGTAGG 3' Amination \$\$768I_Inv GGCACACGTGGGGGTGTCCCACGT 3' Amination \$\$768I_Nv GGCACACGTGGGGGTGTCCCACGT 3' Amination \$\$7790M_\$1f CGCGCCGAGGGGGGAGATGAGGGGCACGGTG 3' Amination \$\$7790M_Inv GCAGCCGAAGGGCATGAGGTGCACGGTG 3' Amination \$\$7790M_Inv GCAGCCGAGGGGCCAAACTGCTG 3' Amination \$\$1858R_\$1f CGCGCCGAGGGGGCCAAACTGCTG 3' Amination \$\$1858R_\$2r ACGGACGCGGAGGGGCCAAACTGCTG 3' Amination \$\$1858R_\$1f CGCGCCGAGGGGGCCAAACTGCTG 3' Amination \$\$1858R_\$2r ACGGACGCGGAGGGGCCAAACTGCTG 3' Amination \$\$1861Q_\$1f CGCGCCGAGGAGTTTGGCCAGCC 3' Amination \$\$1861Q_\$1f CGCGCCGAGGAGTGTTTGGCCAGCC 3' Amination \$\$1861Q_\$1nv GCATGGTATTCTTTCTTCTCTCCGCACCCAGCC 3' Amination \$\$26quencing primers \$\$200000000000000000000000000000000000		as747-delinsS_Inv	CAGAAGGTGAGAAAGTTAAAATTCCCGTCG CTATCAAGGAAC	
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T790M \$T790M_S1f CGCGCCGAGGGGTGATGAGGGTGCACGGTG 3' Amination \$T790M_S2r ACGGACGCGGAGATGATGAGGTGCACGGTG 3' Amination \$T790M_Inv GCAGCCGAAGGGCATGAGGTGCACGGTG 3' Amination L858R asL858R_S1f CGCGCCGAGGTGGCCAAACTGCTG 3' Amination asL858R_S2r ACGGACGCGGAGGGGCCAAACTGCTG 3' Amination asL858R_S1f CGCGCCGAGGAGGGGCCAAACTGCTG 3' Amination asL858R_S2r ACGGACGCGGAGGGGCCAAACTGCTG 3' Amination asL858R_Inv CCGCAGCATGTCAAGATCACAGATTTTGGGCC 3' Amination L861Q \$L861Q_S1f CGCGCCGAGGAGTGTTTGGCCAGCC 3' Amination \$L861Q_S2r ACGGACGCGGAGTGTTTGGCCAGCC 3' Amination \$L861Q_Inv GCATGGTATTCTTTCTCTTCCGCACCCAGCC 3' Amination Lesion Name Sequence Sequencing primers E E Exon 18 EGFR18F CATGCCGTGGCTGCTGGTCC E EGFR18R AGTAGATGATGGAAATATACAGCTTGCA E		sS768I Inv	GGCACACGTGGGGGGTTGTCCACGT	
sT790M_S2r ACGGACGCGGAGATGATGAGGTGCACGGTG 3' Amination sT790M_Inv GCAGCCGAAGGGCATGAGCTGCT 3' Amination L858R asL858R_S1f CGCGCCGAGGTGGCCAAACTGCTG 3' Amination asL858R_S2r ACGGACGCGGAGGGGGCCAAACTGCTG 3' Amination asL858R_S2r ACGGACGCGGAGGGGGCCAAACTGCTG 3' Amination asL858R_Inv CCGCAGCATGTCAAGATCACAGATTTTGGGCC 3' Amination L861Q sL861Q_S1f CGCGCCGAGGAGTGTTTGGCCAGCC 3' Amination sL861Q_S2r ACGGACGCGGAGTGTTTGGCCAGCC 3' Amination sL861Q_Inv GCATGGTATTCTTTCTCTTCCGCACCCAGCC 3' Amination Sequencing primers E E E Exon 18 EGFR18F CATGCCGTGGCTGCTGGTCC E EGFR18R AGTAGATGATGGAAATATACAGCTTGCA I	T790M	sT790M S1f	CGCGCCGAGGGGTGATGAGGTGCACGGTG	3' Amination
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asL858R_Inv CCGCAGCATGTCAAGATCACAGATTTTGGGCC L861Q sL861Q_S1f CGCGCCGAGGAGTTTGGCCAGCC 3' Amination sL861Q_S2r ACGGACGCGGAGTGTTTGGCCAGCC 3' Amination sL861Q_Inv GCATGGTATTCTTTCTCTTCCGCACCCAGCC 3' Amination Lesion Name Sequence Sequencing primers EGFR18F CATGCCGTGGCTGCTGGTCC EGFR18R AGTAGATGATGGAAATATACAGCTTGCA		asL858R S2r	ACGGACGCGGAGGGGCCAAACTGCTG	3' Amination
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sL861Q_S2r ACGGACGCGGAGTGTTTGGCCAGCC 3' Amination sL861Q_Inv GCATGGTATTCTTTCTCTCCGCACCCAGCC 3' Amination Lesion Name Sequence Sequencing primers EGFR18F CATGCCGTGGCTGCTGGTCC EGFR18R AGTAGATGATGGAAATATACAGCTTGCA	L861O	sL8610 S1f	CGCGCCGAGGAGTTTGGCCAGCC	3' Amination
sL861Q_Inv GCATGGTATTCTTTCTCCGCACCCAGCC Lesion Name Sequencing primers Exon 18 EGFR18F CATGCCGTGGCTGCTGGTCC EGFR18R AGTAGATGGAAATATACAGCTTGCA		sL8610_S2r	ACGGACGCGGAGTGTTTGGCCAGCC	3' Amination
Lesion Name Sequence Sequencing primers Exon 18 EGFR18F CATGCCGTGGCTGCTGGTCC EGFR18R AGTAGATGATGAAATATACAGCTTGCA		sL861Q_Inv	GCATGGTATTCTTTCTCTTCCGCACCCAGCC	
Sequencing primers Exon 18 EGFR18F CATGCCGTGGCTGCTGGTCC EGFR18R AGTAGATGATGGAAATATACAGCTTGCA	Lesion	Name	Sequence	
EGFR18F CATGCCGTGGCTGCTGGTCC EGFR18R AGTAGATGATGATAGATGATGACAGCTTGCA	Sequencing primers			
EGFR18R AGTAGATGATGGAAATATACAGCTTGCA	Exon 18	EGFR18F	CATGCCGTGGCTGCTGGTCC	
		EGFR18R	AGTAGATGATGGAAATATACAGCTTGCA	

Table 1 continued

Lesion	Name	Sequence
Exon 19	EGFR19F	CAGATCACTGGGCAGCATGT
	EGFR19R	AGAGCAGCTGCCAGACATGA
Exon 20	EGFR20F	CCCTCCTTCTGGCCACCATGC
	EGFR20R	CCATGGCAAACTCTTGCTATCC
Exon 21	EGFR21F	AGAGCTTCTTCCCATGATGATCTG
	EGFR21R	ACAGCTAGTGGGAAGGCAGC

Fig. 1 Schematic illustration of the EGFR-invader method. Target DNA is amplified by multiplex PCR. During the primary reaction, an invader oligo and a matched primary probe are annealed to the target PCR product, overlapping at the mutation position (in this case "A"). The cleavase enzyme recognizes this threedimensional structure and releases the 5' flap. If the primary probe does not match the mutation position, cleavase will not act and cleavage of the primary probe will not occur. In the secondary reaction, the 5' flap anneals to the FRET probe and the second cleavage reaction releases the fluorescent dye (Refs. [15-17]). F fluorescein, Q quencher



Table 2	Baseline	patients	characteristics	(n	=	42)
				· · ·			

Age	Median (range)	65 (33-82)
Sex	Male:female	15:27
Histology	Adeno:sqamous:large	38:3:1
Smoking	Never:former:current:unknown	16:12:8:6

Adeno adenocarcinoma, squamous squamous cell carcinoma, large large-cell carcinoma

19 deletion, 10 patients (45.5%) an L858R point mutation, and 1 case (4.5%) a L861Q point mutation. All mutations detected in this study were gefitinib-sensitive mutations and none had the EGFR-TKI resistant T790M mutation.

Comparison of PCR-invader and direct sequencing methods

The PCR-invader method detected EGFR mutations in 5 patients with a negative result by direct sequencing

Table 3	Comparison	of PCR-invad	er method	and direct	sequencing
for detect	tion of EGFR	mutation in 4	2 patients	(a) and 54	samples (b)

	PCR-invader metho	od
	Mutation (+)	Mutation (-)
(a) Patients, <i>n</i> (%)		
Direct sequencing		
Mutation (+)	17 (40%)	1 (2%)
Mutation (–)	5 (12%)	19 (45%)
(b) Samples, <i>n</i> (%)		
Direct sequencing		
Mutation (+)	17 (31%)	1 (2%)
Mutation (–)	11 (20%)	25 (46%)

(Table 3). In contrast, direct sequencing detected a rare but known EGFR mutation (T847I) in one patient (2.4%) with a negative result by use of the PCR-invader method. Direct

sequencing detected EGFR mutations in 18/42 patients (43%) and 19/54 samples (35%).

Sex, smoking, and EGFR mutation status

EGFR mutations were detected in 19/27 females (70%) and in 4/15 males (27%) (Table 4). EGFR mutations were detected in 13/16 non-smokers (81%) and in 5/20 smokers (25%).

 $\label{eq:constraint} \begin{array}{l} \textbf{Table 4} \\ \text{Relationship between EGFR mutation and sex (a) and} \\ \text{smoking status (b)} \end{array}$

Patients, n (%)	Mutation (+)	Mutation (-)
(a)		
Female	19 (45%)	8 (19%)
Male	4 (10%)	11 (26%)
(b)		
Never smoker	13 (36%)	3 (8%)
Smoker (current/former)	5 (1/4) (14%)	15 (7/8) (42%)

Mutation positive (+) means positive results from the PCR-invader method or from direct sequencing, or both

Table 5 EGFR mutations with different specimens

EGFR mutations detected by use of the PCR-invader method were coincident at different sites and different times in nine out of ten patients (Table 5). In one patient, the primary lung biopsy sample was negative for EGFR mutation, but a pleural effusion sample obtained at the time of relapse was positive for EGFR mutation. More precisely, the PCR-invader method yielded the same results for 9 different samples from 4 EGFR mutation-positive patients, and for 10 samples from 5 EGFR mutation-negative patients. The reproducibility of different times and different sites was verified in most cases by use of the PCRinvader method.

On the other hand, direct sequencing furnished coincident results for different specimens for only one patient out of five EGFR mutation-positive patients.

Treatment with EGFR-TKI

Twenty-four patients were treated with EGFR-TKI (gefitinib) at some point during the treatment courses (from 1st line to 6th line) (Table 6). In the EGFR mutation-positive patients (n = 17), RR was 41%, and the disease control

Pt	Sex	Sample	Date	EGFR mut	ation		
				Mutation	PCR invader result	Sequencing result	K-ras
1	F	Surgery	March 1999	(+)	L858R	(-)	ND
		TBLB	January 2006	(+)	L858R	L858R	ND
		Supra clavicular LN	June 2007	(+)	L858R	(-)	ND
2	F	Pleural effusion	May 2007	(+)	E746-A750del type2	(-)	ND
		TBLB	October 2005	(+)	E746-A750del type2	E746-A750del type2	ND
3	М	Surgery (rt-upper lobe)	March 2007	(+)	L858R	(-)	ND
		Surgery (rt-middle lobe)	March 2007	(+)	L858R	(-)	ND
4	F	TBLB	September 2007	Wild type	(-)	(-)	ND
		Pleural effusion	November 2007	(+)	E746-A750del type1	E746-A750del type1	ND
5	F	TBLB	August 2007	(+)	L747-A750del insP	L747-A750del insP	ND
		Pleural effusion	January 2008	(+)	L747-A750del insP	L747-A750del insP	ND
6	F	Upper lobe (partial resection)	February 2007	Wild type	(-)	(-)	Wild type
		Lower lobe	February 2007	Wild type	(-)	(-)	Wild type
7	М	TBLB	September 2006	Wild type	(-)	(-)	ND
		Neck LN	September 2006	Wild type	(-)	(-)	ND
8	М	Surgery	September 2006	Wild type	(-)	(-)	Mutation (GAT)
		Partial resection	May 2007	Wild type	(-)	(-)	Mutation (GAT)
9	М	TBLB	December 2007	Wild type	(-)	(-)	ND
		Pericardial effusion	January 2008	Wild type	(-)	(-)	ND
10	М	TBLB	December 2007	Wild type	(-)	(-)	ND
		Brain metastasis	January 2008	Wild type	(-)	(-)	ND

Pt patient, *date* the time the samples were taken from patients, *mutation* final decision from both PCR-invader result and sequencing result, (+) mutation positive, (-) EGFR wild type, *ND* not determined

rate (DCR; PR + SD) was 94%. The range of treatment duration was 3 weeks to more than 20 months and the median treatment duration was more than 6 months. In the EGFR mutation-negative patients (n = 7), RR was 0% and DCR was 14%. The range of treatment duration was 2 weeks to 2 months and the median treatment duration was 1 month. Patients with EGFR mutation were treated longer and had better disease control with EGFR-TKI.

Assessment of the sensitivity of the PCR-invader method

To show the sensitivity of the PCR-invader method, sensitivity was assessed by use of cell lines with known EGFR mutation status. As shown in Fig. 2, exon 19 deletion was detected in the cell line mixture of 1% (Fig. 2d) to 0.1 % (Fig. 2e) of a mutation-positive cell line (H1650). Also, L858R and T790M were detected in the cell line mixture of 1% (Fig. 2j) to 0.1 % (Fig. 2k) of a mutation-positive cell line (H1975), although with borderline threshold for

T790M in the 0.1% mixture (Fig. 2k). For the exon 19 deletion, the results were positive for both DEL1 and DEL2 in Fig. 2b and f, although the fluorescence level is much higher for DEL1. This suggests cross-reaction might be a problem for exon 19 deletion, especially for amounts of mutation-positive cells as high as 50% (Fig. 2b, f). According to the results, the PCR-invader method can detect the known EGFR mutation in lung cancer cells at 100–1000-fold dilution.

Discussion

The PCR-invader method detected EGFR mutations in clinical lung cancer specimens more effectively than direct sequencing. Direct sequencing detected a rare but known mutation in one patient. Most important mutations can be detected by use of the PCR-invader method. By use of the PCR-invader method, EGFR mutations were coincident in samples obtained from the same patients at different sites

Table 6 Comparison between EGFR mutation status and response to EGFR tyrosine kinase inhibitor

Pt	Sex	Histology	EGFR mutation	PCR-invader	Sequencing	EGFR-TKI	Response	Duration ^a
1	F	Ad	(+)	DEL1	DEL1	1st	PR	7M
2	F	Ad	(+)	DEL1	DEL1	2nd	PR	6M
3	М	Ad	(+)	DEL2	DEL2	2nd	PR	10M
4	F	Ad	(+)	L747-T751del ^b	L747-T751del	2nd	PR	7M
5	F	Ad	(+)	L858R	L858R	1st	PR	6M
6	F	Ad	(+)	L858R	L858R	2nd	PR	8M
7	F	Ad	(+)	L858R	L858R	5th	PR	3M
8	М	Ad	(+)	DEL1	(-)	4th	SD	20M
9	F	Ad	(+)	DEL2	(-)	3rd	SD	4M
10	F	Ad	(+)	DEL1	DEL1	1st	SD	7M
11	F	Ad	(+)	DEL1	DEL1	3rd	SD	4M
12	F	Ad	(+)	DEL2	DEL2	2nd	SD	21M
13	F	Ad	(+)	L858R	L858R	1st	SD	1.5M
14	F	Ad	(+)	L858R	L858R	2nd	SD	7M
15	М	Ad	(+)	L858R	L858R	6th	SD	4M
16	F	Ad	(+)	L861Q	L861Q	4th	SD	4M
17	F	Ad	(+)	del insP ^b	del insP	2nd	PD	0.75M
1	М	Ad	(-)			2nd	PD	0.75M
2	F	Ad	(-)			2nd	PD	1M
3	F	Sq	(-)			2nd	PD	1M
4	F	Ad	(-)			3rd	PD	0.5M
5	М	Ad	(-)			3rd	PD	0.5M
6	М	Ad	(-)			3rd	PD	1M
7	М	Sq	(-)			3rd	SD	2M

Ad adenocarcinoma, Sq squamous cell carcinoma, EGFR-TKI the line of the chemotherapy when the EGFR-TKI was used, DEL1 E746-A750del (2235-2249del GGAATTAAGAGAAGCA), DEL2 E746-A750del (2236-2250del GAATTAAGAGAAGCA), del insP L747-A750del insP

^a Treatment duration with the EGFR-TKI (months)

^b Detected on the basis of multiple bands in electrophoresis of PCR product, and after sequencing



Fig. 2 Sensitivity assay of the PCR-invader method. Sensitivity assay was done using cell lines with known EGFR mutation status, H1650 (exon 19 del: DEL1), H1975 (L858R and T790M), and SK-MES-1 (wild type) (Refs. [8, 19]). Cell lines were mixed in different ratios. For **a** to **f**, H1650 and SK-MES-1 were mixed 0:1, 0.5:0.5, 0.1:0.9, 0.01:0.99, 0.001:0.999, and 1:0. For **g** to **l** H1975 and SK-MES-1 were mixed 0:1, 0.5:0.5, 0.1:0.9, 0.001:0.999, and 1:0. White squares show quality control and a value above zero means PCR quality is good: the value = (fluorescence of the samples with detection probe for wild type sequences) – ([fluorescence of the

and different times in nine out of ten patients. Time-independent and site-independent reproducibility were verified. By using the EGFR-invader method, we can only detect known targeted mutations. However, most of the important mutations are covered and other studies such as the IPASS study mainly targeted the major mutations [5].

It is not possible to draw any definite conclusions from the current results regarding the effect with EGFR-TKI, because the study design was not prospective. The RR of our study with mutation-positive patients was relatively low (41%) compared with reported clinical trials (RR 71.2–95%) [20–25]. Several possible reasons for this are the heterogeneity of our patients including treatment line (from 1st line to 6th line), and the fact it was a non-prospective study, which means evaluation to decide a RE-CIST response may not be sufficient. However, the DCR in this study (94%) was almost the same as that in recent reports (81–96.5%) [5, 20–25]. This higher DCR and longer treatment duration compared with EGFR mutation-



normal control with the same probe] \times 0.8). Values of *solid squares* above zero means positive results with the detection probe for mutation sequences excluding false positives: the value = (fluorescence of the samples with detection probe for mutation sequences) – ([fluorescence of the normal control with the same probe] \times 2). To exclude false positivity, this criterion was introduced and used in clinical laboratories. Normal control is the mixture of white blood cell DNA from normal human volunteers. According to the results, PCR-invader methods could detect a known EGFR mutation of lung cancer cell lines diluted 100 to 1000-fold

negative patients suggested these are meaningful differences and that there are biological differences between these two groups classified according to the presence of EGFR mutation. EGFR mutation detected by the PCRinvader method can be a predictive marker of the effect with EGFR-TKI.

As shown in Table 6, association between results of treatment with EGFR-TKI and EGFR mutation status by the two methods (i.e. PCR-invader/direct sequencing, +/+ vs. +/-) are not evident. There are two EGFR mutation +/- (invader +, direct sequencing –) patients with the treatment effect of SD treated for 4 and 20 months with EGFR-TKI. On the other hand, 15 patients with EGFR mutation +/+ (invader +, direct sequencing +) were treated with EGFR-TKI and resulted in 7 PR, 7 SD, and 1 PD. Because the patients with EGFR mutation +/- had reasonable results (SD) with EGFR-TKI, it seems the EGFR mutation results with PCR-invader method are predictive of treatment.



Fig. 2 continued

There are several limitations in this study. First, the work was carried out at a single institution in Japan. Samples were collected for the purpose of general clinical practice and some samples were several years old, so sample quality may be heterogeneous. No one knows the "true" positive rate for EGFR mutation in our samples. Treatments were also heterogeneous and were not prospectively planned. Even though there were sample-quality issues, we were able to detect a fairly reasonable occurrence of mutation in this study. In cases with negative results, the possibility of false negative (because of quality of samples, etc.) should be evaluated, but positive results obtained by this method should be regarded as truly positive.

One of the issues regarding the sensitivity of mutation detection with archived specimens is the quality of the formalin-fixed samples. Previous reports suggested the feasibility of combining conventional DNA extraction and the PCR-invader method using formalin-fixed paraffin wax tissues [26]. In our hands, we could not elucidate the direct effect of formalin fixation on the quality of DNA and the sensitivity of mutation detection method, even though we used the same samples with the PCR-invader method and with direct sequencing. Further study is warranted to clarify the direct effect of formalin fixation on the sensitivity of the PCR-invader method and direct sequencing.

Exon 20 insertion was not detected by the PCR-invader method, because the PCR primers were not constructed to detect those mutations. Exon 20 insertion is thought to be a resistant mutation [27]. In a review paper, the frequency reported is approximately 3% [28]. On the other hand, in the phase III study with 1st line gefitinib treatment, EGFR mutation was checked by the ARMS method, and no exon 20 insertion was detected in 132 EGFR mutation-positive patients (IPASS trial) [5]. In recent phase III studies done in Japan with 1st line gefitinib against EGFR mutationpositive NSCLC [29, 30], the targeted patients were mainly exon 19 deletion and L858R without T790M resistant mutation. Neither study has counted the exon 20 insertion mutation. One study used results from several detection methods including the PCR-invader method [29]. Exon 20 insertion may be important but from those large scale data and clinical trials, in treatment decision making with EGFR-TKI, the inclusion of exon 20 insertion detection does not seem to be mandatory.

For detection of "exon 19 deletion", it is very important that the "exon 19 deletion" are in fact several types as mentioned in "Patients and methods". In the 11 patients with "exon 19 deletion", 8 patients were diagnosed as DEL1 and DEL2 and none had INS-S. One patient was diagnosed as DEL2 by the PCR-invader method, but the sequencing results revealed the exact mutation was

E746-T751del insA, one base different from DEL2. Two other patients had L747-T751del and L747-A750del insP, those were noticed by electrophoresis of the exon 19 PCR product with negative results with the invader assay probes specific to the three "deletion" mutations. Table 7 shows the frequency of different types of exon 19 deletion from several papers. Summation of these reports revealed that the percentage of the same exon 19 deletions detected in this study was 88.6%. Even with other exon 19 deletions which were not seen in our patients, the PCR-invader method with electrophoresis and subsequent sequencing may enable us to check other rare mutations.

It is very important to address false positivity in highly specific techniques. Basically, the PCR-invader method has always been performed with positive and negative controls for quality assurance. Furthermore, detection was conducted with pre-specified thresholds as follows. If the fluorescence of the sample with the mutation detection invader probe was more than twice that of normal control DNA with same probe, the samples were regarded as mutation positive (as shown in Fig. 2). To exclude false positivity, this criterion was introduced and has been used in general practice in Japan. In the sensitivity assay, average of fluorescence values of normal control DNA with mutation detection invader probe were 21-89 according to the probes (data not shown). On the other hand, actual fluorescence values of sample with mutation detection invader probe were approximately 1000-100. For example, in the sensitivity assay with H1975 cell line (Fig. 2g-l) the actual fluorescence values of the sample with L858R probe were 1013-142.5, and that with T790M were 966-79.5 according to the mixture rate with SK-MES-1. In contrast, normal control values were 42.5 for L858R and 39.0 for T790M. So the actual values were always more than twice as high as controls in this situation with up to 1:1000 cell mixture (Fig. 2). Even though we could not eliminate the possibility of contamination, for example sample carry over, the above mentioned threshold seems good enough to eliminate the non-specific false positive results.

Our results with relatively low RR might be because of hidden mutation (for example resistant mutation of exon 19 ins), and/or other clinical factors. We believe that the most likely reasons are the retrospective nature of this study and the patients' characteristics (mainly heavily treated patients). Further validation study in a prospective setting is warranted.

Several different sensitive EGFR mutation-detection methods are available in clinics in Japan. Direct comparisons of these sensitive methods are needed. However, on the basis of the results of this study, which compared the PCR-invader method and direct sequencing, which is the "current standard", the former can be regarded as a standard and reliable sensitive method.

Table 7 Frequency of different types of EGFR exon19 deletion

Repots	Current	Tanaka (Cancer Sci 2007)	Sequist	Gow (Ann Oncol 2008)	Shigematsu	Tamura (Br J Cancer 2008) 1281	Total
Method No. of Pt with del	PCR-invader $n = 11$	PNA-LNA-PCR clampn = 29	Sequencing $n = 18$	Sequencing $n = 15$	Sequencing $n = 62$	Sequencing $n = 14$	n=149
Mutation type							
E746-A750del	8 (72.7%)	20 (69.0%)	14 (77.8%)	9 (60.0%)	42 (67.7%)	10(71.4%)	103 (69.1%)
L747-P753del insS	(0.0%)	3 (10.3%)	1(5.6%)	1 (6.7%)	3 (4.8%)	1 (7.1%)	(0.0%) (6.0%)
L747-T751del	1 (9.1%)	1 (3.4%)	1(5.6%)	2 (13.3%)	4 (5.6%)	2 (14.3%)	11 (7.4%)
E746-T751del insA	1 (9.1%)	0 (0%)	0(0%)	0 (0%)	0 (0%)	1 (7.1%)	2 (1.3%)
L747-A750del insP	1 (9.1%)	2 (6.9%)	1(5.6%)	0 (0%)	3 (4.8%)	0 (0%)	7 (4.7%)
Others	(0.0%)	3 (10.3%)	1(5.6%)	3 (20.0%)	10 (16.1%)	0 (0%)	17 (11.4%)

In conclusion, the PCR-invader method detected EGFR mutations in clinical lung cancer specimens more effectively than direct sequencing. Time-independent and site-independent reproducibility was verified.

Acknowledgments The authors wish to thank Mr Toshikazu Yamaguchi of BML Inc. for his technical support with the mutation analyses. This work was presented in part at the 33rd European Society of Medical Oncology (ESMO) Congress in Stockholm, Sweden, 12–16 September 2008. This study was partly supported by a 33rd ESMO Congress travel grant.

Conflict of interest No author has any conflict of interest.

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