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Most T790M mutations are present on the same *EGFR* allele as activating mutations in patients with non–small cell lung cancer

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Abstract

Objectives: The T790M and C797S mutations of the epidermal growth factor receptor gene (*EGFR*) confer resistance to first- and third-generation EGFR tyrosine kinase inhibitors (TKIs), respectively, in patients with non–small cell lung cancer (NSCLC) harboring activating mutations of *EGFR*. C797S has been identified in cis or in trans with T790M in tumor specimens from patients who experienced treatment failure with first- and third-generation EGFR-TKIs. The allelic relation between T790M and activating mutations of *EGFR* has not been well characterized, however. We have now developed a digital polymerase chain reaction (dPCR)–based method for determination of the allelic relation between two types of *EGFR* mutation (T790M and either C797S or an activating mutation).

Materials and Methods: Seven clinical NSCLC specimens and two NSCLC cell lines harboring both an activating mutation and T790M were analyzed with this new method to identify the allelic relation between these *EGFR* mutations.

Results: The median ratio of the number of alleles positive for both an activating mutation and T790M to the number of T790M-positive alleles was 97.1% (range, 90.0–100%). Confirmatory analysis by next-generation sequencing yielded a corresponding value of 96.7% (range, 89.1–99.5%). Our dPCR method thus reliably identifies the allelic relation between two *EGFR* mutations in a quantitative manner.

Conclusion: Almost all T790M mutations were detected in cis with activating mutations of *EGFR* regardless of the de novo or acquired status of T790M, with cancer cells harboring T790M and activating mutations on the same allele appearing to be selected and enriched during EGFR-TKI treatment.

Key words: epidermal growth factor receptor (EGFR), mutation, allele, digital PCR, lung cancer

1. Introduction

Tyrosine kinase inhibitors (TKIs) specific for the epidermal growth factor receptor (EGFR) show pronounced clinical activity in individuals with non–small cell lung cancer (NSCLC) positive for an activating mutation of *EGFR* [1-7]. Despite showing an initial response to these drugs, however, most patients experience progressive disease within 1 to 2 years [8, 9]. The emergence of a secondary mutation of threonine-790 to methionine (T790M) of *EGFR* is largely responsible for resistance to the first-generation EGFR-TKIs gefitinib and erlotinib [9-12] as well as a cause of resistance to irreversible EGFR-TKIs such as the second-generation drug afatinib [13, 14].

Tumor cells harboring the T790M mutation of *EGFR* have been found to be present in small numbers even before EGFR-TKI administration in some patients [15-22], with these cells undergoing selection and enrichment during EGFR-TKI treatment and eventually giving rise to drug resistance [21, 23-25]. Alternatively, T790M can appear during EGFR-TKI therapy in cells that are initially negative for this mutation [26, 27].

Third-generation EGFR-TKIs such as osimertinib and rociletinib are effective in patients with NSCLC positive for an activating mutation of *EGFR* who have acquired resistance to first-generation EGFR-TKIs as a result of the emergence of T790M [28-31]. However, the emergence of an additional mutation of cysteine-797 to serine (C797S) confers resistance to these third-generation EGFR-TKIs [32-34]. The C797S mutation has been identified in cis or in trans with T790M in tumor specimens from patients who experienced treatment failure with first- and third-generation EGFR-TKIs [32]. On the other hand, the allelic relation between T790M and activating mutations of *EGFR* has not been well characterized in NSCLC cells with either an acquired or de novo T790M mutation [35].

Digital polymerase chain reaction (dPCR) analysis entails the addition of diluted template DNA to a panel with many hundreds of chambers such that each reaction chamber receives one or zero copies of the target molecule. Performance of allele-specific PCR analysis with a single template molecule in each chamber allows quantitative and highly sensitive detection of target molecules [36]. We have now developed a method based on dPCR to evaluate whether two *EGFR* mutations (T790M and either an activating mutation or C797S) are present in cis or in trans. Using this technique, we have evaluated whether T790M occurs in cis or in trans with an activating mutation in clinical specimens.

2. Materials and Methods

2.1. Patients and specimen collection

Among the patients with NSCLC who were treated at Kyushu University Hospital between January 2013 and December 2015, a frozen tumor specimen with both a common activating mutation (an exon 19 deletion [Ex19del] or L858R point mutation) and T790M mutation of *EGFR* detected by conventional *EGFR* mutation testing was available for 10 patients, including one specimen obtained before EGFR-TKI treatment and nine specimens obtained after the development of progressive disease during such treatment. *EGFR* activating mutation status for the 10 specimens revealed by conventional testing was as follows: L858R (n = 5), Ex19del (E746–A750 [nucleotides 2235–2249], n = 2), Ex19del (E746–A750 [nucleotides 2236–2250], n = 1), and Ex19del (other types, n = 2). Seven clinical specimens—L858R (n = 5) and Ex19del (E746–A750 [nucleotides 2235–2249], n = 2)—and two human NSCLC cell lines (H1975, PC9-GR) were examined for the allelic relation between an activating mutation of *EGFR* and T790M by dPCR and next-generation sequencing (NGS). The three remaining clinical specimens with an Ex19del other than E746–A750 (nucleotides 2235–2249) were examined only by NGS. The study was conducted in accordance with the provisions of the Declaration of Helsinki and was approved by the Ethics Committee of Kyushu University.

2.2. Cell culture and reagents

H1975 (positive for L858R and T790M mutations of *EGFR*) and PC9 (positive for Ex19del [E746–A750, nucleotides 2235–2249]) cell lines were obtained from American Type Culture Collection and European Collection of Authenticated Cell Cultures, respectively. Both cell lines were cultured under 5% CO_2 at 37°C in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (1 μg/ml).

2.3. Generation of a gefitinib-resistant PC9 cell subline

To generate a cell line that harbors T790M and is resistant to gefitinib, we exposed PC9 cells to this EGFR-TKI at a concentration of 1 μM for 6 months [37]. We then performed single-cell cloning and established the T790M-positive, gefitinib-resistant subline PC9-GR.

2.4. Preparation of DNA for analysis by dPCR and NGS

Total RNA was isolated from cell lines and frozen clinical specimens with the use of an AllPrep DNA/RNA Mini Kit (Qiagen K.K., Tokyo, Japan) and was subjected to reverse transcription (RT) with the use of PrimeScript RT Master Mix (Takara Bio, Shiga, Japan). The resulting cDNA was subjected to PCR in order to amplify a region of *EGFR* encompassing exons 18 to 23 (which includes both sites of activating mutations and

T790M), yielding amplicons of 563 bp (L858R) or 548 bp (Ex19del, E746–A750). Detailed protocols for PCR are provided in Supplementary Material, with primer sequences being shown in Supplementary Table S1. The final DNA samples were then analyzed by dPCR and NGS.

2.5. Plasmid construction

Plasmids encoding wild-type (pBabe-EGFR-WT), Ex19del (E746–A750 [nucleotides 2235–2249]) (pBabe-19del), T790M (pBabe-T790M), or Ex19del + T790M (pBabe-19del+T790M) forms of human EGFR were obtained from Addgene (catalog nos. #11011, #32062, #32070, and #32072, respectively). Plasmids encoding L858R (pBabe-L858R) or C797S (pBabe-C797S) forms of EGFR were generated from pBabe-EGFR-WT with the use of a QuickChange Lightning Site-Directed Mutagenesis Kit (Aglient Technologies, Santa Clara, CA). Plasmids encoding L858R + T790M (pBabe-L858R+T790M) or C797S + T790M (pBabe-C797S+T790M) forms of EGFR were generated from pBabe-T790M. Primer sequences for introduction of the L858R or C797S mutations are shown in Supplementary Table S1.

2.6. dPCR analysis

We performed dPCR analysis with a nanofluidic dPCR system (BioMark HD; Fluidigm, South San Francisco, CA) equipped with a Fluidigm digital chip for quantitation of target DNA molecules. Experimental details are provided in Supplementary Material, with primer and probe sequences being shown in Supplementary Table S1.

2.7. NGS analysis

Libraries were prepared from PCR products with the use of a NEBNext Ultra DNA Library Prep Kit (New England Biolabs Japan, Tokyo, Japan) and were sequenced with an Illumina MiSeq machine to generate 300-nucleotide paired-end reads. The resulting reads were aligned with the human *EGFR* mRNA sequence (GenBank accession no. X00588.1). Only aligned reads with a mapping quality score of 60 or above were analyzed, and those with or without Ex19del, L858R, or T790M were counted.

2.8. Statistical analysis

Linear regression analysis was applied to evaluate both the relation between the amount of input plasmid and the number of alleles estimated by dPCR as well as the relation between the ratio of the number of T790M-positive alleles to the number of activating mutation–positive alleles (T/A ratio) determined by dPCR and that determined by NGS. Statistical analysis was performed with the use of GraphPad Prism for Windows (GraphPad Software, La Jolla, CA).

3. Results

3.1. Development of a method for evaluation of the allelic relation between two EGFR mutations by dPCR

Analysis by dPCR was performed with mutation-specific probes targeting Ex19del (E746–A750 [nucleotides 2235–2249]), L858R, or C797S of *EGFR* (each labeled with 6-carboxyfluorescein [FAM]) and with a probe targeting T790M (labeled with VIC [Life Technologies Japan, Tokyo, Japan]). Primer sets were designed to generate PCR amplicons that include the regions targeted by pairs of the mutation-specific probes (Ex19del and T790M, L858R and T790M, or C797S and T790M) (Figure 1A, B). The PCR mixtures were applied to panels with 770 separate chambers for dPCR analysis, with template DNA being diluted sufficiently to ensure that each chamber contains zero or one target molecule, and allele-specific PCR was then performed in each chamber. To reduce the possibility that a chamber contains more than one target allele, the amount of template applied to each panel was adjusted to fall within the range of 0.05×10^{-6} to 1.7×10^{-6} ng, with the sum of the estimated number of activating mutations and that of T790M being <80.

A plasmid encoding EGFR positive for both Ex19del and T790M (pBabe-19del+T790M) was used as a model template for mutations in cis and yielded positive signals for both Ex19del and T790M in the same chambers (Figure 1A, C). When a 1:1 mixture of two plasmids (pBabe-19del and pBabe-T790M) was used as a model for mutations in trans, the signals for Ex19del and T790M were detected in different chambers (Figure 1B, D). These results indicated that our method is able to determine whether Ex19del and T790M are present in cis or in trans. Similar results were obtained with plasmids designed to identify the allelic relation between L858R and T790M (data not shown) or between C797S and T790M (Supplementary Figure S1).

Analysis of a plasmid encoding wild-type (WT) EGFR (pBabe-EGFR-WT) at up to 1.0×10^5 copies per reaction as the template did not yield positive signals with probes specific for Ex19del, L858R, C797S, or T790M (Supplementary Table S2). Analysis of serial dilutions of pBabe-L858R+T790M or pBabe-19del+T790M as templates revealed a positive linear correlation between the amount of input plasmid and the estimated number of double-positive alleles (R = 0.9864 and 0.9839, respectively) (Figure 2A, B). Together, these findings indicated that allele-specific dPCR analysis is indeed specific and that quantitative evaluation of *EGFR* double-mutant alleles by this approach is reliable.

The relation between the known input copy number of pBabe-C797S+T790M and the estimated copy number by dPCR was also linear (R = 0.9912) for 2, 5, 25, and 50

input copies of the plasmid in a sample also including 5000 copies of pBabe-EGFR-WT (Figure 2C), indicating that the quantitative evaluation of alleles positive for both T790M and C797S is reliable and has a sensitivity that allows the detection of such a double-positive allele present at a frequency of at least 0.04%.

3.2. Evaluation of the allelic relation between EGFR activating mutations and T790M in NSCLC specimens and cell lines

Complementary DNA was obtained from seven clinical specimens of NSCLC and the NSCLC cell lines H1975 and PC9-GR, all of which were found to be positive for an activating mutation and T790M by conventional *EGFR* mutation testing, and was examined for the allelic relation between these mutations by dPCR. One clinical specimen (patient no. 1) was obtained before treatment with an EGFR-TKI, and the H1975 cell line was established before development of these drugs, indicating that these two samples harbor a de novo T790M mutation. The other six clinical specimens and PC9-GR were obtained or established after the failure of EGFR-TKI treatment (acquired resistance).

Representative results for duplicate assays—panels (1) and (2)—of one of these clinical specimens (patient no. 6) harboring Ex19del and T790M are shown in Figure 3. The number of signals was counted by the system software (raw data), the number of target alleles applied to the panels was estimated from the raw data with the use of the Poisson distribution (estimated number of target alleles), and the values for the duplicate assays were summed as (1) + (2). The ratio of the number of double-positive alleles (cis) to the number of T790M-positive alleles (C/T ratio) was 100% (27/27). The C/T ratio (%) for all nine samples evaluated by dPCR is shown in Table 1, with the median value being 97.1% (range, 90.0–100%), indicating that, regardless of the de novo or acquired status of T790M, almost all T790M mutations were present together with the activating mutation on the same allele.

To confirm these dPCR results, we sequenced libraries prepared from a total of 12 samples, consisting of the nine samples analyzed by dPCR and three additional NSCLC specimens harboring a different type of Ex19del (E746–A750 [nucleotide 2236–2250], $n = 1$; types other than E746–A750, $n = 2$) (Table 1). NGS detected four different types of reads (alleles): a read positive for both an activating mutation and T790M (A+T+), a read positive for an activating mutation and negative for T790M (A+T–), a read negative for an activating mutation and positive for T790M (A–T+), and a read negative for both an activating mutation and T790M (A–T–). For example, in the case of patient no. 6, the numbers of these reads were 3993 (35.0%), 6952 (60.9%), 22 (0.2%), and 443

(3.9%), respectively, yielding estimated numbers of alleles with an activating mutation (Ex19del), with T790M, and with Ex19del and T790M in cis of 10,945, 4015, and 3993, respectively. The C/T ratio for this sample as determined by NGS was thus 99.5% (3993/4015). The median C/T ratio determined by NGS was 96.7% (range, 89.1–99.5%), consistent with the dPCR data.

3.3. Evaluation of the ratio of the number of T790M alleles to the number of activating mutation alleles

To evaluate the contribution of T790M to resistance to first-generation EGFR-TKIs, we calculated the ratio of the number of T790M-positive alleles to the number of activating mutation–positive alleles (T/A ratio) as previously described [21]. The T/A ratio (%) for the nine samples analyzed by dPCR and the 12 samples analyzed by NGS is shown in Table 1. A positive linear relation (R = 0.9618) between the T/A ratio (%) evaluated by dPCR and that evaluated by NGS was apparent (Figure 4), and both methods showed that the T/A ratio for the samples with a de novo T790M mutation (patient no. 1 and H1975) was higher than that for the samples with acquired resistance (mean of 84.2% versus 34.9% by dPCR and of 94.7% versus 39.3% by NGS).

4. Discussion

We have here developed a method based on dPCR for determination of whether activating mutations or C797S of *EGFR* are present on the same allele as T790M. Subcloning of RT-PCR products followed by Sanger sequencing has previously been applied to evaluation of the allelic relation between activating mutations and T790M in several studies [10, 38-40], with T790M having been identified in cis or in trans with activating mutations in tumor specimens [10, 38]. The low throughput of this approach has limited the analysis to only a small number of samples and a small number of clones per sample, however, which makes it difficult to assess how frequently T790M exists together with an activating mutation on the same allele. In the present study, we analyzed a greater number of alleles with our newly developed method and found that the median value of the C/T ratio was 97.1% (range, 90.0–100%), showing that almost all T790M mutations were present on the same allele as activating mutations of *EGFR*.

Highly sensitive methods such as dPCR have detected T790M in a large proportion of specimens of activation mutation–positive NSCLC obtained from patients before EGFR-TKI treatment [15-22]. We previously found that the T/A ratio was low (median, 0.99%) in most pre–EGFR-TKI samples in which T790M was identified by dPCR but not detected by conventional *EGFR* testing, with first-generation EGFR-TKIs showing

substantial antitumor efficacy in such patients [21]. On the other hand, conventional *EGFR* mutation testing detected T790M in only 1.4% of specimens obtained from activating mutation–positive lung adenocarcinoma patients before EGFR-TKI treatment [41], and such tumors are thought to be intrinsically resistant to first-generation EGFR-TKIs. In the present study, conventional *EGFR* mutation testing detected T790M in a pre–EGFR-TKI specimen obtained from patient no. 1, and the T/A ratio as determined for this specimen by dPCR or NGS was high (80.0% and 90.1%, respectively). T790M was not detected by dPCR in normal leukocytes obtained from this patient (data not shown), indicating that the high T/A ratio in the pre–EGFR-TKI tumor sample was not due to the existence of a T790M germline mutation. A high T/A ratio (88.3% by dPCR, 99.2% by NGS) was also obtained for the H1975 cell line, which was established long before the development of EGFR-TKIs. Even for these pre–EGFR-TKI samples, almost all T790M mutations were detected on the same allele as the activating mutations. These findings suggest that a secondary T790M mutation appeared on the same allele as the activating mutation at an early stage of carcinogenesis for these tumors, giving rise to the high T/A ratio and likely conferring intrinsic resistance to first-generation EGFR-TKIs.

Germline T790M mutations have been identified in 0.54% of nonsmoking patients with lung adenocarcinoma [42]. Subcloning of RT-PCR products followed by sequencing revealed that a secondary somatic activating mutation of *EGFR* appeared in cis with the constitutional T790M mutation in such patients [43]. Together with our results, this finding suggests that, unlike in the cis configuration, T790M cannot effectively cooperate with activating mutations of *EGFR* in the trans configuration, and that enhancement of the catalytic activity of EGFR harboring an activating mutation by T790M may lead to the selection of cell clones with both types of mutation on the same allele [24, 44, 45].

The combination of first- and third-generation EGFR-TKIs has been found to restore EGFR inhibition in patients with both T790M and C797S when these two mutations are present on different alleles [46]. In the present study, we found that our dPCR method is also applicable to evaluation of whether C797S is present in cis or in trans with T790M. A study applying our dPCR technique to cell lines that are resistant to first- and third-generation EGFR-TKIs as well as to clinical samples from patients who experience failure of treatment with these drugs is warranted. Given that dPCR is more sensitive and allows a higher throughput of samples for detection of specific mutations compared with NGS [47-49], our technique has the potential to be applied to the analysis of circulating tumor DNA as well as to tumor tissue specimens obtained

from such patients.

In conclusion, we have developed a method that is able to evaluate whether an activating mutation or C797S is present on the same *EGFR* allele as T790M. Using this method, we found that almost all T790M mutations are present in cis with activating mutations regardless of whether tumor resistance to first-generation EGFR-TKIs caused by T790M is intrinsic or acquired. Cancer cells with T790M in cis with an activating mutation thus appear to be selected and enriched during treatment with EGFR-TKIs.

Author Contributions

Conception and design: E.I., T.H., and I.O. Development of methodology: N.H., E.I., K.M., T.H., and I.O. Acquisition of data: N.H., E.I., N.K., and H.S. Analysis and interpretation of data: N.H., E.I., N.K., T.H., K.M., K.T., and I.O. Writing, review, or revision of the manuscript: N.H., E.I., N.K., T.H., K.T., I.O., E.B., and Y.N. Administrative, technical, or material support: E.I., T.H., M.K., I.O., E.B., K.A., H.S., and Y.N. Study supervision: E.I., T.H., K.T., I.O., and Y.N.

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Conflict of Interest

The authors declare no conflicts of interest.

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Figure Legends

Figure 1. Design of a dPCR-based method to evaluate the allelic relation between two *EGFR* mutations. (**A**, **B**) Primers and probes were designed for the detection of an activating mutation or C797S (FAM labeled, red) as well as for that of T790M (VIC labeled, blue) in cis (A) or in trans (B). (**C**, **D**) Representative heat-map results for dPCR analysis of a plasmid encoding EGFR positive for both Ex19del and T790M (pBabe-19del+T790M) or of a 1:1 mixture of plasmids encoding EGFR positive for Ex19del (pBabe-19del) or for T790M (pBabe-T790M) as models for mutations in cis (C) or in trans (D), respectively.

Figure 2. Quantitative performance of dPCR assays for the detection of double-mutant *EGFR* alleles. (**A**, **B**) Estimated number of alleles positive for both mutations versus the input plasmid concentration for pBabe-L858R+T790M (A) or pBabe-19del+T790M (B). (**C**) Estimated number of alleles positive for both mutations versus the input copy number of pBabe-C797S+T790M against a background of 5000 copies of pBabe-EGFR-WT. All data are means ± SEM for three separate experiments, and the correlation coefficient (*R*) is shown.

Figure 3. Representative results for dPCR analysis of the clinical specimen from patient no. 6 (positive for Ex19del and T790M). The dPCR assay was performed in duplicate [panels (1) and (2)]. The numbers of chambers positive for Ex19del (red), T790M (blue), and both mutations (red and blue) were counted by the system software (raw data), and the estimated numbers of target alleles were determined with the use of the Poisson distribution.

Figure 4. Correlation between the T/A ratio (%) determined by dPCR and that determined by NGS for the nine clinical and cell line samples analyzed by both assays. The two samples with a de novo T790M mutation are indicated by open circles. The correlation coefficient (*R*) is shown.

Supplementary Figure S1. Representative heat-map results for dPCR analysis of a plasmid encoding EGFR positive for both C797S and T790M mutations (pBabe-C797S+T790M) or of a 1:1 mixture of plasmids encoding EGFR positive for C797S (pBabe-C797S) or for T790M (pBabe-T790M) as models for mutations in cis (**A**) or in trans (**B**), respectively. See Figure 1.

Figure 1.

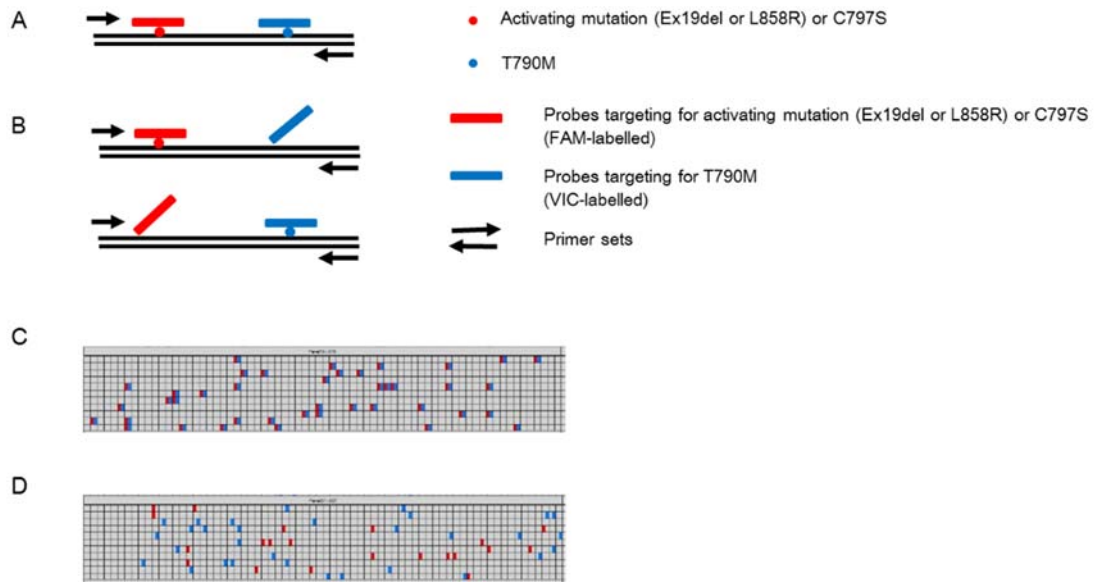


Figure 2.

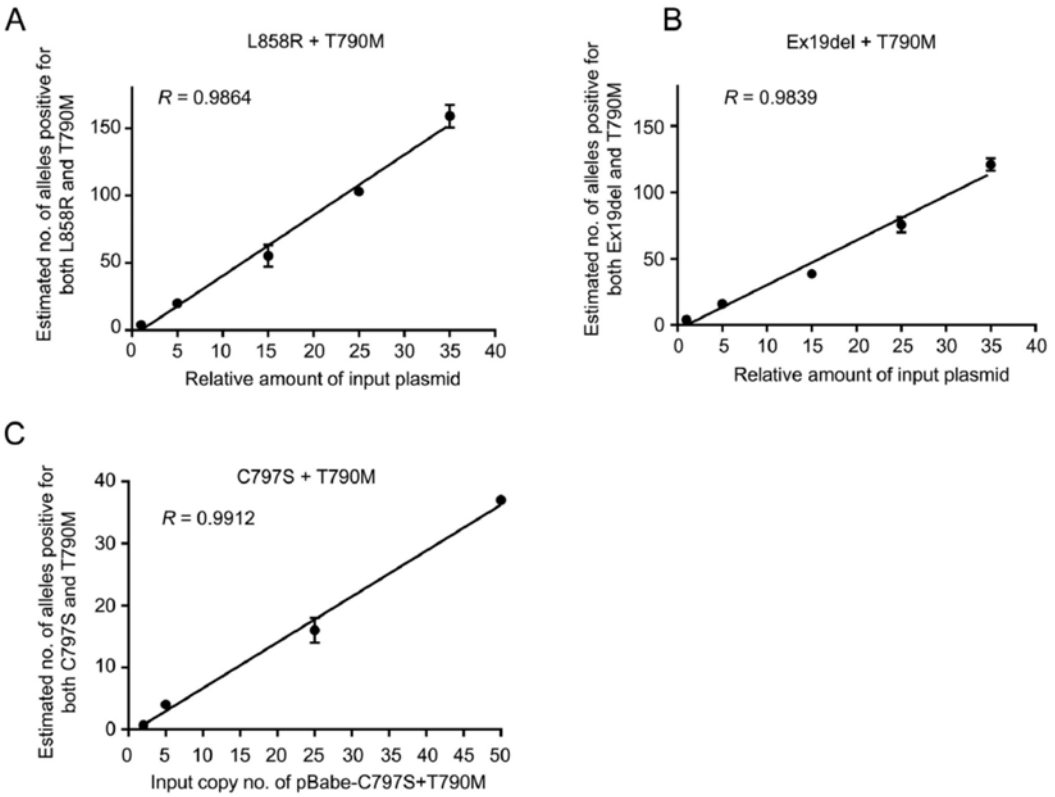
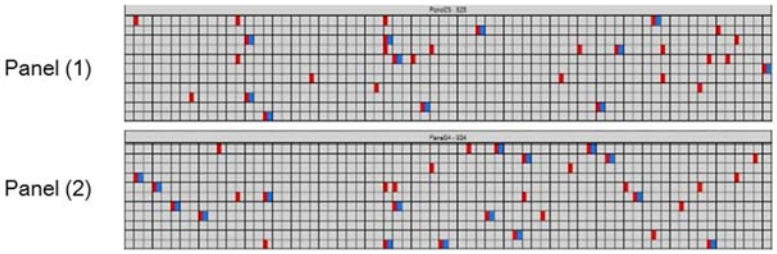


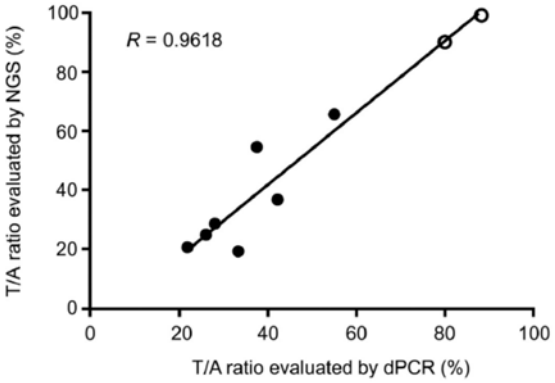
Figure 3.



Estimate number of target alleles (by Poisson distribution)

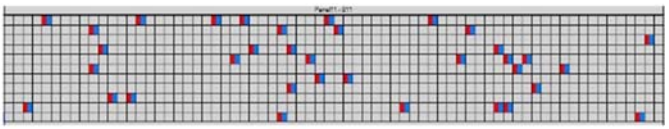
	Ex 19 del	T790M	Ex 19 del + T790M (cis)
Panel (1)	31	11	11
Panel (2)	33	16	16
Total	64	27	27

Figure 4.



Supplementary Figure S1.

A



B

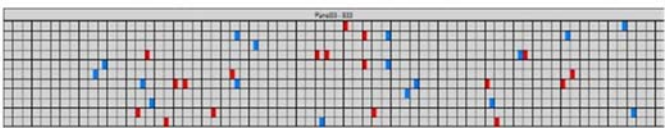


Table 1. Evaluation of the positional relation between activating mutations (AMs) and T790M of *EGFR* by dPCR and NGS.

		dPCR analysis					NGS analysis							
		Estimate no. of target alleles applied to panels (1) + (2)			Ratio of estimated no. of alleles (%)		No. of reads (%) by NGS				Estimated no. of alleles		Ratio of estimated no. of alleles (%)	
Patient no. or cell line	Type of AM	AM	T790M	Cis	T/A	C/T	A+T+ (cis)	A+T–	A–T+	A–T–	AM	T790M	T/A	C/T
1	L858R	25	20	18	80.0	90.0	1148 (36.3)	281 (8.9)	140 (4.4)	1592 (50.4)	1429	1288	90.1	89.1
2	L858R	60	33	32	55.0	97.0	208 (27.4)	123 (16.2)	9 (1.2)	420 (55.3)	331	217	65.6	95.9
3	L858R	55	12	11	21.8	91.7	4144 (15.6)	16,879 (63.6)	170 (0.6)	5338 (20.1)	21,023	4314	20.5	96.1
4	L858R	24	9	9	37.5	100	1085 (22.2)	1081 (22.1)	96 (2.0)	443 (3.9)	2166	1181	54.5	91.9
5	L858R	25	7	7	28.0	100	82 (11.2)	223 (30.6)	5 (0.7)	419 (57.5)	305	87	28.5	94.3
6	Ex19del[a]	64	27	27	42.2	100	3993 (35.0)	6952 (60.9)	22 (0.2)	443 (3.9)	10,945	4015	36.7	99.5
7	Ex19del[a]	102	34	33	33.3	97.1	933 (14.5)	4058 (63.0)	26 (0.40)	1421 (22.1)	4991	959	19.2	97.3
8	Ex19del[b]						1518 (33.9)	1771 (39.6)	21 (0.5)	1162 (26.0)	3289	1539	46.8	98.6
9	Ex19del[c]						1380 (21.5)	1719 (26.8)	74 (1.2)	3234 (0.5)	3099	1454	46.9	94.9
10	Ex19del[c]						1825 (37.6)	1964 (40.5)	35 (0.7)	1031 (21.2)	3789	1860	49.1	98.1
H1975	L858R	60	53	51	88.3	96.2	3164 (73.7)	185 (4.3)	158 (3.7)	784 (18.3)	3349	3322	99.2	95.2
PC9-GR	Ex19del[a]	77	20	20	26.0	100	2740 (20.5)	8479 (63.3)	44 (0.3)	2126 (15.9)	11,219	2784	24.8	98.4

[a]Ex19del [E746–A750 (nucleotides 2235–2249)], [b] Ex19del [E746–A750 (nucleotides 2236–2250)], [c] Ex19del (other deletion type). Samples obtained before treatment with EGFR-TKIs (harboring a de novo T790M mutation) are shaded gray.

Supplementary Table S1. Primer and probe sequences for the present study.

Target	Primer	Sequence
Primers for mutagenesis		
L858R	Fw	5'-TCACAGATTTTGGGCGGGCCAAACTGCTGGG-3'
	Rev	5'-CCCAGCAGTTTGGCCCGCCCAAAATCTGTGA-3'
C797S (T2389A)	Fw	5'-CATGCCCTTCGGCAGCCTCCTGGACTA-3'
	Rev	5'-TAGTCCAGGAGGCTGCCGAAGGGCATG-3'
Primers for initial amplification of EGFR		
Exons 18–23	Fw	5'-AGATCAAAGTGCTGGGCTCC-3'
	Rev	5'-CCCGTAGCTCCAGACATCAC-3'
Primers for detection of target mutations by dPCR		
Ex19del + T790M	Fw	5'-GCGTTCGGCACGGTGTATA-3'
	Rev	5'-GTCTTTGTGTTCCCGGACATAGTC-3'
L858R + T790M	Fw	5'-CGCCTGCTGGGCATCTG-3'
	Rev	5'-ACTTTGCCTCCTTCTGCATGG-3'
C797S + T790M	Fw	5'-CGCCTGCTGGGCATCTG-3'
	Rev	5'-GTCTTTGTGTTCCCGGACATAGTC-3'
Allele-specific probes for detection of target alleles by dPCR		
Ex19del		5'-FAM-CGCTATCAAAACATCTCCGA-MGB-3'
L858R		5'-FAM-AGTTTGGCCCGCCCAA-MGB-3'
T790M		5'-VIC-ATGAGCTGCATGATGAG-MGB-3'
C797S (T2389A)		5'-FAM-TTCGGCAGCCTCC-MGB-3'

MGB, Minor Groove Binder

Supplementary Table S2. Specificity of dPCR for the detection of various types of *EGFR* mutation.

Input copy number for pBabe- EGFR-WT	Estimated no. of target alleles			
(n = 3)	Ex19del	L858R	C797S	T790M
1.0×10^3	0	0	0	0
1.0×10^4	0	0	0	0
1.0×10^5	0	0	0	0

Supplementary Method

PCR protocols for initial amplification of *EGFR*

The PCR mixture included 1 µl of a 1/10 dilution of cDNA, 0.2 mM of each deoxynucleoside triphosphate, 1.5 mM $MgSO_4$, 300 nM of each primer (Ex18-23 Fw and Rev), and 0.4 U of KOD-Plus-Ver.2 (Toyobo, Osaka, Japan) in a total volume of 20 µl. Primer sequences are shown in Supplementary Table S1. The cycling protocol included an initial incubation at 94°C for 2 min followed by 40 cycles of 98°C for 10 min, 50°C for 30 s, and 68°C for 30 s. The PCR products were purified with the use of a QIAquick PCR Purification Kit (Qiagen K.K.), fractionated by agarose gel electrophoresis on a 1% gel, and eluted from the gel with the use of a QIAquick Gel Extraction Kit (Qiagen K.K.). The concentration of the eluted DNA was determined with a Qubit dsDNA HS Assay Kit and Qubit 3.0 fluorometer (Thermo Fisher Scientific K.K., Kanagawa, Japan).

Nanofluidic dPCR analysis

A nanofluidic dPCR system (BioMark HD; Fluidigm) and a Fluidigm digital chip were used for quantitation of target DNA molecules. The digital chip delivers up to 48 mixtures of samples and PCR reagents to 48 individual panels each containing 770 independent 0.84-nl chambers. The PCR mixture for each panel comprised template DNA (PCR products or plasmids), 0.27 µl of 20× sample loading buffer, 2.7 µl of Taqman Universal PCR Master Mix (Thermo Fisher Scientific K.K.), 300 nM of each primer, and 250 nM probe in a total volume of 5.4 µl. The cycling conditions for simultaneous detection of activating and T790M mutations included initial incubations at 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 100 s, whereas those for simultaneous detection of T790M and C797S included initial incubations at 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 60 s.