Application of mutant enrichment technologies to improve the clinical sensitivity of plasma epidermal growth factor receptor testing in non-small cell lung cancer patients

Boyeon Kim

Department of Medicine

The Graduate School, Yonsei University

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Directed by Professor Kyung-A Lee

The Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Doctor of Philosophy of Medical Science

Boyeon Kim

December 2020

This certifies that the Doctoral Dissertation of Boyeon Kim is approved.

Thesis Supervisor: Kyung-A Lee

Thesis Committee Member#1: Jae Yong Cho

Thesis Committee Member#2: Sook Won Ryu

Thesis Committee Member#3: Lark Kyun Kim

Thesis Committee Member#4: Sungsoo Lee

The Graduate School
Yonsei University

December 2020

ACKNOWLEDGEMENTS

First, I would like to express my deepest appreciation and profound respect to my supervisor Prof. Kyung-A Lee for her invaluable guidance, encouragement, and patience throughout my years of study and during the process of writing this thesis.

I would also like to acknowledge the rest of my thesis committee, Prof. Jae Yong Cho, Prof. Sook Won Ryu, Prof. Lark Kyun Kim, and Prof. Sungsoo Lee, for their insightful comments and supervision.

In addition, I am extremely grateful to my dearest Prof. Yoonjung Kim for her ingenious guidance and immeasurable support. Without her advice, I would not have been able to complete this thesis. I would also like to express special thanks to the gifted laboratory technicians Miss. Boram Choi and Miss. Hyunji Kim for their tremendous technical support.

Finally, and most importantly, I would like to express my huge thanks to my caring, loving, and supportive family: my parents, my husband, and my son. It was a great comfort and relief to know that you always stand by my side and give me your unwavering love and encouragement.

Boyeon Kim

December 2020

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ABSTRACT

Application of mutant enrichment technologies to improve the clinical sensitivity of plasma epidermal growth factor receptor testing in non-small cell lung cancer patients

Boyeon Kim

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Epidermal growth factor receptor (*EGFR*)-tyrosine kinase inhibitor (TKI) has provided clinical benefits for non-small cell lung cancer (NSCLC) patients with the EGFR mutation; however, acquired resistance frequently appears after a median period of 8-18 months of TKI treatment. Sensitive detection of the p.Thr790Met (T790M) mutation is particularly important for patients who do not respond to first-line TKI because T790M-targeted therapy can be used as a second-line treatment. Although many technical platforms targeting circulating tumor DNA (ctDNA) are already being implemented in clinical practice, highly fragmented and low quantity ctDNA is an obstacle for detecting *EGFR* mutations in NSCLC patients. Therefore, there is a need for strategies to improve the detection capability for clinically significant mutant alleles with exceptionally low copy number among circulating nucleic acids.

Recently, a clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein 9 (cas9) system was introduced to the molecular diagnostic field as a mutant enrichment method. Here, we report a new mutant enrichment technology, CRISPR system combined post-polymerase chain reaction (PCR) cell-free DNA (cfDNA) (CRISPR-CPPC) to detect T790M mutation from the cfDNA of NSCLC patients with extremely low mutant allele

copies (<10 copies/mL).

The CRISPR-CPPC process is comprised of the following three steps: (1) cfDNA PCR, (2) assembly of post-PCR cfDNA and cas9 complex, and (3) droplet digital PCR (ddPCR). We preformed optimization and validation of CRISPR-CPPC using reference cfDNA materials and cfDNA from NSCLC patients who underwent TKI therapy. Then, we compared the detection sensitivity of CRISPR-CPPC with the results of real-time PCR (qPCR), and with the results of ddPCR without CRISPR-CPPC.

Using CRISPR-CPPC, T790M mutant copies were sensitively detected by ddPCR, achieving about 13-fold increase in detected allele frequency. CRISPR-CPPC can detect T790M with 93.9% sensitivity and 100% specificity in patients with a progressive disease.

When tested to patients with a progressive disease, CRISPR-CPPC's performance is exceptionally higher than other currently available methods. This technology can be used to confirm the result of qPCR, which may facilitate selection of optimal treatment strategies, and provide extra opportunities to patients to receive T790M-targeted therapy.

Key words: cell-free DNA, *EGFR* gene, liquid biopsy, CRISPR-Cas systems, non-small cell lung cancer

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I. INTRODUCTION

Worldwide, the incidence of lung cancer continues to increase, and it is the most common cause of cancer death.[1] Non-small cell lung cancer (NSCLC), a heterogeneous class of tumors, accounts for 85% of all lung cancers.[2] The discovery of activating mutations in the tyrosine kinase (TK) domain of the epidermal growth factor receptor (*EGFR*) gene has led to the development of EGFR-Tyrosine kinase inhibitor (TKI) resulting in better treatment outcomes in NSCLC patients. The EGFR is a transmembrane protein that belongs to a family of receptor TKs and is highly expressed in epithelial tumors, including lung cancer. When EGFR is stimulated, the transmembrane receptors trigger a cascade of intracellular signaling which affects cellular proliferation, angiogenesis, and apoptosis.[3,4]

EGFR-TKI mutations are present in 10-17% of NSCLC patients in North America and Europe, and up to 50% in Asia.[5,6] Activating *EGFR* mutations are found in exon 18 through 21 of the TK domain.[7] The most common activating mutations (termed 'classical mutations') in the *EGFR* gene are exon 19 deletions and p.Leu858Arg (L858R) point mutation in exon 21.[8] Reported uncommon

activating *EGFR* mutations include p.Gly719XXX (G719X), a point mutation of substituting the glycine at position 719 to other residues such as alanine, cysteine, and serine in exon 18; p.Ser768Ile (S768I) a point mutation in exon 20; and p.Leu861Gln (L861Q), a point mutation in exon 21.[9,10]

Treatment with an EGFR-TKI is recommended when *EGFR* activating mutations are detected in patients with advanced or metastatic NSCLC.[11] Compared with platinum-based chemotherapy, first-generation TKIs, gefitinib (Iressa®, AstraZeneca Pharmaceuticals, London, United Kingdom) and erlotinib (Tarceva®, F. Hoffmann-La Roche, Basel, Switzerland); and second-generation TKI, afatinib (Giotrif®, Boehringer Ingelheim, Germany), have demonstrated improved progression-free survival (PFS) and quality of life in patients with *EGFR*-mutant NSCLC.[12-14]

Despite these promising outcomes, acquired resistance frequently appears after a median period of 8-18 months of TKI treatment.[14] The p.Thr790Met (T790M) point mutation in exon 20 of *EGFR* is the most common resistance mutation. About 50-60% of secondary resistance to primary EGFR-TKI therapy is caused by acquired T790M mutation.[15] Several studies have reported *EGFR*-T790M as a secondary *EGFR* resistance mutation, as well as a de novo mutation, arising from pretreatment of TKIs.[16,17]

Sensitive detection of T790M mutation is important, particularly for patients who received first-line TKI but show PD, because third-generation EGFR-TKIs can be used as a second-line treatment.[18,19] Generally, more than ≥10 copies/mL of T790M can be detected by currently available method, but most patients have a low T790M copy number (<10 copies/mL), making T790M difficult to detect.[20] Nevertheless, patients with a low T790M copy number (<10 copies/mL) have a similar response to third-generation EGFR-TKI as those with a higher T790M copy number (≥10 copies/mL).[21] Therefore, developing more sensitive T790M mutation detection method could result in identifying more

patients who can receive third-generation EGFR-TKI treatment.

Many third-generation EGFR-TKIs targeting both EGFR-TKI sensitizing and resistance mutations (T790M) have been developed.[22] Clinical trials are still underway on many third-generation TKIs. Osimertinib (Tagrisso®, AstraZeneca Pharmaceuticals, London, United Kingdom) is currently the most advanced TKI in clinical development. It has shown clearly superior efficacy as a first-line treatment in patients with *EGFR*-mutant NSCLC and in those with de novo *EGFR* T790M in pretreatment tumors.[19,23-26] Furthermore, osimertinib resulted in tumor regression in NSCLC patients with central nervous system metastases, due to its ability to penetrate the blood brain barrier.[25,27,28] Recently, the FLAURA trial reported a better PFS for osimertinib compared to erlotinib or gefitinib as a first-line treatment for NSCLC patients with L858R or exon 19 deletions.[26] Based on these results, osimertinib may be considered as a standard treatment for metastatic NSCLC patients with *EGFR*-sensitizing mutations.[29]

In patients with *EGFR*-sensitizing mutations receiving third-generation TKIs as first-line treatment, testing for T790M resistance mutation may be obsolete. However, testing is particularly important in NSCLC patients pre-first- or second-generation TKI treatment because if T790M is detected, further options for use of third-generation TKIs become available. Furthermore, for patients without TKI treatment with T790M, further options for use of third-generation TKIs become available and unnecessary first- or second-line TKI treatments can be avoided. Moreover, health insurance in many countries, including South Korea, does not cover the use of third-generation TKIs unless T790M is detected. Therefore, sensitive detection of T790M is crucial.

It is often difficult to obtain a tissue biopsy from patients with advanced stage NSCLC due to poor patient condition or tumor localization, and for other reasons. Even if the tissue is acquired, insufficient sample size and lesion heterogeneity makes molecular analysis challenging. Liquid biopsy for detecting

circulating tumor DNA (ctDNA) has been most commonly implemented for detecting *EGFR* mutation in NSCLC.[30] Currently, real-time polymerase chain reaction (PCR), droplet digital PCR (ddPCR), and next-generation sequencing (NGS) are available to detect *EGFR* mutation in ctDNA. While real-time PCR (qPCR) is currently widely used in the clinical setting for its ease-of-use and relatively low cost, but its detection ability for low copy *EGFR*-mutant is lower than ddPCR and NGS.[31] NGS and ddPCR have been known as highly sensitive techniques for detecting mutant allele.[32-34] However, NGS is labor intensive and time-consuming, despite its availability for simultaneous detection of multiple gene mutations.

Watanabe et al. reported the experience of detecting T790M from formalin-fixed paraffin-embedded tumor tissue using ddPCR, and showed that approximately 80% of pretreatment NSCLC patients were T790M mutant-positive. This indicates the mutant allele frequency is below 0.1%, which is very difficult to detect by qPCR.[35] Therefore detection of low copy EGFR-mutant should be achieved using different techniques.

Although many technical platforms targeting ctDNA are already being implemented in clinical practice, highly fragmented and low quantity ctDNA is an obstacle for detecting *EGFR* mutations in NSCLC patients.[36] The currently available platforms do not produce sufficiently reliable results in NSCLC cell-free DNA (cfDNA) samples with scant *EGFR*-mutant copies. Mutations with less than 0.1% allele frequency can be randomly detected with the current techniques, but this approach is unreliable.[37] Therefore, there is a need for strategies to improve the detection capability for clinically significant mutant alleles with exceptionally low copy number among circulating nucleic acids.[33]

Recently, a clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein 9 (Cas9) system was introduced to the molecular diagnostic field as a mutant enrichment method. The enriched ctDNAs

obtained using the CRISPR-Cas9 system demonstrated increased number of mutant allele copies compared to those of conventionally extracted ctDNA.[38,39] The active CRISPR-Cas9 is a versatile and precise tool for gene editing and gene-targeting.[40] The final 20 base pairs of the single guide RNA (sgRNA) can be designed to target sites that contain protospacer-adjacent motif (PAM) without any significant cross-reactivity or off-target effects.[41,42]

Some approaches using the CRISPR-Cas9 system have increased the analytical sensitivity of detecting targeted mutation by specifically cleaving the wild-type DNA sequences.[43,44] For the first time, here, we report a new mutant enrichment technology called CRISPR system combined post-PCR cfDNA (CRISPR-CPPC) to detect T790M mutation from samples with extremely low mutant allele copies extracted from NSCLC patients.

II. MATERIALS AND METHODS

1. Study design

We developed new mutant enrichment technology, CRISPR-CPPC, and optimized it to increase diagnostic sensitivity. We validated CRISPR-CPPC with reference materials with mutant alleles and cfDNA of NSCLC patients who had clinically progressed during or after EGFR-TKI. The sensitivity of CRISPR-CPPC for T790M detection was analyzed by comparing CRISPR-CPPC results to ddPCR without CRISPR-CPPC results. A study flow chart is shown in Figure 1. The CRISPR-CPPC is comprised of the following three steps: (1) cfDNA PCR, (2) assembly of post-PCR cfDNA and cas9 complex, and (3) ddPCR. A schematic representation of CRISPR-CPPC and sgRNA target positions is shown in Figure 2.

2. Patients

A total of 60 samples were collected from 51 patients who required *EGFR* gene mutation test using Roche cobas® EGFR Mutation Test v2 (Roche Molecular Systems, Pleasanton, CA, USA). The patients were in two hospitals: Gangnam Severance Hospital and Severance Hospital located in Seoul, South Korea, from June 2018 to October 2020. Only patients with advanced *EGFR*-mutated NSCLC who had clinically progressed during or after at least one first- or second-generation EGFR-TKI treatment cycle were included. Eight patients had one or two follow-up *EGFR* gene mutation tests. For all patients, *EGFR* genotyping was performed from the initial tissue biopsy taken at diagnosis. The study was approved by the Institutional Review Board of Gangnam Severance Hospital (IRB no. 3-2019-0393) and Severance Hospital (IRB no. 1-2019-0092). All patients provided general informed written consent for specimen collection and genetic analysis. The need for informed consent of the participants for reviewing medical records was waived on the condition that the research involves no more

than minimal risk to the patients and the patient's privacy.

3. Preparation of cfDNA

Blood samples (8 mL each) collected in either vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA) or cfDNA collection tubes with cell stabilizer, Cell-Free DNA BCT (Streck, La Vista, NE, USA). Plasma was prepared from blood collected in EDTA tubes within 2 hours (hr) of collection. Cell-Free DNA BCT could be stored for up to 7 days in room temperature due to the stability of cfDNA. Blood samples were centrifuged at 1,600 g for 10 minutes (min) followed by a second high-spin centrifugation at 16,000 g for 10 min to separate the plasma from the peripheral blood cells. Plasma supernatant was stored at -80℃ until cfDNA extraction. The MagMAX Cell-Free DNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to extract cfDNA. The Concentration and size distribution of nucleic acid were assessed by using a 2200 TapeStation Instrument (Agilent Technologies, Santa Clara, CA, USA) with the Agilent High Sensitivity D1000 ScreenTape System (Agilent Technologies, Santa Clara, CA, USA).

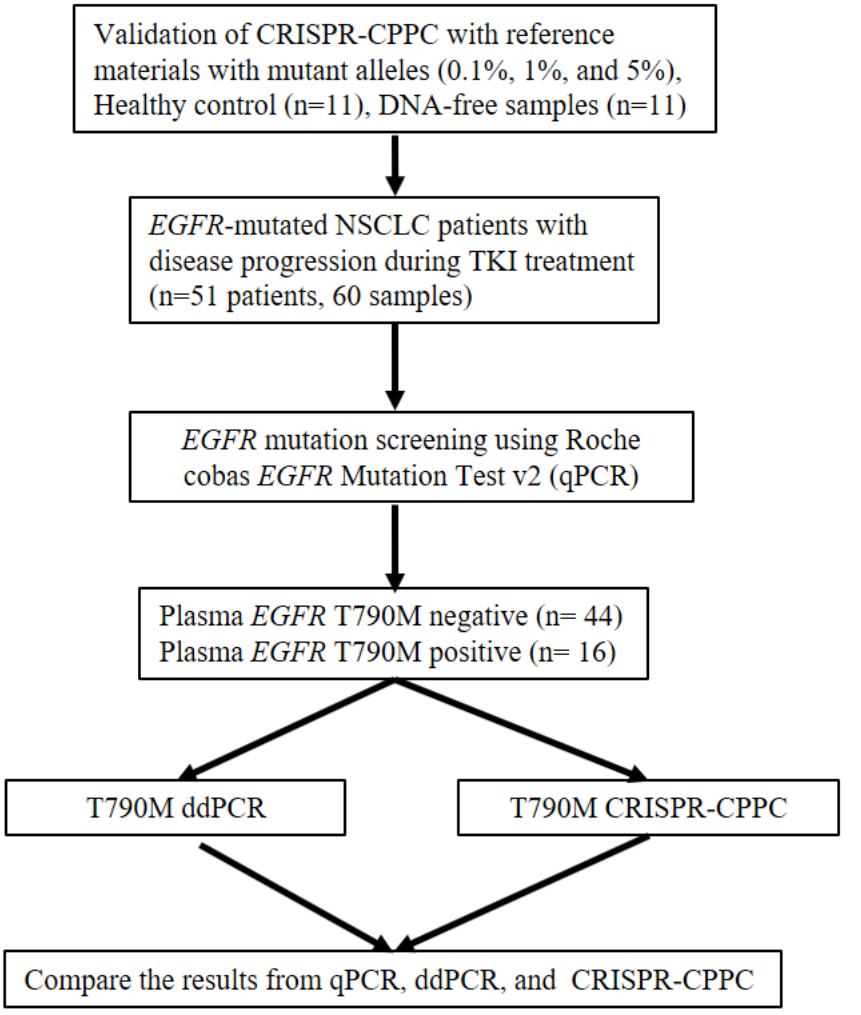
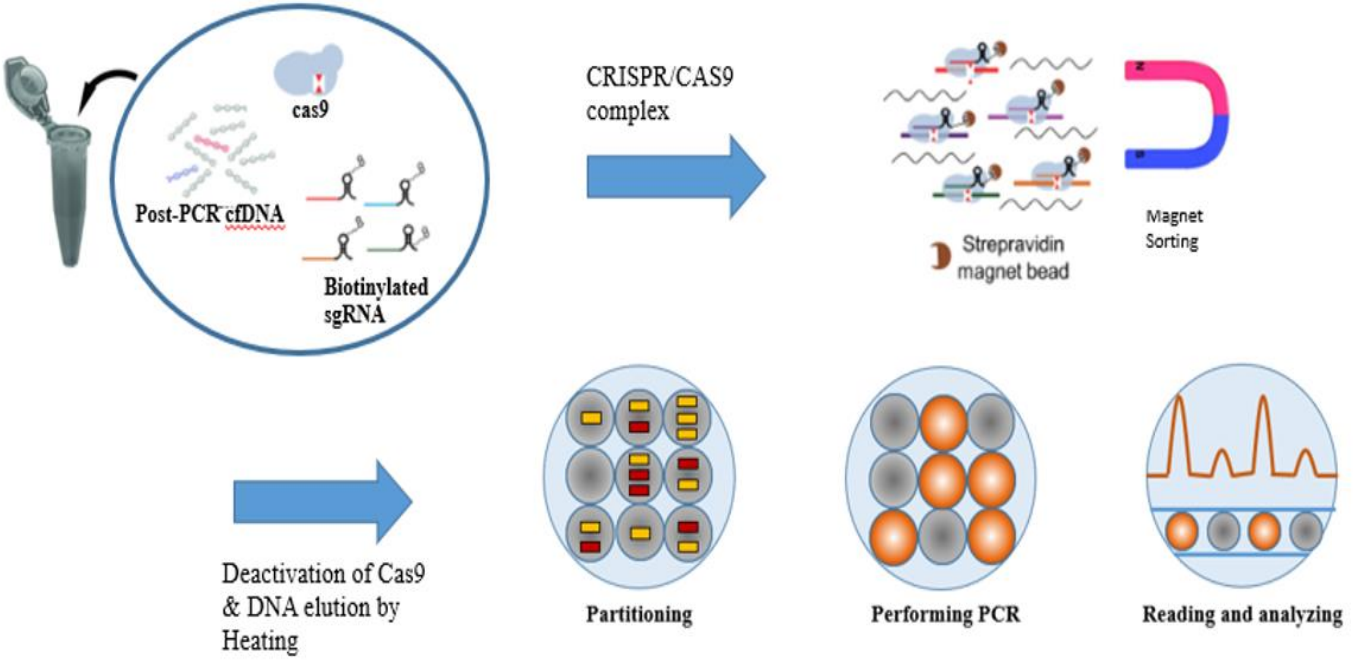


Figure 1. Study flow chart

Abbreviations: CRISPR-CPPC, a clustered regularly interspaced short palindromic repeats system combined post-PCR cell free DNA; ddPCR, droplet digital PCR; EGFR, epidermal growth factor receptor; NSCLC, Non-small cell lung cancer; PCR, polymerase chain reaction; qPCR, real-time PCR; TKI, tyrosine kinase inhibitor;

(A)



(B)

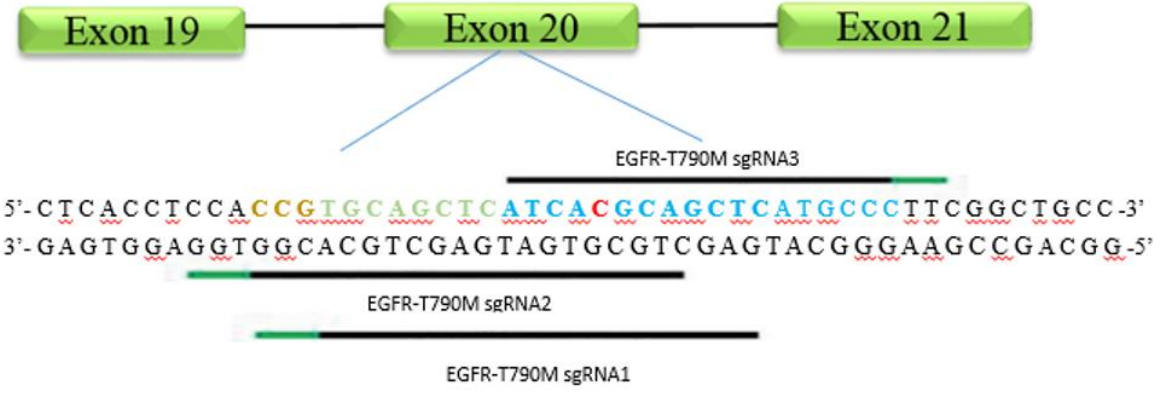


Figure 2. Schematic diagram of CRISPR-CPPC and sgRNA target positions. (A) Schematic diagram of CRISPR-CPPC procedure. In the assembly step, Cas9, a biotinylated sgRNA, and post-PCR cfDNA are mixed and incubated until magnetic sorting is applied. After binding to streptavidin magnetic beads, cas9 is deactivated by heating, and target cfDNA is released from the complexes. (B) Schematic illustration of the CRISPR target site around the human *EGFR* T790 locus. The three tested target sequences are indicated by horizontal lines. Protospacer-adjacent motifs (PAMs) are marked in green.
Abbreviations: sgRNA, single guide RNA; Cas9, CRISPR-associated protein 9; cfDNA, cell-free DNA

4. Optimization of CRISPR-CPPC

A. Biotinylated sgRNA construction

We designed the T790M primer sets for sgRNA and cfDNA PCR. The primer information is presented in Table 1. The SgRNA template was synthesized and purified using the GeneArt™ Precision gRNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions, with the exception that the incubation time was elongated to 4 hr for in vitro gRNA transcription. The yield of sgRNA was measured by using the Qubit RNA BR Assay kit (Thermo Fisher Scientific, Waltham, MA, USA) to confirm its yield was within the 10-40 ug range. The 3' end of sgRNA was biotinylated using the Pierce™ RNA 3' End Biotinylation Kit (Thermo Fisher Scientific, Waltham, MA, USA). Reactions were incubated overnight at 16℃ to increase efficiency.

B. The cfDNA PCR

Cell-free DNA samples were processed with PCR, before reacting with CRISPR-CAS9. Two *EGFR* T790M primers were designed and compared (Table 1) for optimization of CRISPR-CPPC. PCR conditions were as follows: 5 min at 95℃, followed by 35 repeated cycles of 95℃ for 30 s, 62℃ for 30 s, and 72℃ for 1 min.

C. CRISPR/CAS9 complex with post-PCR cfDNA

A CRISPR/Cas9 complex was made using Cas9 Nuclease, *S. pyogenes* (New England Biolabs, Ipswich, MA, USA) and the PCR product of the cfDNA samples. Cas9-sgRNA complexes were formed by mixing biotinylated sgRNA and Cas9 protein at a 5:1 ratio with NEB3 buffer, and incubating at room temperature for 10 min. Complexes were then incubated at a final concentration of 100 nmol/L with 0.5 ng post-PCR cfDNA at 37℃ for 2 hr in a thermocycler. Afterwards, Cas9 complexes containing the target DNA was bound to the the

Dynabeads® MyOne™ Streptavidin C1 superparamagnetic beads (Thermo Fisher Scientific, Waltham, MA, USA) and released by heating to 65℃.

5. The ddPCR assay

The number of T790M mutant copies in cfDNA samples before and after CRISPR-CPPC were quantified by using ddPCR with the PrimePCR ddPCR Mutation Detection Assay kit (Bio-Rad, Hercules, CA, USA) according to manufacturer's instructions. Amplification was carried out in a reaction volume of 20 uL on a QX100 Droplet Digital PCR System (Bio-Rad, Hercules, CA, USA). The PCR mix was composed of 10 uL Bio-Rad Super mix TaqMan, 2 uL of T790M primer/probe mix, and 8 uL of post-CRISPR-CPPC cfDNA. The thermal cycling conditions were as follows: 10 min at 95℃, followed by 40 repeated cycles of 95℃ for 30 seconds (s) and 55℃ for 60 s. Results were analyzed with Quantasoft v.1.7.2 software (Bio-Rad, Hercules, CA, USA).

6. Validation of CRISPR-CPPC

Before using CRISPR-CPPC for patient sample cfDNA the method was validated by using cfDNA from Multiplex I cfDNA Reference Standards (Horizon Discovery, Cambridge, United Kingdom) which included wild-type cfDNA with mutant allele frequencies of 5%, 1%, and 0.1%. Healthy control samples and DNA-free samples were also analyzed. After performing the entire process of CRISPR-CPPC, ddPCR was implemented.

7. Data analysis

Quantification of the number of T790M molecules in the reaction was achieved by counting the number of positive and negative droplets. The limit of blank (LOB) was determined by the frequency of positive droplets measured in standard deviation (SD) of DNA-free samples, and determined using the

following equation: $Mean_{copy\ number\ blank} + 1.645 \times SD_{copy\ number\ blank}$. Then the limit of detection (LOD) was defined as the lowest copy number concentration that could be distinguished from the LOB with a 95% confidence interval (CI) of the wild type control using the following equation: $LOD = LOB + 1.645 \times SD_{low\ mutant\ copy\ number\ sample}$. Because the number of PCR-positive droplets was below 20, the 95% CI was determined using the Poisson model [45-47]. The ddPCR assays were considered positive if the measured event was ≥2 events/assay and negative if the events within a gated region were <2 events/assay.[48]

8. Statistical analysis

Statistical analysis was performed using Microsoft Excel 2016 (Microsoft Corporation, Redmond, WA, USA). Data are presented using a 95% CI and 2-sided *P* value. P values < 0.05 were considered to be statistically significant.

Table 1. *EGFR* T790M primer information

sgRNA*	Primer	forward	5′-TAATACGACTCACTATAGATCATGCAGCTCATGCCC-3′
		reverse	5′- TTCTAGCTCTAAAACAAGGGCATGAGCTGCATGAT-3′
cfDNA PCR†	Primer 1	forward	5′-CATGCGAAGCCACACTGAC-3′
		reverse	5′- CGGACATAGTCCAGGAGGCA-3′
	Primer 2	forward	5′-CTCCAGGAAGCCTACGTGAT-3′
		reverse	5′- GTCTTTGTGTTCCCGGACAT-3′

*EGFR T790M primer for sgRNA

† Primer set for cfDNA PCR. Expected product size is 164 and 144 for primer 1 and primer 2, respectively.

Abbreviation: cfDNA, cell-free DNA; PCR, polymerase chain reaction; sgRNA, single guide RNA

III. RESULTS

1. Patient characteristics

Patient characteristics are described in Table 2. The median age was 62 years old (range, 39-83 years), and 36 patients (70.6%) were female. Thirty patients (58.8%) had exon 19 deletion; 18 patients (35.3%) had L858R point mutation, two patients (3.9%) had S768I point mutation, one patient (2.0%) had L861Q point mutation, and one patient (2.0%) had G719S point mutation. Ten patients (19.6%) received erlotinib therapy, 13 (25.5%) received afatinib, and 27 (52.9%) received gefitinib therapy. One patient (2.0%) received gefitinib and erlotinib therapies at different points in time.

2. Optimization of CRISPR-CPPC

A. cfDNA PCR and ddPCR

We designed two different T790M primers for use during cfDNA PCR. We compared the applicability of these primers to select the best one. As shown in Figure 3, only T790M positive patient samples were well amplified by both primers. Therefore, both primers could be used for CRISPR-CPPC.

To set the ddPCR condition, we used patient samples with T790M mutation with a semiquantitative index (SQI) value of 17.2 measured with Roche cobas® EGFR Mutation Test v2 (Roche Molecular Systems, Pleasanton, CA, USA). Both primers were used for cfDNA PCR. When the cfDNA of post-CRISPR-CPPC was quantified with ddPCR, it was difficult to separate false positive and true positive, so we diluted the cfDNA of post-CRISPR-CPPC 10^3 times, 10^4 times, and 10^5 times. As shown in Figure 4, higher dilution led to a wider interval between false positive and true positive. To confirm this phenomenon, we used the same patient sample but only used primer 2 for cfDNA PCR and diluted the post-CRISPR-CPPC cfDNA 100 times and 1000 times for ddPCR. The ratio of positive copies to wild type copies were almost the same for

the 100 times and 1000 times diluted ddPCR (Figure 4). Because the patient sample had a high number of T790M mutant copies, different patient samples with one to three mutant copies of T790M were required for testing.

B. Setting optimal quantity of PCR-product for CRISPR-CPPC

The Cas9 Nuclease, *S. pyogenes* (New England Biolabs, Ipswich, MA, USA) protocol specifies that 50~200 ng of DNA should be reacted to cas9 complex. Thus, we diluted the PCR product to around 50 ng first. For this study, we used two patient samples. When the nascent cfDNA of the patient samples with ddPCR were measured, three positive events were detected for patient A and one positive event was detected for patient B (Figure 5.a).

After cfDNA PCR, we reacted PCR product with cas9 complex according to the manufacturer's instructions. However, as shown in Figure 5.b, the true positive and false positive were hardly distinguishable from each other. We then tested different molar ratios of post-PCR cfDNA to Cas9 complex, ranging from 1:4 to 1:400. As shown in Figure 5.c, the more highly diluted PCR product resulted in true positives placed in the upper amplitude. Then we diluted post-CRISPR-CPPC cfDNA 100 times before ddPCR. As shown in Figure 5.d, the most optimal enrichment condition for CRISPR-CPPC appeared to be a 1:400 ratio of post-PCR cfDNA to Cas9 complex. When detected by ddPCR, 100 times diluted CRISPR-CPPC product was recognizable. After discovering the optimal condition, we compared the ratio of sgRNA to Cas9 which can affect the sensitivity of CRISPR-CPPC. While the protocol of Cas9 Nuclease, *S. pyogenes* (New England Biolabs, Ipswich, MA, USA) recommends a 10:10:1 ratio of sgRNA:Cas9:target (Figure 5.e), we found that the best condition of biotinylated sgRNA to cas9 protein was 5:1 ratio in 20 ul of cas9 reaction.

Table 2. Patient characteristics

Characteristics	No. of Patients n= 51 (100%)*
Age (years)	62 (39-83)
Gender	
Female	36 (70.6%)
Male	15 (29.4%)
Tissue EGFR genotyping	
Exon 19 deletion†	30 (58.8%)
L858R†	18 (35.3%)
S768I	2 (3.9%)
L861Q	1 (2.0%)
G719S	1 (2.0%)
Previous EGFR-TKI therapy	
Erlotinib	10 (19.6%)
Afatinib	13 (25.5%)
Gefitinib	27 (52.9%)
>1 EGFR-TKIs	1 (2.0%)

Abbreviation: TKI, tyrosine kinase inhibitor

*Percentages may not total 100% because of rounding

† 1 patient had both exon 19 deletion and L858R

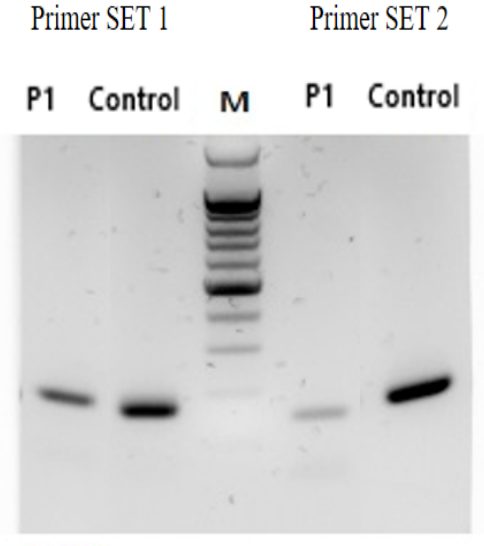
(a) **Primer set 1: PRODUCT SIZE: 164**

		TM
LEFT PRIMER	catgcgaagccacactgac	61.49
RIGHT PRIMER	CGGACATAGTCCAGGAGGCA	63.49

Primer set 2: PRODUCT SIZE: 144

		TM
LEFT PRIMER	ctccagGAAGCCTACGTGAT	59.31
RIGHT PRIMER	GTCTTTGTGTTCCCGGACAT	59.83

(b)



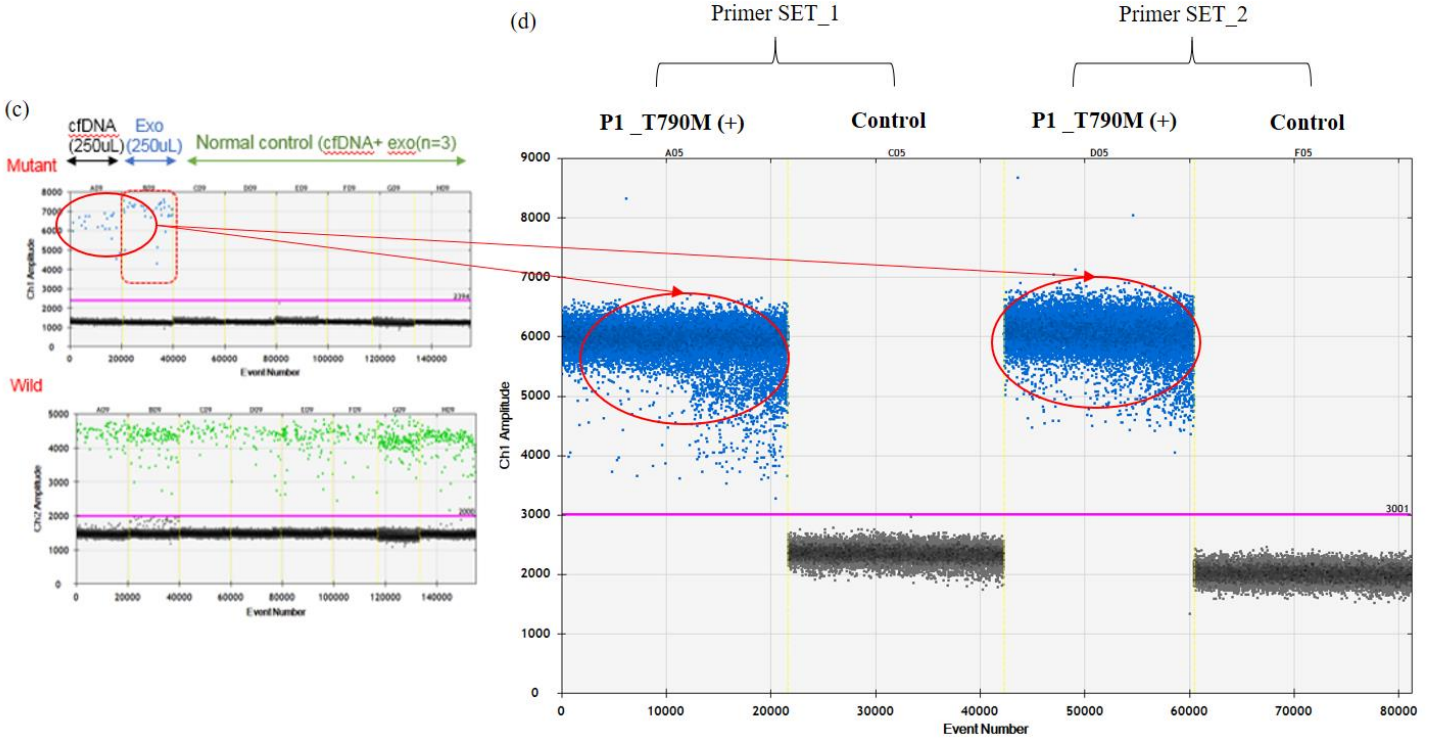


Figure 3. Comparison between T790M primer 1 and primer 2: (a) Primer 1 and Primer 2 information (b) PCR band of patient cfDNA and healthy control (c) Pre-CRISPR-CPPC T790M mutant patient cfDNA was measured with ddPCR, and positive

signals are shown at amplitude ~6000. (d) Post-CRISPR-CPPC T790M mutant patient cfDNA and healthy control were measured with ddPCR. The number of mutant copies placed at the same amplitude ~6000 was increased.

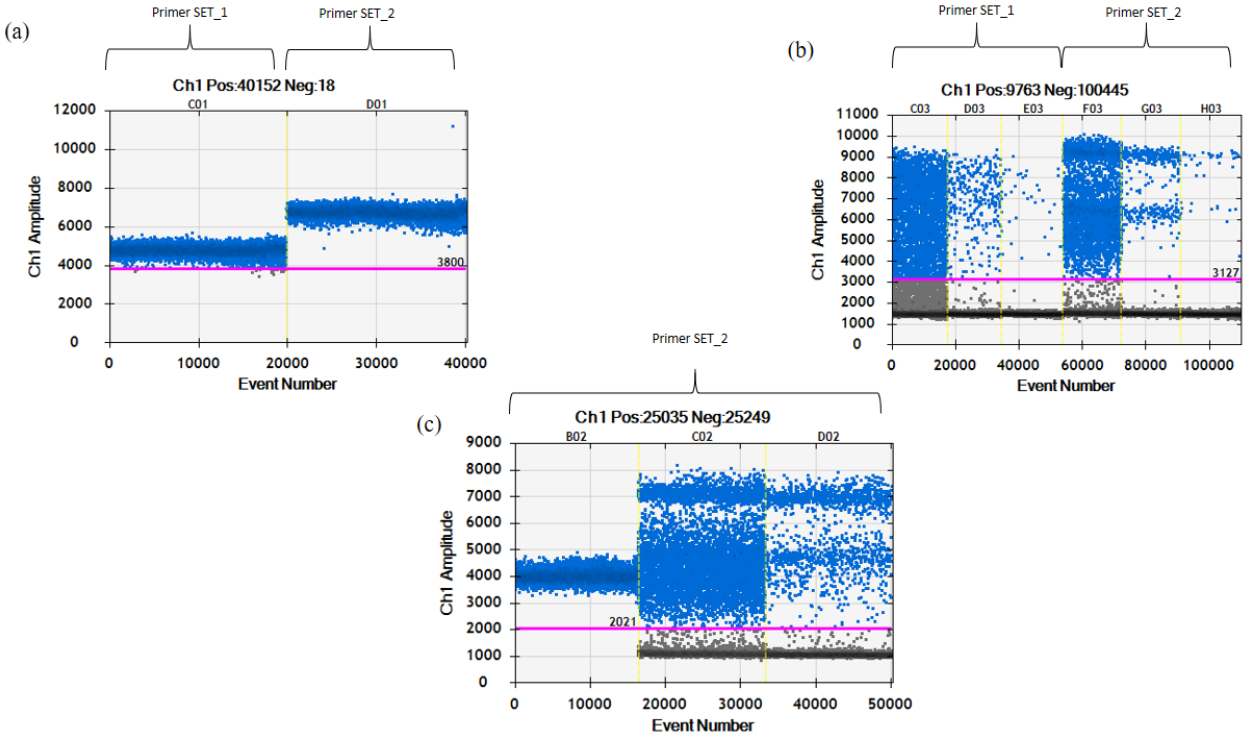


Figure 4. Comparison of different ddPCR conditions: **(**a) cfDNA of patient sample (24% of T790M mutants, SQI 17.2) with CRISPR-CPPC using primer 1 and primer 2 (b) Post-CRISPR-CPPC cfDNA with ddPCR at $10^3\times$,$10^4\times$, and $10^5\times$ dilution (c) Post-CRISPR-CPPC cfDNA with ddPCR at 100× and 1000× dilution

(a)

	T790M SQI*	T790M ddPCR (ctDNA)			T790M ddPCR (exo-NA)		
		Postive	Wild	Alle frequency (%)	Positive	Wild	Alle frequency (%)
Patient A	-	3	560	0.53	2	557	0.36
Patient B	8.45	1	238	0.42	6	199	2.93

* SQI was measured with Roche cobas® EGFR Mutation Test v2

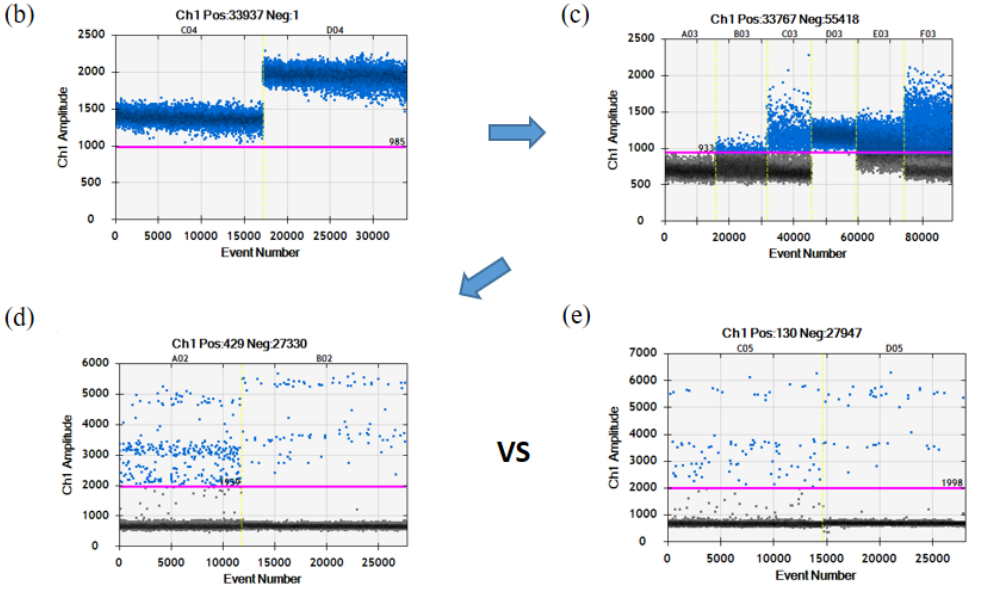


Figure 5. Discovering optimal conditions for CRISPR-CPPC using T790M positive patient samples (1) 385.4 ng/ul, 0.5% (2)

384.1 ng/ul, 0.4%): (a) Patient information (b) 50 ng DNA with ddPCR (c) 38 ng, 3.8 ng, 0.38 ng DNA with ddPCR (d) 50pmol sgRNA + cas9 + 0.38ng DNA with ddPCR at 100× dilution (e) 10:10:1 ratio of sgRNA:cas9:0.38ng DNA with ddPCR at 100× dilution

3. Assessment of LOB, LOD and sensitivity of CRISPR-CPPC

Table 3 shows the raw data for LOB and LOD analysis. From this data, CRISPR-CPPC assays were considered positive if the measured events were ≥6 events/assay and negative if the events within a gated region were <6 events/assay.

The analytical sensitivity of the CRISPR-CPPC was evaluated using Multiplex I cfDNA Reference Standard with allele frequencies of 5%, 1%, and 0.1% (Horizon Discovery, Cambridge, United Kingdom). The expected copy number of mutant alleles (3-109 copies) and actual copy number of mutant alleles observed in these samples are presented in Table 4. Positive detections of mutant DNA after CRISPR-CPPC were around two to six times higher than the expected copies of mutant DNA. Because of mutant enrichment, the allele frequency was about two to four times higher than the expected allele frequency.

Table 3. The LOB and LOD of CRISPR-CPPC on T790M mutation

Number of Samples	Blank*	T790M (-) Healthy control cfDNA†
	Positive events	Positive events
1	0	1
2	0	0
3	0	2
4	0	2
5	0	0
6	0	1
7	0	1
8	0	5
9	0	2
10	0	2
11	0	5
Event	0	21

Mean	0	1.9
SD	0	1.6
LOB (Mean+2*SD)	0	
LOD (CLSI EP17-A2, LOB+1.645*SD)		3
95% CI upper bound (one-tail Poisson distribution)		2.5

*Analysis of post CRISPR-CPPC blank samples without DNA (n=11)

†11 healthy subjects were anonymized and studied as control samples

Abbreviations: cfDNA, cell-free DNA; CI, confidence interval; LOB, limit of blank; LOD, limit of detection; SD, standard deviation

Table 4. Analytical sensitivity of CRISPR-CPPC on *EGFR* T790M mutation

Reference Materials (T790M)	Expected allele frequency (%)*	Expected copies of mutant DNA per sample*	Expected copies of wild-type DNA per sample*	1st ddPCR after CRISPR-CPPC Detection positive (≥6 events/assay)	
				Copies of mutant DNA per sample	Copies of wild-type DNA per sample
5% Multiplex I cfDNA Reference Standard (HD777), 20ng/ul	4.8901	109	2120	231	2409
1% Multiplex I cfDNA Reference Standard (HD778), 20ng/ul	1.0526	24	2256	60	3376
0.1% Multiplex I cfDNA Reference Standard (HD779), 20ng/ul	0.1345	3	2228	19	3842

* Expected allele frequency and copy number of wild-type and mutant DNA measured using ddPCR were provided by the manufacturer. Expected copy numbers of diluted reference materials were calculated.
Abbreviations: cfDNA. Cell-free DNA; CRISPR-CPPC, CRISPR system combined post-PCR cfDNA; ddPCR, droplet digital polymerase chain reaction

4. CRISPR-CPPC for detecting *EGFR* T790M in patient samples

Sixty samples from 51 patients were tested. With the use of Cobas qPCR, T790M was detected from 16 samples and not detected from 44 samples. All samples were tested with ddPCR before CRISPR-CPPC was applied. A comparison of allele frequency and positive calls of these samples are shown in Table 5. Most samples showed about 1.2 to 13 times higher allele frequencies with the use of CRISPR-CPPC. In addition, about 1.6 to 562 times more positive calls were detected with the use of CRISPR-CPPC. The copy number comparison between pairs was statistically significant with a p-value of <0.0001 by Wilcoxon signed rank test.

The T790M positive copy number differences between CRISPR-CPPC and ddPCR are represented in Figure 6. Seven samples with a copy difference below 1 were not expressed on the log-10 scaled y-axis. Figure 6 shows that CRISPR-CPPC increase the T790M positive copy numbers compared to ddPCR except for sample number 47.

Among 51 patients, 8 patients had one or two follow-up *EGFR* gene mutation tests using Roche cobas® EGFR Mutation Test v2. As shown in Table 6, patient E, G, and H had a follow-up test to detect T790M, but qPCR was unable to detect T790M. With the use of ddPCR, 0, 3, 0 positive calls with the respective allele frequencies of 0, 0.3, and 0 were detected. With the use of CRISPR-CPPC in the first sample from patient H, T790M variant was detected with six positive calls with an allele frequency of 0.1. In the second samples from patients E and G, T790M was detected with eight, and nine positive calls and an allele frequency

of 0.2, and 0.3, respectively. These results indicate that patients with exceptionally low copies of T790M may be easily missed when using qPCR or ddPCR.

Table 5. Application of CRISPR-CPPC on patient samples

Sample No.	T790M qPCR	T790M ddPCR (cfDNA) Detection positive (≥2 events/assay)			T790M CRISPR-CPPC (cfDNA) Detection positive (≥6 events/assay)		
	Result	Positive	Wild	Allele frequency (%)	Positive	Wild	Allele frequency (%)
1	Positive	17	293	5.5	224	1257	15.1
2	-	0	423	0.0	5	2336	0.2
3	-	3	560	0.5	353	4570	7.2
4	Positive	1	238	0.4	102	1811	5.3
5	Positive	22	423	4.9	47	2727	1.7
6	Positive	758	1864	28.9	6932	12176	36.3
7	Positive	0	533	0.0	6	1228	0.5
8	Positive	3	430	0.7	7	1556	0.4
9	Positive	5	438	1.1	8	1215	0.7
10	Positive	16	362	4.2	88	2333	3.6
11	-	1	191	0.5	3	3138	0.1
12*	-	3	194	1.5	3	2579	0.1
13	Positive	18	2068	0.9	62	2402	2.5
14	-	0	648	0.0	4	2536	0.2

15	Positive	42	1221	3.3	87	807	9.7
16	-	0	32	0.0	4	683	0.6
17	-	0	401	0.0	2	1870	0.1
18	-	0	590	0.0	5	2071	0.2
19	-	0	602	0.0	4	3796	0.1
20	-	0	146	0.0	4	3036	0.1
21	-	0	412	0.0	8	3589	0.2
22	-	0	1886	0.0	4	872	0.5
23	-	0	5416	0.0	1	1181	0.1
24	-	0	292	0.0	2	3114	0.1
25	-	0	481	0.0	14	2776	0.5
26	-	0	513	0.0	0	1180	0.0
27	-	0	39	0.0	1	736	0.1
28	-	0	29	0.0	4	1012	0.4
29	-	3	1170	0.3	9	2568	0.3
30	-	2	386	0.5	38	2766	1.4
31	-	0	162	0.0	6	3995	0.1
32	-	0	302	0.0	2	1978	0.1
33	-	0	542	0.0	4	4108	0.1
34	-	1	310	0.3	24	3816	0.6
35	-	1	2432	0.0	4	2013	0.2
36	-	0	1111	0.0	5	4803	0.1

37	-	0	134	0.0	0	1326	0.0
38	-	0	155	0.0	5	2460	0.2
39	-	1	207	0.5	2	1440	0.1
40	-	0	172	0.0	12	1394	0.9
41	Positive	51	721	6.6	91	912	9.1
42	-	0	601	0.0	4	8345	0.0
43	-	0	110	0.0	6	5712	0.1
44	-	0	1541	0.0	8	9580	0.1
45	Positive	6	205	2.8	28	1220	2.2
46	Positive	2	114	1.7	158	7316	2.1
47†	-	12	410	2.8	5	4709	0.1
48	-	0	71	0.0	7	2119	0.3
49	-	0	98	0.0	2	3618	0.1
50	Positive	28	762	3.5	319	3713	7.9
51	-	0	112	0.0	2	726	0.3
52	-	0	44	0.0	2	2870	0.1
53	-	0	44	0.0	3	4128	0.1
54	Positive	0	31	0.0	274	3874	6.6
55	-	0	63	0.0	13	7324	0.2
56	-	0	201	0.0	16	7243	0.2
57	-	0	19	0.0	5	3641	0.1
58	-	0	139	0.0	12	6052	0.2

59	-	0	11	0.0	10	5294	0.2
60	Positive	1	137	0.7	562	6343	8.1

* T790M was negative by next generation sequencing (NGS)

† T790M was positive with an allele frequency of 0.2% by NGS

Abbreviations: cfDNA, Cell-free DNA; CRISPR-CPPC, CRISPR system combined post-PCR cfDNA; ddPCR, droplet digital polymerase chain reaction; qPCR, real-time PCR; SQI, semiquantitative index

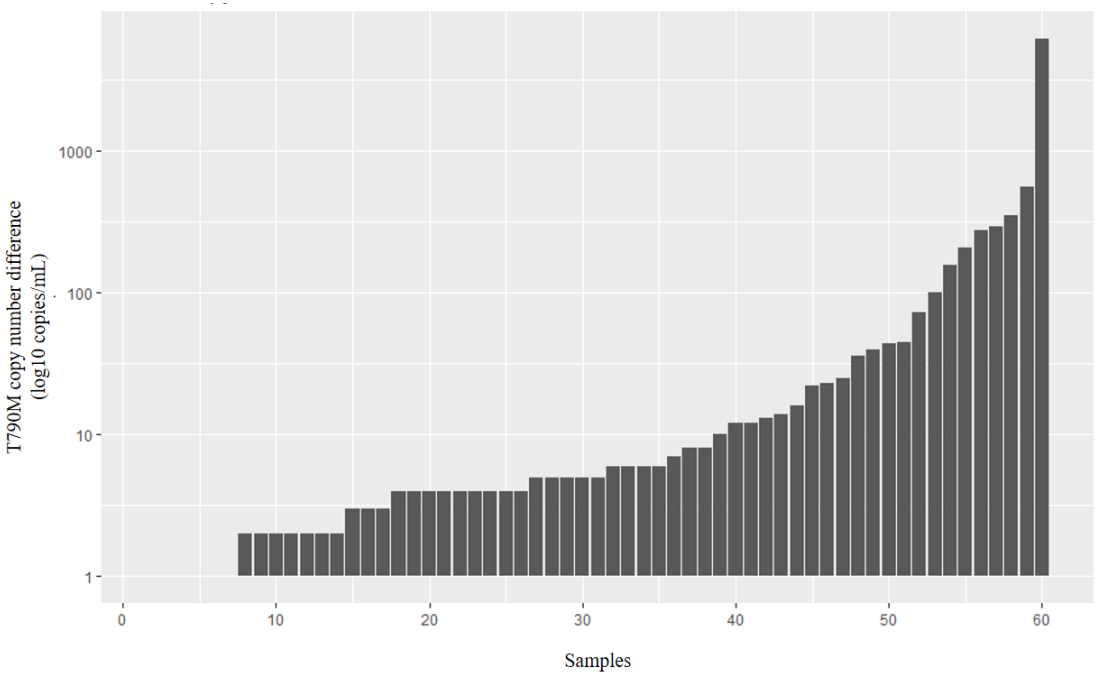


Figure 6. T790M positive copy number differences between CRISPR-CPPC and ddPCR in all 60 samples: CRISPR-CPPC increased the T790M positive copy numbers except for sample number 47. Seven samples with a copy difference below 1 were not expressed on the log-10 scaled y-axis.

Table 6. Application of CRISPR-CPPC on follow-up patient samples

	T790M qPCR	T790M ddPCR (cfDNA)			T790M CRISPR-CPPC (cfDNA)		
	SQI*	Positive	Wild	Allele frequency (%)	Positive	Wild	Allele frequency (%)
Patient A	-	1	191	0.5	3	3138	0.1
Patient A	-	3	194	1.5	3	2579	0.1
Patient A	9.29	18	2068	0.9	62	2402	2.5
Patient B	-	0	648	0.0	4	2536	0.2
Patient B	10.74	42	1221	3.3	87	807	9.7
Patient C	-	0	32	0.0	4	683	0.6
Patient C	-	0	401	0.0	2	1870	0.1
Patient D	-	0	590	0.0	5	2071	0.2
Patient D	-	0	602	0.0	4	3796	0.1
Patient E	-	0	146	0.0	4	3036	0.1
Patient E	-	0	412	0.0	8	3589	0.2
Patient F	-	0	1886	0.0	4	872	0.5
Patient F	-	0	5416	0.0	1	1181	0.1
Patient G	-	0	29	0.0	4	1012	0.4
Patient G	-	3	1170	0.3	9	2568	0.3
Patient H	-	0	110	0.0	6	5712	0.1

Patient H	-	0	1541	0.0	8	9580	0.1

* SQI was measured with Roche cobas® EGFR Mutation Test v2

Abbreviations: cfDNA, Cell-free DNA; CRISPR-CPPC, CRISPR system combined post-PCR cfDNA; ddPCR, droplet digital polymerase chain reaction; qPCR, real-time PCR; SQI, semiquantitative index

5. Diagnostic performance of CRISPR-CPPC

The concordance rate, sensitivity, and specificity of CRISPR-CPPC and ddPCR compared to the results of qPCR are represented in Table 7. Compared to qPCR, CRISPR-CPPC showed 75% concordance rate and 100% sensitivity. Furthermore, CRISPR-CPPC detected T790M variants from 15 samples whose T790M were undetected by qPCR. Table 8 shows that CRISPR-CPPC detected T790M from 16 samples in which T790M was undetected by ddPCR. In two samples, T790M was positively detected by ddPCR but not by CRISPR-CPPC. These two samples underwent further testing by NGS which showed that one sample was T790M positive with an allele frequency of 0.2%, and the other was T790M negative.

Table 9 shows the clinical histories and *EGFR* mutation-detection test results for 18 patients, of whom 16 had positive CRISPR-CPPC tests. Of the 7 patients who received NGS test, 2 tested negative for T790M while 5 tested positive. The qPCR test was run on tissue samples from 2 patients, both of which were positive for T790M. Additional imaging interpretation was conducted on CRISPR-CPPC positive samples to confirm that the T790M detected by CRISPR-CPPC were not false positives. Image interpretation was not conducted on T790M negative samples because their disease may have progressed for other reasons and the image might be changed by something other than T790M.

Samples which tested positive for T790M through two or more of the experimental methods (qPCR from cfDNA or tissue, NGS, ddPCR, and CRISPR-CPPC) were considered to be true positive. Based on the results of multiple assays, the sensitivities of CRISPR-CPPC and ddPCR was 92.0% and 64.0%, respectively (Table 10). Table 11 presents the analytical performance of CRIPSR-CPPC and ddPCR based on the results of multiple assays and clinical diagnoses. T790M-positive by CRISPR-CPPC were subject to image

interpretation, which increased the sensitivity and specificity of CRISPR-CPPC to 93.9% and 100.0, respectively.

Table 7. Comparison of test results of qPCR to CRISPR-CPPC and ddPCR for *EGFR* T790M in cell-free plasma DNA

		qPCR				
		Positive (%)	Negative (%)	Total		
CRISPR-CPPC	Positive (%)	16 (26.7)	15 (25.0)	31	Concordance rate	75.0
	Negative (%)	0 (0.0)	29 (48.3)	29	Sensitivity	100.0
	Total	16 (26.7)	44 (73.3)	60	Specificity	65.9
ddPCR	Positive (%)	12 (20.0)	5 (8.3)	17	Concordance rate	85.0
	Negative (%)	4 (6.7)	39 (65.0)	43	Sensitivity	75.0
	Total	16 (26.7)	44 (73.3)	60	Specificity	88.6

Abbreviations: CRISPR-CPPC, CRISPR system combined post-PCR cfDNA; ddPCR, droplet digital polymerase chain reaction; qPCR, real-time PCR

Table 8. Comparison of test results of ddPCR to CRISPR-CPPC for *EGFR* T790M in cell-free plasma DNA

		ddPCR				
		Positive (%)	Negative (%)	Total		
CRISPR-CPPC	Positive (%)	15 (25.0)	16 (26.7)	31	Concordance rate	70.0
	Negative (%)	2* (3.3)	27 (45.0)	29	Sensitivity	88.2
	Total	17 (28.3)	43 (71.7)	60	Specificity	62.8

* NGS test was implemented and confirmed that one sample was T790M positive with an allele frequency of 0.2%, and the other sample was T790M negative.
Abbreviations: CRISPR-CPPC, CRISPR system combined post-PCR cfDNA; ddPCR, droplet digital polymerase chain reaction

Table 9. Cases which had different ddPCR and CRISPR-CPPC results

Sample No.	Sex	Age	First-or second-generation EGFR TKIs	Tissue Genotyping at diagnosis	DNA input (ng)	Plasma collection date (Months since TKI)	CRISPR-CPPC			ddPCR			NGS	Tissue genotyping at disease progression	Image (CT, MRI, PET-CT) interpretation at disease progression*
							Positive event (≥6 events/assay)	Wild event	AF (%)	qPCR (SQI)	Positive event (≥2 events/assay)				
CRISPR-CPPC-Positive															
4	M	55	Afatinib	Exon19 deletion	0.96	14	Pos	102	1811	5.3	Pos (8.45)	-	1	T790M 2.4%	N/T	
7	M	77	Gefitinib	L858R	0.58	7	Pos	6	1228	0.5	Pos (4.00)	-	0	N/T	N/T	
21*	F	69	Afatinib	Exon19 deletion	1.41	8	Pos	8	3589	0.2	-	-	0	N/T	N/T	Hepatic metz, R/O bone metz

25*	F	59	Afatinib	L858R	1.07	12	Pos	14	2776	0.5	-	-	0	N/T	N/T	New metz at right adrenal gland, R/O malig-nant pleural effusi-on
31*	F	75	Gefitinib	L858R	0.58	18	Pos	6	3995	0.1	-	-	0	N/T	N/T	Aggra-vation of lung cancer, Pleural seedin-g metz with malig-nant effusi-on,

															Metz in both lungs
34	F	85	Gefitinib	Exon19 deletion	1.28	16	Pos	24	3816	0.6	-	-	1	N/T	T790M Pos	
40*	F	68	Gefitinib	Exon19 deletion	0.71	17	Pos	12	1394	0.9	-	-	0	N/T	N/T	Pleural seedin-g at metast-atic lesions
43*†	M	43	Gefitinib	Exon19 deletion	2.26	46	Pos	6	5712	0.1	-	-	0	N/T	N/T	PD, Brain metz
44*†	M	44	Gefitinib	Exon19 deletion	14.5	48	Pos	8	9580	0.1	-	-	0	N/T	N/T	PD

48	F	59	Gefitinib	Exon19 deletion	1.66	14	Pos	7	2119	0.3	-	-	0	T790M 0.1%	N/T	
54	F	72	Gefitinib	L858R	<0.1	31	Pos	274	3874	6.6	Pos (4.99)	-	0	N/T	N/T	
55*	M	59	Gefitinib	L858R	<0.1	9	Pos	13	7324	0.2	-	-	0	N/T	N/T	R/O metz at brain, Diffus-ed metz in entire both lungs.
56*	F	70	Gefitinib	G719S	1.38	13	Pos	16	7243	0.2	-	-	0	N/T	N/T	PD, Endob-ronchi-al invasi-on and pleural seedin-

																g metz, Left pleural metz with pleural effusi-on, Increa-sed metas-tatic lesion in the left tempo-ral lobe of brain.
58	F	46	Afatinib	Exon19 deletion	<0.1	8	Pos	12	6052	0.2	-	-	0	T790M 0.2%	N/T	
59	F	49	Afatinib	L858R	<0.1	54	Pos	10	5294	0.2	-	-	0	-	T790M Pos	

60	F	60	Afatinib	Exon19 deletion	0.68	11	Pos	562	6343	8.1	Pos (8.36)	-	1	T790M 2.1%	N/T
CRISPR-CPPC-Negative															
12	F	61	Gefitinib	L858R	1.09	24	-	3	2579	0.1	-	Pos	3	-	N/T
47	M	57	Gefitinib	Exon19 deletion	<0.1	6	-	5	4709	0.1	-	Pos	12	T790M 0.2%	N/T

* Clinical history and image interpretation could be supported that a positive CRISPR-CPPC T790M result would be close to the true positive
† Same patient. The second test was done 3 months after the first test
Abbreviations: AF, allele frequency; CRISPR-CPPC, CRISPR system combined post-PCR cfDNA; CT, computed tomography; ddPCR, droplet digital polymerase chain reaction; metz, metastasis; MRI, magnetic resonance imaging; NGS, next-generation sequencing; N/T, not tested; PD, progressive disease; PET-CT, positron emission tomography-computed tomography; Pos, positive; qPCR, real-time PCR; R/O, rule out; SQI, semiquantitative index; TKI, tyrosine kinase inhibitor;

Table 10. Performance of T790M mutation-detecting assays at cfDNA in NSCLC patients with disease progression after receiving first- or second-generation EGFR-TKI.

Method	T790M mutation detected*			Sensitivity (95%CI)	Specificity (95%CI)	Accuracy (95%CI)
	Results	Pos (n=25)	Neg (n=35)			
ddPCR	Pos	16	1	64.0% (42.5% to 82.0%)	97.1% (85.1% to 99.9%)	83.3% (71.5% to 91.7%)
	Neg	9	34			
CRISPR-CPPC	Pos	23	8	92.0% (74.0% to 99.0%)	77.1% (59.9% to 89.6%)	83.3% (71.5% to 91.7%)
	Neg	2	27			

*Samples in which T790M was detected by more than two methods (qPCR from cfDNA or tissue, NGS, ddPCR, and CRISPR-CPPC) were considered to be true positive.

Abbreviations: cfDNA, cell-free DNA; CI, confidence interval; CRISPR-CPPC, CRISPR system combined post-PCR cfDNA; ddPCR, droplet digital polymerase chain reaction; EGFR-TKI, epidermal growth factor receptor-tyrosine kinase inhibitor; Neg, negative; NGS, next-generation sequencing; NSCLC, non-small cell lung cancer; Pos, positive; qPCR, real-time PCR

Table 11. Performance of T790M mutation-detecting assays by clinical diagnoses

Method	T790M mutation detected with clinical diagnosis*			Sensitivity (95%CI)	Specificity (95%CI)	Accuracy (95%CI)
	Results	Pos (n=33)	Neg (n=27)			
ddPCR	Pos	16	1	48.5%	96.3%	70.0%
	Neg	17	26	(30.8% to 66.5%)	(81.0% to 99.9%)	(56.8% to 81.2%)
CRISPR-CPPC	Pos	31	0	93.9%	100.0%	96.7%
	Neg	2	27	(79.8% to 99.3%)	(87.2% to 100.0%)	(88.5% to 99.6%)

*Samples in which T790M was detected by more than two methods (qPCR from cfDNA or tissue, NGS, ddPCR, and CRISPR-CPPC) were considered to be true positive. Samples which tested only positive by CRISPR-CPPC also had to be confirmed through imaging to be considered a true positive.

Abbreviations: CI, confidence interval; CRISPR-CPPC, CRISPR system combined post-PCR cfDNA; ddPCR, droplet digital polymerase chain reaction; NGS, next-generation sequencing; Neg, negative; Pos, positive; qPCR, real-time PCR

IV. DISCUSSION

Cell-free DNA is currently widely used to establish the genomic profile of tumors; however, T790M mutant detection is difficult due to the low levels and the rapid clearance of cfDNA.[49] For NSCLC patients, the FDA-proved Roche cobas® EGFR Mutation Test v2 (Roche Molecular Systems, Pleasanton, CA, USA) is widely used to facilitate treatment selection. This tool is very easy to use and is suitable for routine use. However, the test requires at least 100 copies/mL of specific *EGFR* mutants for the sensitive detection of mutations.[31] Many researchers suggest that sensitive detection of cfDNA mutation can be accomplished with the use of ddPCR. However, mutations with less than 0.1% allele frequency cannot be reliably detected by ddPCR.[37] Therefore, mutant enrichment technology combined with a sensitive detection tool could provide a plausible solution.

In this study, we described a CRISPR-CPPC method for T790M mutant enrichment in cfDNA and demonstrated significant improvements in mutation detection capability in both commercial cfDNA reference standards and patient samples. There have been several approaches to mutation enrichment using CRISPR/CAS9,[38,39] but no standard protocol has been published. However, CRISPR-CPPC approach has demonstrated several advantages: First, it is easy to use as long as the target primer is designed. Second, it successfully enriched samples with low number of mutant copies (<10 copies/mL). Third, it can clarify results in samples which have previously had borderline results. Fourth, all enrichment reactions were performed with the same amount (about 0.4 ng) of post-PCR cfDNA, meaning that CRISPR-CPPC can become a standardized process. Finally, CRISPR-CPPC compensated for DNA loss by adding the PCR step of cfDNA.

We also evaluated the clinical applicability of CRISPR-CPPC by establishing a clinical cutoff. CRISPR-CPPC technology does not miss T790M

variants detected by qPCR. In addition, it allows detection of T790M in patients with low copy numbers which T790M had not been previously detected by qPCR. Using CRISPR-CPPC, T790M was detected from an additional 15 (25%) samples (Table 7). Furthermore, CRISPR-CPPC detected T790M mutation in 16 samples that had been previously identified as T790M mutation negative by ddPCR. Two samples showed discordant result from ddPCR and CRISPR-CPPC; both were positive by ddPCR but negative by CRISPR-CPPC. NGS results indicated that one sample was T790M negative, and the other sample was T790M positive with an allele frequency of 0.2% (Table 8). CRISPR-CPPC may not have detected T790M in the second sample because less than 0.1ng of DNA was used during PCR step despite the sample had low concentration of extracted cfDNA. T790M mutant copy might have not been amplified due to the less amount of DNA input for the PCR step (Table 9). If a sufficient amount of cfDNA had been used, CRISPR-CPPC may have been able to detect T790M in the second sample.

Furthermore, patients' clinical histories and image interpretations were analyzed to evaluate cases which had different ddPCR and CRISPR-CPPC results. Disease progression may be caused by factors other than T790M, so image interpretations of CRISPR-CPPC-positive cases were used as evidence to confirm the presence of T790M. NGS tests were only conducted on some discordant cases to determine whether they were true positives (Table 9). One case (sample number 32) was shown to be T790M negative by both ddPCR and CRISPR-CPPC (Table 5), but NGS and tissue genotyping showed that the case was T790M positive with an allele frequency of 0.2%. Both ddPCR and CRISPR-CPPC failed to detect T790M in this case was likely because cfDNA samples extracted from stored plasma had degraded. In this study, stored plasma was used and so it was not as fresh as when the NGS test was conducted. The cfDNA extracted from the fresh plasma may improve the sensitivity of CRISPR-CPPC.

The evaluation of the cases with discordant results between ddPCR and

CRISPR-CPPC indicate that CRISPR-CPPC can detect T790M with 93.9% sensitivity and 100% specificity in patients with a progressive disease with the proof of imaging interpretation. Further investigation by matching the results of NGS and CRISPR-CPPC from more samples should be conducted to confirm the diagnostic utility of CRISPR-CPPC.

Like other enrichment method, CRISPR-CPPC technology requires further refinement prior to routine use. The step of cfDNA-PCR before CRISPR-CPPC compensated DNA loss, but the possibility of contamination still remains. Careful handling is required which can be burdensome for general laboratories. Furthermore, along with amplification of the mutant copies, wild copies are also amplified, which has led to the inclusion of the dilution step for reacting post-PCR cfDNA with cas9 complex. We tried to eliminate the dilution step by changing the PCR conditions (such as decreasing number of cycles); however, the optimal condition for mutant enrichment was not met without retaining the PCR and dilution steps. Finally, CRISPR-CPPC cannot be used for patient monitoring yet because its quantitative application has not been evaluated. Therefore, at present, the results of CRISPR-CPPC should only be considered qualitatively. Although this approach met the study's original purpose of enriching low mutant copies to render them detectable, it needs to be developed as a quantitative tool to be used for diagnostic purposes as well as for monitoring patient care. Incorporating dead cas9 (dcas9) into CRISPR-CPPC may solve the problem, but further study is still required.

CRISPR system can be used for mutant enrichment as well as gene editing. There have been many approaches to use CRISPR system to integrate with the detection assays to improve the diagnostic ability. Instead of using CRISPR/Cas9, Kellner et al. used Cas13 and developed the protocol for Specific High Sensitivity Enzymatic Reporter UnLOCKing (SHERLOCK) nucleic acid detection; however, that system lacks the ability of absolute digital

quantification.[50] Despite of needs of further refinement of CRISPR-CPPC, this technology demonstrated that CRISPR/Cas9 can be used as mutant enrichment tool with the capability of absolute quantification due to its integration with ddPCR. Therefore, CRISPR-CPPC can be clinically adapted to facilitate gene expression profiling, diagnosis, and the selection of appropriate treatment regiments. This study proves that CRISPR-CPPC's performance is exceptionally higher than any other currently available methods that it can be easily used in clinical settings, and costs less than NGS.

Recently, clinical trials are still underway on many third-generation TKIs. Osimertinib-based combination targeted therapy has been demonstrated to be an effective and compelling approach that supports the upfront use of third-generation TKIs.[51] Therefore, further studies to sensitively detect *EGFR* mutations using new enrichment technologies, CRISPR-CPPC, should be undertaken.

V. CONCLUSION

The proposed CRISPR-CPPC technology is a useful mutant enrichment tool for the sensitive detection of target mutation when used with ddPCR. This approach can be used for patients with TKI resistance that is possibly caused by T790M mutation, but is undetected by current FDA-approved methods. CRISPR-CPPC can be easily used in clinical settings and costs less than NGS. Thus, this technology may be used to confirm results of currently available methods to facilitate treatment selection, and to provide additional opportunities for patients to receive third-generation TKIs.

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ABSTRACT(IN KOREAN)

비소세포성 폐암 환자의 혈장에서 *EGFR* 유전자 변이 검출의 임상적 민감도 개선을 위한 변이 증강 기술의 적용

<지도교수 이 경 아>

연세대학교 대학원 의학과

김 보 연

표피성장인자수용체(epidermal growth factor receptor, EGFR)-티로신 키나아제 억제제(tyrosine kinase inhibitor, TKI)는 *EGFR* 돌연변이를 가진 비소세포폐암(non-small cell lung cancer, NSCLC) 환자의 치료로 사용될 수 있다. 그러나 TKI 치료를 받은 환자들 중 치료 뒤 약 8~18개월 후에 치료 효과가 더 이상 나타나지 않는 현상이 나타나는 경우가 있으며 이는 주로 *EGFR*에서 exon20의 790번째 자리에 위치한 트레오닌이 메티오닌으로 대체되는 변이(p.Thr790Met, T790M)로 인해 발생하게 된다. T790M 변이가 있을 경우 T790M 표적치료제를 2차 치료로 사용할 수 있으므로 1차 TKI 치료를 받았으나 치료 효과가 없는 환자들로부터 T790M 변이를 민감하게 검출하는 것이 중요하다. 순환종양 DNA (circulating tumor DNA, ctDNA)를 검출하기 위한 많은 기술적 플랫폼이 구현되고 있으나 ctDNA는 매우 적은 양의 DNA 조각으로 존재하므로 NSCLC 환자의 ctDNA로부터 *EGFR* 돌연변이를 검출하는 데에 어려움이 있다. 따라서 돌연변이 카피수가 유난히

적은 순환 핵산에서도 T790M과 같은 *EGFR* 돌연변이를 검출할 수 있도록 검출 능력이 향상된 기술의 필요성이 대두되고 있다.

최근 주기적으로 간격을 띠고 분포하는 짧은 회문구조 반복서열이라는 이름이 붙은 크리스퍼(clustered regularly interspaced short palindromic repeats, CRISPR)와 크리스퍼연관 단백질 카스9(CRISPR-associated protein 9, cas9) 시스템을 사용하여 돌연변이 증폭을 시킨 방법들이 분자 진단 분야에 소개된 바 있다. 이 논문에서는 T790M 카피수를 극도로 적게 가지고 있는(<10 copies/mL) NSCLC 환자의 세포유리 DNA(cell-free DNA, cfDNA)에서 T790M 돌연변이를 검출하기 위해 중합효소 연쇄반응(polymerase chain reaction, PCR)과 크리스퍼-카스9 시스템을 결합한 새로운 돌연변이 증폭 테크놀로지인 CRISPR-CPPC (CRISPR system combined post-PCR cfDNA)를 소개하였다.

CRISPR-CPPC는 1) cfDNA PCR, 2) 미리 PCR 전처리 된 cfDNA (post-PCR cfDNA)와 cas9의 복합체로 조립, 3) 디지털 미세방울 방식 PCR (droplet digital PCR, ddPCR)인 세가지 스텝으로 이루어져 있다. CRISR-CPPC의 최적화 및 유효성검사는 참조 cfDNA 및 TKI 치료를 이미 받았으나 질환이 진행된 NSCLC환자의 cfDNA를 사용하여 수행하였다. 그리고 CRISPR-CPPC의 검출 민감도를 확인하기 위해 실시간 중합효소 연쇄반응(real time PCR, qPCR) 및 ddPCR 결과를 CRISPR-CPPC 결과와 비교하였다.

CRISPR-CPPC의 사용으로 T790M 돌연변이 카피수 증폭이 가능하였으며 그 외 대립유전자 빈도 (allele frequency) 또한 최대 약 13배 증가하게 되어 T790M 돌연변이를 민감하게 검출 할 수 있었다. 또한 질환이 진행된 NSCLC 환자에게서 CRISPR-CPPC 를

사용하여 T790M 변이를 검출하였을 때 CRISPR-CPPC의 민감도는 93.9%, 특이도는 100%로 확인되었다.

CRISPR-CPPC의 성능은 현재 이용 가능한 다른 방법보다 훨씬 높다. 따라서 질환이 진행된 환자에게서 T790M 변이를 더 민감하게 검출할 수 있도록 CRISPR-CPPC 기술을 사용하는 것은 임상의들에게는 NSCLC 환자를 치료하는데 적절한 치료를 선택할 수 있도록 도움을 줄 수 있으며, 환자에게는 T790M 표적 치료를 받을 수 있는 기회를 한번 더 제공해줄 수 있다는 점에 의의가 있다.

핵심되는 말 : 세포유리 DNA, *EGFR* 유전자, 액체생검, 크리스퍼-카스 시스템, 비소세포폐암