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Plasma epidermal growth factor receptor mutation testing with a chip-based digital PCR system in patients with advanced non-small cell lung cancer

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ABSTRACT

Objectives: Epidermal growth factor receptor (*EGFR*) mutation testing is a companion diagnostic to determine eligibility for treatment with EGFR tyrosine kinase inhibitors (EGFR-TKIs) in non-small cell lung cancer (NSCLC). Recently, plasma-based EGFR testing by digital polymerase chain reaction (dPCR), which enables accurate quantification of target DNA, has shown promise as a minimally invasive diagnostic. Here, we aimed to evaluate the accuracy of a plasma-based *EGFR* mutation test developed using chip-based dPCR-based detection of 3 *EGFR* mutations (exon 19 deletions, L858R in exon 21, and T790M in exon 20).

Materials and methods: Forty-nine patients with NSCLC harboring EGFR-activating mutations were enrolled, and circulating free DNAs (cfDNAs) were extracted from the plasma of 21 and 28 patients before treatment and after progression following EGFR-TKI treatment, respectively.

Results: Using reference genomic DNA containing each mutation, the detection limit of each assay was determined to be 0.1%. The sensitivity and specificity of detecting exon 19 deletions and L858R mutations, calculated by comparing the mutation status in the corresponding tumors, were 70.6% and 93.3%, and 66.7% and 100%, respectively, showing similar results compared with previous studies. T790M was detected in 43% of 28 cfDNAs after progression with EGFR-TKI treatment, but in no cfDNAs before the start of the treatment.

Conclusion: This chip-based dPCR assay can facilitate detection of EGFR mutations in cfDNA as a minimally invasive method in clinical settings.

Abbreviations: ARMS, amplification refractory mutation system; BEAMing, beads, emulsions, amplification and magnetics; cfDNA, circulating free DNA; CI, confidence interval; ddPCR, droplet digital polymerase chain reaction; dPCR, digital polymerase chain reaction; EGFR, epidermal growth factor receptor; EGFR-TKI, epidermal growth factor receptor tyrosine kinase inhibitors; JAK2, Janus kinase 2; NSCLC, non-small cell lung cancer; PFS, progression-free survival; RR, response rate; SD, standard deviation.

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1. Introduction

Epidermal growth factor receptor (*EGFR*) mutation testing is essential for treatment decisions for patients with advanced non-small cell lung cancer (NSCLC). EGFR tyrosine kinase inhibitors (EGFR-TKIs), including gefitinib, erlotinib, and afatinib, are effective against patients with NSCLC harboring EGFR-activating mutations, such as exon 19 deletions or the L858R mutation in exon 21 [1–6]. However, most patients with NSCLC treated with EGFR-TKIs eventually acquire resistance. The T790M mutation in *EGFR* exon 20 causes approximately 50% of acquired resistance to EGFR-TKIs in patients [7,8]. Recently, the third-generation EGFR-TKI osimertinib, which specifically targets EGFR T790M, was approved for use in some countries including the US and Japan, and the importance of serial biopsies for detecting T790M is increasing, as such information determines the appropriateness of osimertinib treatment [9,10]. However, repeated biopsies are sometimes highly invasive and can be difficult to perform without complications [11].

Recently, some studies have reported the efficacy of *EGFR*-activating mutation analysis with circulating free DNA (cfDNA) extracted from the plasma of patients with NSCLC [12–21]. Moreover, *EGFR*-activating mutation analysis with cfDNA was also approved as a companion diagnosis for selecting patients eligible for treatment with gefitinib and osimertinib in the European Union. However, some technical limitations for detecting *EGFR* mutations with cfDNA have been reported. For example, the quantity and quality of circulating tumor-derived DNA varies widely between patients [22]. Moreover, the detectable percentage of the tumor-derived DNA fraction in cfDNA can reach as low as 0.01% [23]. The digital polymerase chain reaction (dPCR), which enables accurate copy-number quantification of target molecules from low-input DNA, is thought to be a promising technology for overcoming the above limitations in mutation testing with cfDNA [24]. The high performance of dPCR is achieved by compartmentalizing a sample at the level of a single DNA molecule by distributing a sample into thousands of separate PCR reactions. Accordingly, the total copy number of targets can be determined by counting the positive and negative partitions. dPCR platforms are classified into 3 types based on the compartmentalization method used, including droplet digital PCR (ddPCR) [20,25–30], BEAMing (beads, emulsions, amplification and magnetics) PCR [19,20,31], and chip-based dPCR [30,32,33]. Plasma-based *EGFR* mutation testing with ddPCR has been evaluated in many institutes. However, chip-based dPCR has not been widely evaluated.

The new chip-based dPCR system, QuantStudio 3D (QS3D) Digital PCR System (Thermo Fisher Scientific, Waltham, MA, USA), was launched. The conventional chip-based BioMark dPCR system (Fluidigm, San Francisco, CA, US) compartmentalizes DNA into 9,180 micropores [32,33], whereas the QS3D Digital PCR system compartmentalizes DNA into 20,000 micropores, which is comparable to the compartmentalization ability of the ddPCR platform, QX100/QX200 Droplet Digital PCR System (Bio-Rad, Hercules, CA, US) broadly used in studies for liquid biopsy [25–29]. Moreover, both PCR and detection processes can be consecutively performed within a hermetically sealed reaction chamber in the QS3D Digital PCR system; however, this is not possible with the ddPCR system. This feature of the QS3D Digital PCR system offers the advantage of requiring fewer pipetting process than those needed for ddPCR. Together, these properties contribute to the reduced risk of cross-contamination. The QS3D Digital PCR system runs each chip individually, indicating suitability of the QS3D Digital PCR system for clinical settings in small institutions without vast amounts of samples. In previously studies, this QS3D Digital PCR system has been used to detect DNA mutations in Janus kinase 2 (JAK2) [34] and coagulation factor V genes [35], microRNAs [36], and viral DNA [37], but not *EGFR* mutations.

Therefore, this study aimed to evaluate plasma-based *EGFR* mutation analysis in patients with advanced NSCLC, using this new chip-based dPCR system, QS3D Digital PCR System.

2. Material and methods

2.1. Patients

All patient samples used in this study were collected from our previous prospective study to evaluate plasma *EGFR* mutation testing with the RNase H-dependent PCR and blocking oligo-dependent PCR methods [38]. The present study was designed to evaluate the performance of the QS3D Digital PCR System in plasma-based *EGFR* mutation analysis, using cfDNA samples archived in that previous study [38]. To be eligible for our previous study, patients needed to have been diagnosed with advanced-stage NSCLC or post-operative recurrence and to have an EGFR-activating mutation in their tumors, as confirmed by the Scorpion ARMS (amplification refractory mutation system) method [39]. In our previous study [38], 49 patients were enrolled from October 2013 to March 2014, and tumor staging was evaluated according to the Seventh Edition of TNM in Lung Cancer [40]. Clinical factors at the time of blood sampling were obtained from the patients' medical records. Based on the timing of blood sampling, patients were divided into 2 groups. One group included 21 patients enrolled before the start of EGFR-TKI treatment, and the other group included 28 patients who showed disease progression after EGFR-TKI treatment. Written informed consent was obtained from all patients. This study was conducted in accordance with the provisions of the Declaration of Helsinki and was approved by the Institutional Review Board of Shizuoka Cancer Center (approval number #20-50-27-2-3).

2.2. Chip-based dPCR

Plasma samples and DNA extraction are described in the Supplementary Materials and Methods. dPCR was performed on the QS3D Digital PCR System (Thermo Fisher Scientific), which was composed of a ProFlex PCR system, a QS3D digital PCR chip loader, and a QS3D chip scanner (all from Thermo Fisher Scientific).

To detect the most common deletions in *EGFR* exon 19, a pair of primers and 2 TaqMan probes were designed, based on Yung's report [32] (Supplementary Fig. 1). As a reference probe, a VIC-labeled probe was designed against a region neighboring the exon 19 deletion sites that lacks reported mutations. A wild-type-specific FAM probe was designed against the region where most exon 19 deletions occur. In samples harboring exon 19 deletions, the FAM probe cannot anneal; thus, only VIC fluorescence is detected. The sequences of the amplification primers and TaqMan probes used are given in Supplementary Table 1. To detect the L858R and T790M mutations, predesigned TaqMan probe and primer sets, AHRSRSV (EGFR 6224) and AHRSROS (EGFR 6240), respectively, were purchased from Thermo Fisher Scientific.

2.3. Assessment of the detection limit for each mutation

The detection limit of each assay was defined as the lowest target concentration that could be specifically detected (i.e., with no false-positive detection in the absence of the target) and was determined using 20 ng sample on each of 2 chips containing HDx Reference Standard DNA (Horizon Discovery, Cambridge, UK), which was validated using a ddPCR system of Bio-Rad. The reference standard DNAs used included HD251 ΔE746-A750 (registered in the COSMIC database as the most frequent exon 19 deletion [http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/]), HD254 for L858R, and HD258 for T790M. Each reference mutant DNA contains each mutant sequence at a frequency of 50%. Each reference mutant DNA