EBioMedicine xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

EBioMedicine

journal homepage: www.ebiomedicine.com

Research Paper

Multiplex Ultrasensitive Genotyping of Patients with Non-Small Cell Lung Cancer for Epidermal Growth Factor Receptor (EGFR) Mutations by Means of Picodroplet Digital PCR

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ARTICLE INFO

Article history:
Received 2 April 2017
Received in revised form 29 May 2017
Accepted 2 June 2017
Available online xxxx

Keywords:
EGFR mutation
Droplet digital PCR
Non–small cell lung cancer

ABSTRACT

Epidermal growth factor receptor (EGFR) mutations have been used as the strongest predictor of effectiveness of treatment with EGFR tyrosine kinase inhibitors (TKIs). Three most common *EGFR* mutations (L858R, exon 19 deletion, and T790M) are known to be major selection markers for EGFR-TKIs therapy. Here, we developed a multiplex picodroplet digital PCR (ddPCR) assay to detect 3 common *EGFR* mutations in 1 reaction. Serial-dilution experiments with genomic DNA harboring EGFR mutations revealed linear performance, with analytical sensitivity ~0.01% for each mutation. All 33 EGFR-activating mutations detected in formalin-fixed paraffin-embedded (FFPE) tissue samples by the conventional method were also detected by this multiplex assay. Owing to the higher sensitivity, an additional mutation (T790M; including an ultra-low-level mutation, <0.1%) was detected in the same reaction. Regression analysis of the duplex assay and multiplex assay showed a correlation coefficient (R^2) of 0.9986 for L858R, 0.9844 for an exon 19 deletion, and 0.9959 for T790M. Using ddPCR, we designed a multiplex ultrasensitive genotyping platform for 3 common *EGFR* mutations. Results of this proof-of-principle study on clinical samples indicate clinical utility of multiplex ddPCR for screening for multiple EGFR mutations concurrently with an ultra-rare pretreatment mutation (T790M).

Abbreviations: NSCLC, non–small cell lung cancer; TKI, tyrosine kinase inhibitor; PFS, progression-free survival; SARMS, Scorpion Amplification Refractory Mutation System; dPCR, digital polymerase chain reaction; ddPCR, picodroplet dPCR; mCRC, metastatic colorectal cancer; JME, Japan Molecular Epidemiology for Lung Cancer; FFPE, formalin-fixed paraffin-embedded; TET, tetrachlorofluorescein; FAM, 6-carboxyfluorescein; LOB, limit of blank.

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http://dx.doi.org/10.1016/j.ebiom.2017.06.003

1. Introduction

Targeted molecular therapy has improved the treatment of non–small cell lung cancer (NSCLC). Superiority of epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) to platinum-based chemotherapy in terms of progression-free survival (PFS) in EGFR-mutated lung cancers has been reported in several phase III trials as a first-line treatment (Zhou et al., 2011; Rosell et al., 2012; Mok et al., 2009; Mitsudomi et al., 2010; Maemondo et al., 2010). EGFR-TKIs (gefitinib, erlotinib, or afatinib) have been demonstrated to be effective for NSCLC patients with EGFR-activating mutations such as exon19 deletion or exon 21 L858R mutations (Lynch et al., 2004; Paez et al., 2004). Evidence shows, however, that most responders eventually develop acquired resistance to EGFR-TKIs (Kobayashi et al., 2005; Yu et al., 2013; Ohashi et al., 2013). Among these patients, a secondary missense T790M mutation is observed in nearly half of all cases resistant to EGFR-TKIs (Ohashi et al., 2013).

This T790M mutation was also detected in tumors as a minor cellular clone before exposure to EGFR-TKIs and was found concurrently with other EGFR-activating mutations (Inukai et al., 2006). This "pretreatment T790M mutation" is present in 1–8% of cases according to conventional DNA sequencing like Sanger sequencing (Wu et al., 2011; Sequist et al., 2008; Li et al., 2014; Fujita et al., 2012) and in 2–79% of cases according to more sensitive detection methods like Scorpion Amplification Refractory Mutation System (SARMS) technology with an EGFR-activating mutation (Su et al., 2012; Rosell et al., 2011; Maheswaran et al., 2008; Costa et al., 2014; Yu et al., 2014). Patients with pretreatment T790M mutation detected by less sensitive methods show a lower response rate and shorter PFS (Inukai et al., 2006; Wu et al., 2011; Sequist et al., 2008). Recent studies revealed that patients with a pretreatment T790M mutation detected by a highly sensitive method also have shorter PFS (Su et al., 2012; Rosell et al., 2011; Maheswaran et al., 2008; Costa et al., 2014; Ding et al., 2014), suggesting that a low-level pretreatment T790M mutation can be used for optimizing treatment with EGFR-TKIs. Therefore, the ability of molecular analytical technologies to detect EGFR mutants at the subclone level before EGFR-TKI treatment is critically important for enabling more personalized therapies in NSCLC.

Picodroplet digital PCR (ddPCR) recently emerged as a highly sensitive method for detection of gene mutations and is based on compartmentalization of DNA into picoliter-size droplets (Taly et al., 2012). Our previous report showed detection of 0.001% prevalence of the *EGFR* T790M mutation among tumor cells (Watanabe et al., 2015). Several examples of ddPCR application to highly sensitive detection of mutations were published recently (Pekin et al., 2011; Oxnard et al., 2014; Ono et al., 2014; Iwama et al., 2015; Sacher et al., 2016). Multiplexing of mutation detection in a single assay is desirable for genotype testing in the clinic; promising results have also been demonstrated using ddPCR (Zhong et al., 2011; Didelot et al., 2013; Taly et al., 2013; Laurent-Puig et al., 2015; Zonta et al., 2016). The multiplex procedure has been adapted to quantitative detection of 7 common mutations of *KRAS* (in codons 12 and 13) in plasma samples and primary tumor samples from patients with metastatic colorectal cancer (mCRC) (Taly et al., 2013; Laurent-Puig et al., 2015). Zonta et al., developed several multiplex panels for EGFR (several three- and four-plex) in reference standard DNA samples. Here, we report the advantage of our 6-plex ddPCR assay that detects 3 clinically relevant mutations of EGFR (L858R, exon 19 deletion, and T790M mutations) and corresponding wild-type allele at an ultra-low level by using DNA samples of surgically resected primary tumors from 45 NSCLC patients.

2. Materials and Methods

2.1. Study Design and Patients

We used this test system to assess multiplex detection of 3 *EGFR* mutations in 45 samples of surgically resected primary tumors from NSCLC patients enrolled in the Japan Molecular Epidemiology for Lung Cancer Cases (JME) study (Kawaguchi et al., 2016). That study (UMIN000008177) is a prospective, multicenter molecular epidemiological analysis designed to address associations between driver mutations and smoking and other environmental factors. Eligible subjects are patients with newly diagnosed NSCLC of stage I to IIIB who have received surgical treatment. Full details of the study design were published elsewhere (Kawaguchi et al., 2016).

The present study was approved by the Institutional Review Board of the National Hospital Organization of Japan. All patients provided written informed consent. From July 2012 to December 2013, 958 patients were recruited from 43 institutions, and 901 samples were successfully analyzed.

Genomic DNA extraction from formalin-fixed, paraffin-embedded (FFPE) specimens of surgically resected tissue was performed in an independent clinical laboratory (SRL, Tokyo, Japan). Genomic DNA concentration was measured using the PicoGreen dsDNA quantitation assay (Life Technologies, Carlsbad, CA) as per the manufacturer's recommendation. Fluorescent intensity from double strand DNA was measured by GloMax-Multi Microplate Multimode Reader (Progega, Madison, WI). Somatic mutations in *EGFR* and *KRAS* were validated by sensitive PCR methods in an independent clinical laboratory (SRL).

2.2. DNA Controls

Positive and negative control plasmids for the EGFR assay were prepared by cloning DNA fragments containing wild-type or the EGFR mutations were using a TOPO TA Cloning Kit (Life Technologies). The appropriate concentration of plasmid DNA was determined empirically to yield a mixture in which the number of copies of mutant DNA was ca. 0.01–1.000% of the number of wild-type EGFR fragments.

Tumor cell lines H1975, PC-9/ZD, and A549 are a gift from Dr. Fumiaki Koizumi (Tokyo Metropolitan Cancer and Infectious Diseases Center Komagome Hospital, Tokyo, Japan). Genomic DNA was extracted using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Wild-type human genomic DNA was purchased from Clontech (Mountain View, CA). Genomic-DNA samples were digested with CviQ1 (New England Biolabs, Ipswich, MA), and DNA concentration was determined using a Qubit® fluorometer (Life Technologies). Digested-DNA controls were used to quantitatively assess each EGFR mutant sequence and in the multiplex assay panels. In total, 400 ng digested DNA was used for control experiments to determine the limit of blank (LOB).

Genomic DNA of each mutation-specific cell line was serially diluted with wild-type human genomic DNA to attain mutation prevalence between 0.01% and 1%. Evaluation of the linearity and lower limit of mutation detection of each probe was also performed for multiplex ddPCR assays.

2.3. Probes and Primers for Digital PCR

Primers and probes were acquired from MBL-IDT K.K (Nagoya, Japan). Fluorescent probes targeting wild-type and mutant sequences were respectively conjugated to tetrachlorofluorescein (TET; λ_{ex} 522 nm, λ_{em} 539 nm) or 6-carboxyfluorescein (FAM; λ_{ex} 494 nm, λ_{em} 522 nm) fluorophores with the ZEN/IABkFQ double quencher. Sequences of primers and probes for detection of *EGFR* mutations are given in Supplemental Table 1.

2.4. EGFR Mutation Detection

This duplex assay is based on parallel amplification of wild-type and specific mutant sequences. In a pre-PCR setup, 20.0 μL (mm^3) TaqMan Genotyping Master Mix (Life Technologies) was mixed with the assay solution containing 2.0 μL of 10 μM (i.e., $10^{-2} \times mol/m^3$) forward and reverse primers, 2.0 μL of 4 μM FAM and TET labeled-probes, 4.0 μL Droplet Stabilizer (RainDance Technologies, Billerica, MA), 4.0 μL sterile DNase- and RNase-free water (Life Technologies), and 4 μL genomic

Please cite this article as: Watanabe, M., et al., Multiplex Ultrasensitive Genotyping of Patients with Non-Small Cell Lung Cancer for Epidermal Growth Factor Receptor (EGFR) Mutati..., EBioMedicine (2017), http://dx.doi.org/10.1016/j.ebiom.2017.06.003