Plasma-based early screening and monitoring of \( \text{EGFR} \) mutations in NSCLC patients by a 3-color digital PCR assay

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**BACKGROUND:** Noninvasive plasma-based detection of \( \text{EGFR} \) mutations using digital PCR promises a fast, sensitive and reliable approach to predicting the efficiency of \( \text{EGFR-TKI} \). However, the low throughput and high cost of digital PCR restricts its clinical application.

**METHODS:** We designed a digital PCR assay, which can simultaneously detect 39 mutations of exons 18–21 of the \( \text{EGFR} \) gene. To assess overall performance, retrospective FFPE tissues from 30 NSCLC patients and plasma from 33 NSCLC patients were collected and analysed.

**RESULTS:** The LoD of the \( \text{EGFR} \) mutations was as low as 0.308 copies/\( \mu \)L, and the linear correlation between the detected and expected values at different concentrations (0.01–10%) was as well. Compared to ARMS-PCR in FFPE, the accuracy values of the \( \text{dEGFR39} \) assay in plasma from 33 patients was 87.88% (29/33, 95% CI 72.67–95.18%). While monitoring the 33 patients, the \( \text{EGFR} \) mutation load as assessed by \( \text{dEGFR39} \) was associated with the objective response to treatment. Thirteen samples from eight patients were identified by \( \text{dEGFR39} \) to harbour the T790M mutation over time; of these patients, only nine (69%) were detected using SuperARMS.

**CONCLUSION:** Our results indicate that \( \text{dEGFR39} \) assay is reliable, sensitive and cost-efficient. This method is beneficial for profiling \( \text{EGFR} \) mutations for precision therapy and prognosis after \( \text{TKI} \) treatment, especially in patients with insufficient tissue biopsy samples.

*British Journal of Cancer* (2020) 123:1437–1444; https://doi.org/10.1038/s41416-020-1024-2

**BACKGROUND**

In patients with non-small-cell lung cancer (NSCLC), the possibility of treatment with tyrosine kinase inhibitor (TKI) is determined by the presence of mutations on exons 18–21 of the epidermal growth factor receptor (\( \text{EGFR} \)).\(^1\) Clinical evidence heights the fact that \( \text{EGFR} \)-targeted therapy can significantly improve progression-free survival (PFS) and overall survival (OS) in patients.\(^2,3\) Therefore, assessing the status of \( \text{EGFR} \) mutations is important for potential \( \text{EGFR-TKI} \) therapy.

Previous methods of \( \text{EGFR} \) mutation detection have been based on invasive approaches, such as surgical resection of tumours and needle biopsies.\(^4\) However, the heterogeneity of tumour tissue usually confounds the analysis of \( \text{EGFR} \) mutation load.\(^5\) Interestingly, recent data have shown that \( \text{EGFR} \) mutations can be found in plasma-derived cell-free DNA (cfDNA) from NSCLC patients. Moreover, studies have confirmed that \( \text{EGFR} \) mutations from plasma can predict the clinical response to targeted therapy.\(^6,7\)

Based on plasma-derived DNA, noninvasive detection approaches have been developed, such as super amplification refractory mutation system (superARMS) PCR, next-generation sequencing (NGS), and digital PCR.\(^8–10\) Although ARMS-PCR is cost-efficient, its overall performance appears to be least sensitive. The NGS method, on the other hand, is more expensive. The digital PCR method features a quick turn-around and improved sensitivity, and is ideally used for the detection of known mutation types in cancer patients, especially in those whose tissue biopsy samples are insufficient.\(^11–14\) Digital PCR is a new approach to nucleic acid detection. The PCR reaction is first pressurised to partition into 30,000 droplets, each of which contains zero, one or more copies of the target molecule, and then PCR analysis is carried out. During amplification, TaqMan chemistry with dye-labelled probes is used to detect sequence-specific targets. The droplets containing the target molecule will generate fluorescence signals, which are defined as positive, while others are negative. According to the Poisson distribution, the fraction of negative reactions is used to generate an absolute count of the number of target molecules in the sample. Despite the fact that studies have shown good consistency of \( \text{EGFR} \) detection between plasma and tissue using digital PCR,\(^12,15,16\) the low throughput and high cost of digital PCR restricts its clinical application. There is not yet a method that simultaneously assesses all the driver mutations of the \( \text{EGFR} \) gene in plasma using digital PCR.\(^17,18\)

Therefore, we developed an integrated digital PCR assay, named \( \text{dEGFR39} \), which can unambiguously distinguish multiple mutations.
using three different fluorescence channels. This method is based on the need for low cost and small sample input. In this report, we provide evidence that dEGFR39 has excellent performance, which is not only useful to screen EGFR-targeted mutations from plasma of NSCLC patients to guide targeted therapy, but also to evaluate prognosis after treatment. To the best of our knowledge, this is the first report regarding the introduction of digital PCR for the screening and monitoring of all EGFR mutations from patient plasma.

METHODS
Sample collection and processing
The plasmid DNA of EGFR L858R, 19Del and T790M were prepared using the Apexbio plasmid DNA extraction kit, according to the manufacturer’s instructions (Apexbio, Suzhou, China). The human genomic DNA standard HD802 was obtained from Horizon (Horizon, Cambridge, UK). This study was approved by the ethics committee of Cangzhou Central Hospital, Hebei, China and all patients provided written informed consent. Patients were diagnosed with NSCLC and written informed consent. Patients were diagnosed with NSCLC and all patients provided written informed consent. Patients were diagnosed with NSCLC and all patients provided written informed consent. Patients were diagnosed with NSCLC and all patients provided written informed consent. Patients were diagnosed with NSCLC and all patients provided written informed consent.

Detection of EGFR mutations using dEGFR39 assay
Oligonucleotides were synthesised by Sangon Biotech (Shanghai, China). Detection of the EGFR mutation using dEGFR39 assay (Apexbio, Suzhou, China) was carried out in three tubes on the Naica digital PCR system (Stilla Technologies, Villejuif, France) with Sapphire chips (Stilla Technologies, Villejuif, France). The list of 39 mutations found by the dEGFR39 assay is provided in Supplementary Table S1. Twenty-five microlitre of reaction mix in each tube contained 1X PerFecTa Multiplex qPCR ToughMix, 40 nM FITC (Saint Louis, MO, USA), 1 μl of a multiplex mix of primers and probes and 3 μl of DNA. The chip was loaded into the Naica Geode thermocycler to compartmentalise the droplets and to perform the PCR reaction. PCR consisted of 10 min at 95 °C, followed by 45 cycles of 95 °C for 20 s and 60 °C for 30 s. After amplification, the Sapphire chips were imaged using the Naica Prism3 reader.

Limit of blank and limit of detection
The specificity and accuracy of dEGFR39 assay were assessed by wild-type (WT) and mutant DNA of EGFR. The limit of blank (LoB) and limit of detection (LoD) were determined as previously reported. To determine LoB, twenty replicates of a blank sample within two independent runs were carried out. The LoB was set as the highest mutant concentration that might be found when replicates of a blank sample are tested. The LoD was set as the lowest concentration that could be distinguished from the LoB with 95% certainty. The LoB and LoD were calculated with the following formulas:

\[ \text{LoB} = \frac{\text{Mean}_{\text{blank}} + 1.645 \times SD_{\text{blank}}}{N} \]  

\[ \text{LoD} = \text{LoB} + 1.645 \times SD_{\text{low concentration sample}} \]

Accuracy was assessed by testing the DNA Reference Standards and calculating the coefficient of variation and statistical differences.

Data analysis
Raw data from digital PCR were analysed by Crystal Miner software (Stilla Technologies, Villejuif, France) according to the principle of Poisson distribution. In general, when there are \( \lambda \) targets per droplets, the fraction of positive droplets (P) is:

\[ P = 1 - e^{-\lambda} \]  

For each analysis involving data from two channels, where \( \lambda_{c1} \) and \( \lambda_{c2} \) are the average number of loading molecules in these two channels.
channels, with the total number of droplets $N$, the number of double-positive droplets in multiple digital PCR is:

$$N_{\text{Dual}} = N \times (1 - e^{-k_1}) \times (1 - e^{-k_2})$$  \hspace{1cm} (4)$$

NTC and EGFR Gene-Specific Multiplex Reference Standard genomic DNA HD802 were used as negative and positive controls, respectively. Negative and positive droplets were also used to check the fluorescence spill-over compensation. The paired t-test, Bland–Altman, and McNemar’s test were used to compare the consistency of different groups with PPA (sensitivity), NPA (specificity), accuracy (OPA) and Kappa values. The cut-off for statistical significance was $p < 0.05$. All statistical data were analysed by IBM SPSS statistics software 22.0 (IBM, Armonk, NY, USA) and GraphPad Prism 5 (GraphPad, La Jolla, CA, USA).

**RESULTS**

Characteristics of dEGFR39 assay

In this study, dEGFR39 assay was developed to detect up to 39 mutations along exons 18–21 of the EGFR gene, including common mutation sites such as L858R, 19Del and T790M, as well as rare mutation sites such as L861Q, S768I, G719X, C797S and 20ins. To develop dEGFR39 assay, we designed digital PCR into three consecutive reactions. In the first reaction, 19DelREF was labelled with HEX, L858R and L858R were labelled with FAM, and 19DelWT and S768IMU were labelled with CY5 (Fig. 2). In the presence of 19Del, probe 19Del WT could cross-react with the neighbouring nondeleted WT sequences; in the meantime, probe 19DelREF was used to detect the EGFR gene (including 19DelWT and 19DelMU). As a result, 19DelMU molecules were double-positive (CY5$^+$/HEX$^+$), whereas the rest of the signals in the HEX and CY5 channels were only from 19Del MU (CY5$^-$/HEX$^+$) and S768IMU (CY5$^+$/HEX$^-$), respectively. The blue and green dots represent the signal of T790M and C797S in trans configuration with T790M, respectively. The double positive represents C797S in cis configuration with T790M.
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(3+/−/HEX−), respectively. The signals in the FAM channel were generated by L858R_MU and L861Q_MU. Similarly, in the second reaction, G719X_WT and 20insWT were labelled with FAM and CY5, respectively, while 20insMU and G719X_MU were labelled with HEX. 20insWT molecules were consequently double-positive (3+/−/HEX+), and the rest of the signals in the HEX and CY5 channels were only from G719X_MU (3+/−/HEX−) and 20insMU (3+/−/HEX−), respectively. Given that the presence of C797S and T790M, whether in the trans or cis form, would determine the efficacy of TKI treatment, the third reaction was specifically designed to characterise this genotype. T790M_MU and C797S_MU were labelled with FAM and HEX, respectively. Since the amount of plasma-derived DNA was exceedingly low, the positive signal from the HEX channel could only be from C797S_MU in the trans configuration with T790M_MU.

Notably, our assay included the C797S site, a critical mutation recently identified after TKI treatment. We did not detect C797S in patients enrolled in our study, which is consistent with previous reports that C797S does not occur in TKI-naïve NSCLC.

The specificity and sensitivity of dEGFR39

To assess the specificity of the dEGFR39 assay, both WT and mutant (MU) DNA of EGFR were synthesised. Notably, only a specific fluorescent signal could be detected that corresponded to WT and mutant forms (Supplementary Figs. S1–3), indicating the high specificity of the assay. To evaluate the accuracy, we performed the dEGFR39 reaction with a standard DNA HD802 (expected mutant ratio 12.5%). Our data showed the mutation loads of 19Del, T790M, and L858R as being 12.63%, 12.19% and 12.13%, respectively. This assessment was in concordance with the expected values (p > 0.05) (Table 1), strongly suggesting that dEGFR39 assay has good accuracy. Additionally, we tested the dEGFR39 mutations with abundance from 0.01 to 10%, and the slope between measured and expected abundance was close to 1, indicating that the correlation was strikingly significant (Fig. 3 and Supplementary Figs. S4–7). For L858R/L861Q, 19Del, T790M, and C797S, the LoB was 0.2 copies, while the LoB of 20ins and G719X was 0.3 copies (Supplementary Table S2–S4). Next, we assessed the LoD using serial dilutions of mutant DNA following the CLSI EP17 method. The LoD of L858R/L861Q, 19Del and T790M was 0.339, 0.305 and 0.333 copies/μL, respectively. For rare mutations, the LoD of 768I, G719X, 20ins and C797S was 0.311, 0.343, 0.457 and 0.349 copies/μL, respectively (Supplementary Table S5).

The consistence between the dEGFR39 method and ARMS-PCR in FFPE tissues

To compare the performance between dEGFR39 assay and ARMS-PCR, 30 FFPE tissues from NSCLC patients were collected. As revealed by the dEGFR39 assay, the individual mutation load ranged from 0.04 to 43.7%. Among these mutations, a large number had an abundance of less than 1% (ranging from 0.04 to 0.79%) (Supplementary Fig. S8).

In this study, we set the respective LoD of mutations as the cut-off value for dEGFR39 assay. Mutations with a concentration less than the LoD value were defined as negative. Consequently, the overall predictive agreement (OPA), positive predictive value (PPV) and negative predictive agreement (NPA) were assessed. In addition, we summarised the detection results of all sites. Mutations with a lower abundance could not be correctly detected using the ARMS-PCR method. In contrast, dEGFR39 was able to detect that the PPV, NPV and OPA were 94.44% (95% CI 74.24–99.01%), 25% (95% CI 8.89–53.23%) and 66.67% (95% CI 48.78–80.77%), respectively (Table 2 and Supplementary Table S6–8). Altogether, these results demonstrate that dEGFR39 can effectively detect low-abundance EGFR mutations in patients, and is more sensitive than the ARMS-PCR method.

Evaluation of dEGFR39 for detecting EGFR mutations in plasma

To evaluate the performance of dEGFR39 in patient-derived plasma, we analysed 33 matched plasma samples from patients with advanced NSCLC. The diagnosis was previously based on the ARMS-PCR method from FFPE tissues, in which 16 patients were positive for EGFR mutations (six for L858R/L861Q, eight for 19Del and three for T790M, including compound mutation) and 17 were negative for EGFR mutations. The histopathological characteristics of the patients are summarised in Supplementary Table S9. In this analysis, dEGFR39 detected 17 positives and 16 negatives from patient-matched plasma. The PPV, NPV and OPA of EGFR

Table 1. Analysis accuracy of EGFR L858R, 19Del and T790M in dEGFR39 assay was determined by testing the DNA Reference Standards.

|             | 19Del | L858R | T790M |
|-------------|-------|-------|-------|
| Expected mutant ratio | 12.50% | 12.50% | 12.50% |
| Measured mean ± SD | 12.63 ± 1.05% | 12.13 ± 0.97% | 12.19 ± 1.03% |
| CV | 0.08 | 0.08 | 0.08 |
| Paired t-Test | < 0.05 | NO | NO |
| Significantly different (P < 0.05) | NO | NO | NO |
| Bland–Altman analysis | 0.70 | 0.24 | 0.36 |
| Bias ± SD | 0.13 ± 0.1% | 0.37 ± 0.9% | 0.31 ± 1.0% |
| 95% CI | 0.57 to 0.83% | −1.53 to 2.27% | −1.71 to 2.33% |

SD standard deviation, CV coefficient variation, CI confidence interval.
mutations were analysed and showed a lower NPA and OPA due to the higher sensitivity of the dEGFR39 assay. Take T790M as an example: two more positive cases were detected by dEGFR39 than ARMS-PCR, reducing its OPA to 93.81% (95% CI 80.39–98.32%), while the OPA of other mutations was 96.97% (Table 3 and Supplementary Table S10–12). This experiment indicates that the dEGFR39 assay is sensitive and specific enough to detect more EGFR mutations than other methods.

Correlation between EGFR mutations detected by dEGFR39 and response to treatment

We analysed the abundance of EGFR mutations before and after TKI treatment. Prior to treatment, multiple plasma samples were collected for the initial assessment. dEGFR39 assay was subsequently performed to monitor the EGFR mutation undergoing multiple lines of different treatment (Fig. 4 and Supplementary Table S13–15). Although 20ins and L858R were detected by both dEGFR39 assay and superARMS PCR in patient P-07 at the baseline, there was still a great benefit in taking gefitinib, consistent with the dEGFR39 result at 450 days (Fig. 4c). Patient P-04 received first-line treatment with icotinib, and was diagnosed as PD at 45 days after treatment, and the subsequent combination therapy was not effective. The mutant abundance of patient P-12, who initially harboured a 19Del mutation, slowly decreased with treatment while the OPA of other mutations was 96.97% (95% CI 80.39–99.41%), while the OPA of other mutations was 96.97% (Table 3 and Supplementary Table S10–12). This experiment indicates that the dEGFR39 assay is sensitive and specific enough to detect more EGFR mutations than other methods.

Table 2. Concordance of dEGFR39 for EGFR mutations in FFPE tissues as compared to ARMS.

| Mutant type | Patient count | PPA (95% CI) | NPA (95% CI) | OPA (95% CI) |
|-------------|---------------|--------------|--------------|--------------|
| TP | FN | TN | FP |
| L858R/L861Q | 15 | 0 | 14 | 1 | 100 | 93.33 | 96.67 |
| | | | | | 79.61–100% | 70.18–98.81% | 83.33–99.41% |
| S768I | 8 | 0 | 19 | 3 | 100 | 86.36 | 90 |
| | | | | | 67.56–100% | 66.66–95.25% | 74.38–96.54% |
| G719X | 1 | 0 | 29 | 0 | 100 | 88.30–100% | 100 |
| | | | | | 20.65–100% | 100 | 88.65–100% |
| 19Del | 2 | 0 | 28 | 0 | 100 | 87.94–100% | 100 |
| | | | | | 34.24–100% | 100 | 88.65–100% |
| 20ins | 5 | 1 | 19 | 5 | 83.33 | 79.17 | 80.00 |
| | | | | | 43.65–96.99% | 59.53–90.76% | 62.69–90.50% |
| T790M | 1 | 0 | 26 | 3 | 100 | 89.66 | 90 |
| | | | | | 20.65–100% | 73.61–96.42% | 74.38–96.54% |
| Overall | 17 | 1 | 3 | 9 | 94.44 | 25 | 66.67 |
| | | | | | 74.24–99.01% | 8.89–53.23% | 48.78–80.77% |

TP true positive, FN false negative, TN true negative, FP false positive, PPA positive predict agreement, NPA negative predict agreement, OPA overall predict agreement, CI confidence interval.

Table 3. Concordance of dEGFR39 for plasma EGFR mutations compared to ARMS.

| Mutant type | Patient count | PPA (95% CI) | NPA (95% CI) | OPA (95% CI) |
|-------------|---------------|--------------|--------------|--------------|
| TP | FN | TN | FP |
| L858R/L861Q | 6 | 0 | 26 | 1 | 100 | 96.29 | 96.97 |
| | | | | | 60.97–100% | 81.72–99.34% | 84.68–99.46% |
| S768I | 7 | 1 | 25 | 0 | 85.71 | 100 | 96.97 |
| | | | | | 52.91–97.76% | 86.68–100% | 84.68–99.46% |
| G719X | 1 | 0 | 32 | 0 | 100 | 100 | 100 |
| | | | | | 20.65–100% | 89.28–100% | 89.57–100% |
| 19Del | 0 | 0 | 33 | 0 | NA | 100 | 90 |
| | | | | | 89.57–100% | 100 | 89.57–100% |
| 20ins | 1 | 0 | 32 | 0 | 100 | 100 | 100 |
| | | | | | 20.65–100% | 89.28–100% | 89.57–100% |
| T790M | 3 | 0 | 28 | 2 | 100 | 93.33 | 93.94 |
| | | | | | 43.85–100% | 78.68–98.15% | 80.39–98.32% |
| Overall | 15 | 1 | 14 | 3 | 9375.00 | 82.35 | 87.88 |
| | | | | | 71.67–98.89% | 58.97–93.81% | 72.67–95.18% |

TP true positive, FN false negative, TN true negative, FP false positive, PPA positive predict agreement, NPA negative predict agreement, OPA overall predict agreement, CI confidence interval.
Of the 33 enrolled patients with NSCLC, 13 samples from eight patients were identified by dEGFR39 to harbour the T790M mutation over time; of these, only nine (69%) were detected using SuperARMS. In the plasma of patient P-15, L858R and T790M mutations were simultaneously detected after 368 days of Icotinib treatment. SuperARMS PCR, however, failed to detect the T790M mutation; moreover, there was no significant progress evident in imaging until 121 days (Fig. 4b). Similarly, in patient P-25, dEGFR39 results showed an increase in mutant abundance in 19Del and T790M after 251 days of TKI treatment, which was 43 days ahead of that of imaging (Fig. 4e). In patient P-23, the emergence of L858R was initially observed and dropped upon Icotinib treatment. Meanwhile, T790M was detected by dEGFR39, which gradually increased with Icotinib as well as Apatinib plus chemotherapy treatment. In this case, detection of T790M was comparatively delayed by superARMS PCR and CT imaging (Fig. 5). Taken together, this data supports the conclusion that detection of T790M mutations by dEGFR39 occurs relatively earlier than by superARMS PCR and CT imaging, which can play a role in diagnosis and prognosis (Fig. 4f).

**DISCUSSION**

Early detection of *EGFR* mutations promises more precise therapy for patients with NSCLC. Using digital PCR, we developed a plasma-based noninvasive method, named dEGFR39, that detects multiple mutations of *EGFR*. To our knowledge, this is the first report regarding the utilisation of digital PCR for this purpose.

Since digital PCR usually has two channels of fluorescence, the system is primarily used for allele mutations in duplex assays because each specialised fluorescent probe can only recognise single allele mutations. Based on labelling the same fluorescent probe for each mutation, seven common KRAS mutations...
were consistently detected in plasma from patients with colorectal cancer (CRC).\(^{27}\) Moreover, multiple mutations can be recognised by different clusters, which depend on different corresponding concentrations of input primer and probe.\(^{28,29}\) However, it remains challenging to identify the cross region of clusters or define the mutation type when the DNA abundance is low.\(^{30}\) In contrast, the dEGFR39 assay is ideal for detecting samples with low DNA content, such as plasma. When the template content is too high in the reaction, some additional double-positive signals will appear, which can lead to mistakes in calculating mutation abundance. As shown in Supplementary Fig. S5, due to the large amount of template added, a part of S768IMU and 19DelMU are in one droplet, and the fluorescence intensity of these double-positive signals is different from that of true 19DelWT. Therefore, it is clearly divided into two regions, wherein the signal enclosed by the red dotted line is considered to be generated by S768IMU and 19DelMU template amplification. Furthermore, according to the formula provided in this method, the lower the concentration within the confidence range, the lower the frequency of false double-positive signals. The dEGFR39 assay takes advantage of the low abundance of DNA in plasma, making the test results more accurate.

Another study reported a drop-off method to achieve multiple detections; although this method can detect mutations located together, it fails to simultaneously detect other mutation types.\(^{36}\) To overcome these challenges, we have devised a three-step reaction by optimising annealing temperature, combined probe concentrations, and modifying system configuration with fluorescent compensation (Supplementary Fig. S9). After these improvements, dEGFR39 exhibits superior performance to profile the driver mutations of the EGFR gene. It is important to note that our data show that accuracy for dEGFR39 from plasma and ARMS from FFPE is 87.88%, which is similar to previous studies (80.8–94.19\%).\(^{31–33}\)

Importantly, it has been previously reported that digital PCR has technical issues such as the phenomenon of “rain”, which ranges between explicit positive and negative droplets. This issue eventually hinders the correct setting of threshold and consequently leads to failure of the digital PCR experiment.\(^{34,35}\) In this study, we induced a “double positive” discrimination method in the detection of 19Del, 20ins and C797S, which made the distribution of rain primarily in the diagonal area in the 2D plot diagram, thus avoiding interference with the identification of positive droplets. This approach greatly facilitated multiplex detection and led to less background noise (Fig. 2). Although it was minimal, the rain phenomenon still occurred. While detecting 20ins mutant locus from patient-derived FFPE tissues, we observed an increase in LoB and low correlation with the accuracy of 66.67% (95% CI 48.78–80.77\%). Interestingly, while using patient-derived plasma, we observed a significant improvement in the ratio of signal-to-noise. We reason that FFPE, but not plasma, might potentially contain an inhibitor for digital PCR analysis.

Several studies have demonstrated that mutant abundance of EGFR is closely associated with the response to treatment.\(^{36}\) More importantly, dEGFR39 is more sensitive than superARMS PCR and CT imaging, thus allowing early detection of EGFR mutations. Of note, some patients, although positive for EGFR driver mutations, received no clinical benefit from EGFR-TKI treatment (Fig. 4). In these patients, we observed an obvious trend of increasing mutation load.

Another comparative advantage of the dEGFR39 assay is low sample input. Whereas the commercial EGFR kit (ARMS method) requires detection of all EGFR mutations in eight tubes,\(^{37}\) dEGFR39 uses three reactions to sufficiently characterise the EGFR mutation status even from plasma-derived DNA. In future studies, assessment of EGFR, ALK, ROS1 and perhaps other oncogenes can be streamlined in the same digital PCR platform. Although NGS can parallelly analyse multiple variations, including unknown variations, digital PCR is still the most suitable method in clinical testing, because of its higher sensitivity, easier-to-understand results, low turn-around time and low cost.

In conclusion, we developed a noninvasive plasma-based digital PCR method, hereby named dEGFR39, that allows simultaneous detection of multiple mutation sites of the EGFR gene. In this report, we provide evidence that this method is highly sensitive, reliable and cost-efficient, promising efficacy for clinical diagnosis and treatment assessment for patients with NSCLC.

**AUTHOR CONTRIBUTIONS**

Study design: X.S., J.G., L.C. Patient enrolment and patient data collection: X.S., X.L.Z. Performing experiments and data analysis: J.G., X.S., L.C., M.G., X.Y.F., H.H. Manuscript preparation: J.G., X.S., L.C. All authors discussed the results and implications, and critically revised and approved the final manuscript.
ADDITIONAL INFORMATION

Ethics approval and consent to participate This study was approved (cch-BOC-1800020) by the ethics committee of Cangzhou Central Hospital, Hebei, China, and all patients provided written informed consent. This study was conducted in accordance with the Declaration of Helsinki.

Consent to publish Not applicable.

Data availability The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests The authors declare no competing interests.

Funding information This work was funded by CIP program from Stilla technologies Co., Ltd.

Supplementary information is available for this paper at https://doi.org/10.1038/s41416-020-1024-2.

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