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PAPER IN FOREFRONT

Highly sensitive and specific real-time PCR by employing serial invasive reaction as a sequence identifier for quantifying EGFR mutation abundance in cfDNA

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Abstract

Detection of EGFR mutations in circulating cell-free DNA (cfDNA) is beneficial to monitor the therapeutic effect, tumor progression, and drug resistance in real time. However, it requires that the mutation detection method has the ability to quantify the mutation abundance accurately. Although the next-generation sequencing (NGS) and digital PCR showed high sensitivity for quantifying mutations in cfDNA, the use of expensive equipment and the high-cost hampered their applications in the clinic. Herein, we propose a highly sensitive and specific real-time PCR by employing serial invasive reaction as a sequence identifier for quantifying EGFR mutation abundance in cfDNA (termed as qPCR-Invader). The mutation abundance can be quantified by using the difference of Ct values between mutant and wild-type targets without the need of making a standard curve. The method can quantify a mutation level as lower as 0.1% (10 copies/tube). Thirty-six tissue samples from non-small-cell lung cancer (NSCLC) patients were detected by our method and 14/36 tissues gave EGFR L858R mutation-positive results, whereas ARMS-PCR just identified 12 of L858R mutant samples. The two inconsistent samples were confirmed as L858R mutant by pyrophosphorolysis-activated polymerization method, indicating that qPCR-Invader is more sensitive than ARMS-PCR for mutation detection. The L858R mutation abundances of 19 cfDNA samples detected by qPCR-Invader were close to that from NGS, indicating our method can precisely quantify mutation abundance in cfDNA. The qPCR-Invader just needs a common real-time PCR device to accomplish quantification of EGFR mutations, and the fluorescence probes are universal for any target detection. Therefore, it could be used in most laboratories to analyze mutations in cfDNA.

Keywords EGFR mutations · Mutation abundance detection · Real-time PCR · Invader reaction · Circulating cell-free DNA

Introduction

Lung cancer is the leading cause of death among malignant tumors, and non-small-cell lung cancer (NSCLC) accounts for over 80% of the reported deaths [1]. Epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs), including gefitinib, erlotinib, and afatinib, can greatly improve treatment response and prolong progression-free survival (PFS) in NSCLC patients [2, 3]. However, these targeted drugs are only effective in patients with EGFR activating mutations, such as a small in-frame deletions in exon 19 and L858R mutation in exon 21 [4]. Therefore, precisely detecting EGFR mutations is necessary prior to the use of targeted drugs. Conventionally, biopsy of tumor tissue was the main way to identify targeted drug-related mutations [5]. Due to the heterogeneity of the tissue, the mutation detection methods should be very sensitive and specific to pick up mutant targets from a large amount of

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wild-type DNAs. Although many methods had been developed to identify the EGFR mutation types from tumor tissues, such as amplification refractory mutation system-PCR (ARMS-PCR) [6], high-resolution melting (HRM) [7], and pyrosequencing [8], it is very difficult to monitor the mutation changes by using biopsy, because the tumor tissues cannot be obtained in real time.

The discovery of circulating cell-free DNA (cfDNA) makes it possible to monitor mutation biomarkers in real time. By detecting the mutations in cfDNA, we can evaluate the therapeutic effect [9], predict tumor progression [10], and monitor drug resistance [11]. However, detection of the mutations in cfDNA is more difficult than that in tumor tissues; it requires the detection methods not only to accurately identify the type of tumor-related mutations, but also to precisely quantify the mutation abundance. Since only the changes of mutation abundance can reflect the tumor progression and the therapeutic effect.

Currently, digital PCR [12, 13] and next-generation sequencing (NGS) [14, 15] are the main methods to detect the mutation abundance in cfDNA due to their high sensitivity and high specificity, which can accurately quantify as low as 0.1% mutation abundance in cfDNA. However, both methods require specialized instruments, such as digital PCR device and NGS sequencer, and the detection cost is still high. ARMS-PCR is a preferable method for quantitatively detecting mutations, because it is cost effective and just needs a real-time PCR device to accomplish target amplification and results readout. However, it is challengeable for conventional ARMS-PCR to detect mutants in cfDNA due to its insufficient specificity. Usually, the conventional ARMS-PCR can detect as low as 1% mutation abundance [6], but the mutation abundance in cfDNA may be less than 1% [16]. Although some strategies, such as using locked nucleic acid (LNA) [17] or peptide nucleic acid (PNA) [18] probes to clamp wild-type templates amplification, could improve the specificity of ARMS-PCR to achieve highly sensitive detection of mutants in cfDNA, it is difficult for them to quantify the mutation abundance accurately, because the amplification bias exists in the clamp-PCR. To overcome the issue, the mutant and the wild-type templates should be amplified with equal amplification efficiency. The best way to achieve this is to use one pair of primers to amplify both mutant and wild-type templates. However, this requires a highly specific sequence identifier to discriminate and report the mutant and wild-type amplicons.

Invasive reaction catalyzed by flap endonuclease 1 (FEN1), which could recognize the structure of an upstream probe invading one base to the double-strand region formed by a downstream probe and a target and cut the flap fragment of the downstream probe, is an ideal sequence identifier to discriminate the single base difference between wild-type DNA and mutant DNA [19]. Katsuhiko Naoki [20] employed serial invasive reaction to detect EGFR mutations in PCR products by comparing the fluorescence signals from the reactions with wild-type probe and mutant probe. Although the sensitivity reached 1~0.1% mutant alleles in total DNA targets, the endpoint detection of the method could not quantify the mutation abundance. Most importantly, the open-tube operation greatly increases the risk of cross-contamination from PCR amplicons. To realize close-tube detection of EGFR mutations, we previously developed a visualization method for mutation detection by coupling PCR amplification and the invasive reaction with gold nanoparticle-modified DNA probes (GNP) [21]. Beneficial from the high sensitivity of PCR, the high specificity of invasive reaction, and the features of GNP, as low as 0.1% EGFR mutant DNA could be identified from a large amount of wild-type DNA, and the detection limit reached to detect six copies of mutant targets. A small amount of EGFR mutations in cfDNA were successfully detected by naked eyes with the method. However, this method is also an endpoint detection and difficult to achieve quantification of mutation abundance.

Here, we proposed a real-time PCR assay by employing serial invasive reaction as a sequence identifier to identify and report mutant and wild-type PCR amplicons in each PCR cycle (termed as qPCR-Invader) for sensitively and specifically quantifying the abundance of EGFR mutations in cfDNA. The serial invasive reaction could specifically identify the mutant and wild-type PCR amplicons and generate the target-specific fluorescence signals in each PCR cycles, resulting in two real-time amplification curves for mutant and wild-type targets, respectively. Consequently, the relative abundance of mutant targets could be quantified by comparing the Ct values of mutant and wild-type targets without making a standard curve. Our method enables quantifying the EGFR mutations in cfDNA with a common real-time PCR device, and the close-tube reaction effectively reduces the cross-contamination of amplicons. We believe this method is well suitable for detection of the relative content of EGFR mutations in cfDNA.

Materials and methods

Clinical samples and DNA extraction

DNA was extracted from peripheral blood using a Whole Blood Genomic DNA Extraction Kit (TaKaRa, Japan) and from tumor tissues with the QIAamp Fast DNA Tissue Kit (Qiagen, Germany) according to the manufacturer's instructions. PCR products were extracted using the EasyPure PCR Purification Kit (TransGen Biotech, China) and quantified by ultraviolet spectrophotometry (One-drop, OD-1000+, Shanghai, China). Plasma-derived circulating cell-free DNA was extracted using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Germany) using the manufacturer's instructions. All clinical samples were remainders from conventional clinical tests, and used with ethical approval from Nanjing Jinling Hospital.

Primer and probe design

The primers and probes based on target sequence were designed using Universal Invader™ software. All oligonucleotides were synthesized Invitrogen Corporation (Shanghai, China). The sequences were shown in Table 1.

Quantification of the artificial DNA target

The real-time PCR assay was performed on ABI stepone PCR system. The 20 μL PCR reaction mixture contained 1 × SYBR Green qPCR SuperMix (Invitrogen), 500 nM each primer, and Rox dye according to the manufacture's. Real-time PCR conditions were 3 min at 95 °C, followed by 40 cycles of denaturation for 20 s at 95 °C and annealing/extension for 60 s at 70 °C. Human genomic DNA was quantified by UV, and then was gradient diluted as a quantitative standard curve. The concentration of the artificial DNA target was quantified by the standard curves.

qPCR-Invader system and procedure

The qPCR-Invader performed the cascade invader reaction simultaneously in the annealing step of PCR amplification. The reaction mixture (20 μL) contained 10 mmol/L Tris-HCl (pH 8.0), 0.05% (*v*/*v*) NP-40 (Amresco, USA), 0.05% (*v*/*v*) Tween-20 (Amresco, USA), 12 μg/mL BSA (Amresco, USA), 7.5 mmol/L $MgCl_2$, 200 μmol/L of each dNTP (SBS Genetech Co., Ltd., China), 0.4 μmol/L of each primer, 1 U of *Taq* Polymerase (Promega, USA), 6.7 ng/μL *Afu* flap endonuclease (*Afu* flap endonuclease was prepared in our lab [22]), 50 nmol/L invasive oligo, 250 nmol/L of each allele-specific probe, 250 nmol/L of each FRET probe, and 1 μL of the target. The amplification reaction consisted of an initial denaturation at 94 °C for 3 min, followed by 10 cycles of 94 °C for 20 s, 67 °C for 30 s, and 70 °C for 30 s, followed by 35 cycles of 94 °C for 20 s, 63 °C for 60 s, and 70 °C for 30 s. The fluorescence intensity was measured at each annealing step at 63 °C using a Rotor-Gene Q 48 Real-Time PCR System (Qiagen, America).

Table 1 Sequences of primers and probes

Oligo[a]	Sequence(5′-3′)
FRET-FAM	FAM-TCTT (BHQ1) AGCCGGTTTTCCGGCTAAGACTCCGCGTC CGT-C6-NH_2
FRET-VIC	VIC-TCTT (BHQ1) AGCCGGTTTTCCGGCTAAGACCTCGG CGCG-C6-NH_2
L858R-F	GGAACGTACTGGTGAAAACACCGC
L858R-R	TGCATGGTATTCTTTCTCTTCCGCACC
L858R-UP	CATGTCAAGATCACAGATTTTGGGCC
L858R-MP	CGCGCCGAGGGGGCCAAACTGCTG-PO_3
L858R-WP	ACGGACGCGGAGTGGCCAAACTGCTG-PO_3

[a] F, forward PCR primer; R, reverse PCR primer; UP, upstream probe (invasive oligo probe); MP, downstream probe for mutant template; WP, downstream probe for wild template

Results and discussion

Principle of qPCR-Invader

The schematic overview of the qPCR-Invader is demonstrated in Fig. 1. The mutant and wild-type templates are amplified with a pair of primers. After 10 cycles of pre-amplification, in which the reaction temperature is over 67 °C, serial invasive reactions are occurred in each PCR annealing step by lowering the annealing temperature to 63 °C, which is close to the melting temperature of detection probe in serial invasive reaction. In this step, an invader oligo and a detection probe are annealed to the target DNA in PCR amplicons, forming a 3-bases overlapping at the mutation site of the target (in this case "A > G"). The FEN1 recognizes this overlapping structure and cleaves the 5′ flap of the detection probe. As the temperature of the annealing step is close to the melting temperature of the detection probe, an intact detection probe will hybridize to the target DNA again, producing amplified flaps. The mutant and the wild-type target DNA produce flaps with different sequences (flap 1 and flap 2 corresponding to the mutant and the wild-type target DNA, respectively). Then, the released flap 1 and flap 2 can anneal to their corresponding fluorescence resonance energy transfer (FRET) probes to form the overlapping structure again, triggering the second invasive reaction to cut the fluorophores of the FRET probes generating corresponding fluorescence signals (VIC for flap 1 and FAM for flap 2). The fluorescence signals are monitored in each PCR cycle to obtain real-time amplification curves of the mutant and the wild-type target DNA. The mutation abundance can be calculated according to the ΔCt value (Ct (M)-Ct (W)). The Ct values of mutant DNA (Ct (M)) and wild-type DNA (Ct (W)) were expressed as the following formulas: $Ct\ (M) = -1/\log(1 + E_x) * \log X_{0(M)} + \log N_M/\log(1 + E_x)$ (Formula 1), $Ct\ (W) = -1/\log(1 + E_x) * \log X_{0(W)} + \log N_W/\log(1 + E_x)$ (Formula 2) [23], $X_{0(M)}$ and $X_{0(W)}$ are the amounts of the initial mutant template and wild-type template, respectively; N_M and N_W are the amounts of the amplified products of mutant template and wild-type template respectively when the fluorescence intensity reaches the threshold intensity; E_x is the amplification efficiency of PCR, which is same for mutant template and wild-type template due to the use of same primers to amplify the both targets in PCR. Thus, $\Delta Ct = Ct\ (M) - Ct\ (W) = -1/\log\ (1 + E_x) * \log\ (X_{0(M)}/X_{0(W)}) + (\log N_M - \log N_W)/\log\ (1 + E_x)$ (Formula 3). Therefore, the ΔCt is linearly related to the log value of

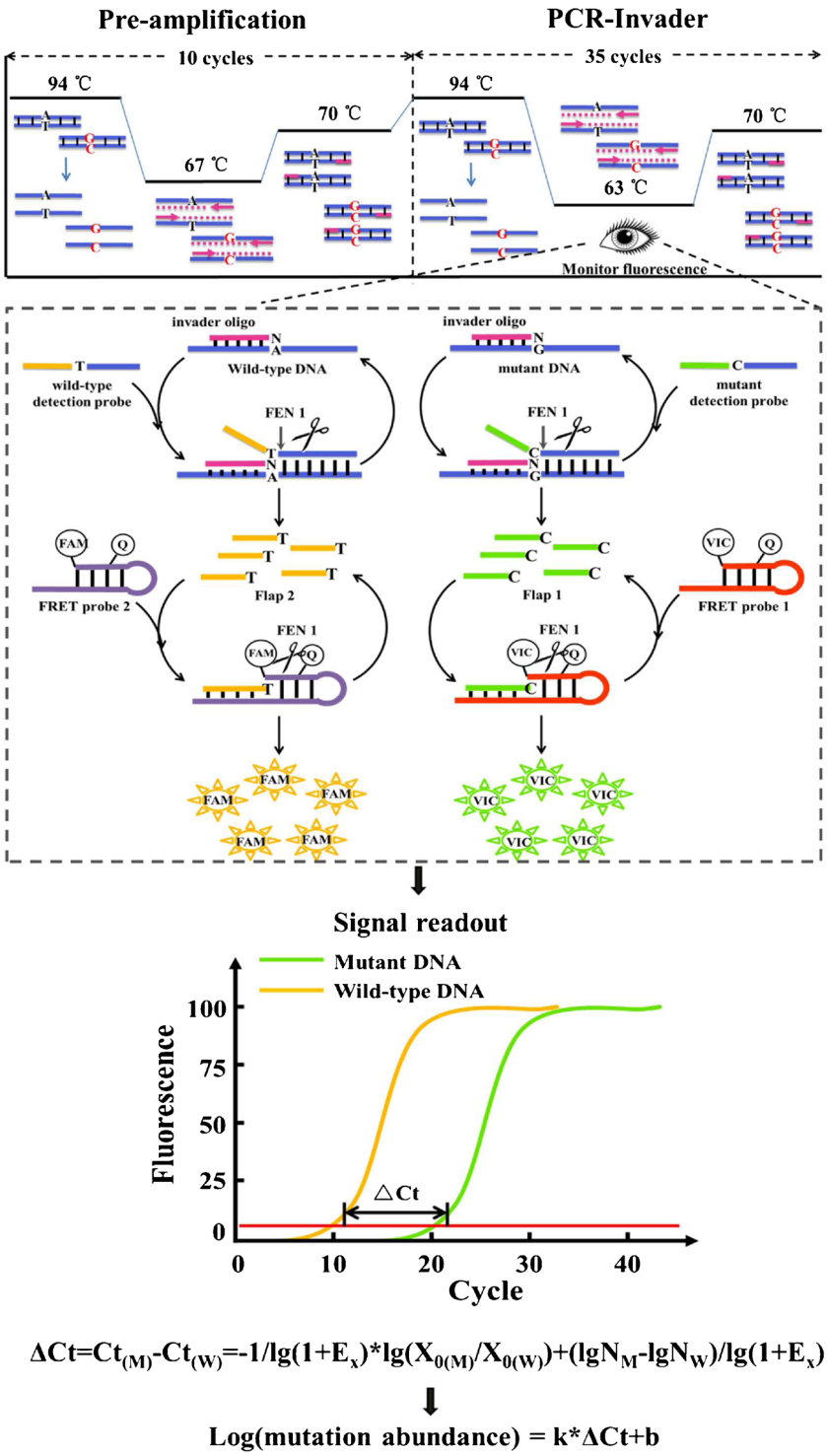

Fig. 1 Schematic of qPCR-Invader method. Mutant DNA and wild-type DNA were pre-amplified with a pair of primers for 10 cycles. Then, serial invasive reactions are occurred in each PCR annealing step by lowering the annealing temperature to 63 °C for 35 cycles. Fluorescence signals (FAM and VIC) generated by the invader reaction are monitored in each PCR annealing step. The amplification curves for mutant and wild-type targets can be obtained after the reaction and the mutation abundance can be quantified according to the △Ct value

the initial template ratio of mutant DNA and wild-type DNA. For an optimized reaction condition, the E_x, N_M, and N_W are constant, so that we can obtain the values of $-1/\log(1+E_x)$ and $(\log N_M - \log N_W)/\log(1+E_x)$ by detecting a serial of artificial templates with different $X_{0(M)}/X_{0(W)}$. The mutation abundance of a sample can be calculated according to the ΔCt value without the need of making a standard curve.

Optimization of qPCR-Invader system

The activity of *Afu* flap endonuclease could affect the recognition capability and cleavage efficiency. We investigated the efficiency of different concentrations of *Afu* flap endonuclease on qPCR-Invader reaction (see Electronic Supplementary Material (ESM) Fig. S1) using L858R mutant DNA. The catalysis efficiency was improved with an increase of *Afu* flap

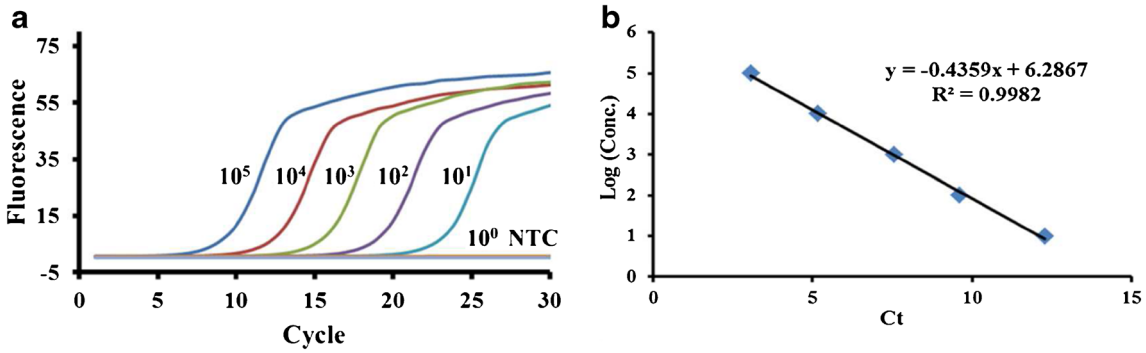

Fig. 2 Sensitivity and linear range of qPCR-Invader. **A** Amplification results of L858R mutant DNA at different concentrations. **B** Results of linear regression ranged from 10 to 10^5 copies of L858R mutant DNA per tube

endonuclease. The number of cycles at the concentration of 160 U was almost the same with the one at the concentration of 80 U. Therefore, we added 80 U of *Afu* flap endonuclease into the 20-μL reaction system.

Optimal concentration of *Taq* DNA polymerase needed for efficient PCR amplification was investigated (see ESM Fig. S2) using L858R mutant DNA as templates. Results showed that the amplification efficiency decreased when increasing *Taq* DNA polymerase, indicating the inhibition of invader reaction caused by *Taq* DNA polymerase. In addition, the amplification efficiency at when using 0.25 U of *Taq* DNA polymerase was lower than when using either 0.5 or 1 U. Therefore, we used 0.5 U of *Taq* DNA polymerase in the 20-μL reaction system.

In the invader reaction, the concentration of detection probes could also affect the recognition capability and cleavage efficiency. We investigated the different concentrations of detection probes on invader reaction efficiency (see ESM Fig. S3) using 1% L858R mutant DNA and L858R wild-type DNA (0% L858R mutant DNA) as templates. Results showed that the amplification efficiency increased when increasing the amount of detection probes in the range of concentrations tested (125 to 500 nM). However, the cleavage efficiency of invader reaction was also affected when the amount of detection probe was increased. Excessive detection probes may produce an X-structure with FRET [19], resulting in a high background signal as observed when target concentration was 500 nM (see ESM Fig. S3C). The optimized concentration of 250 nM of each allele-specific detection probe was used in 20-μL reaction system.

Sensitivity and linear range of qPCR-Invader

To investigate the sensitivity and the linear range of the proposed methodology for detecting mutant DNA, we used serial dilutions of L858R mutation DNA. The mutant templates were diluted using H_2O to achieve 10^5, 10^4, 10^3, 10^2, 10, and 1 copies/μL and 1 μL of the mutant templates was used in 20 μL reaction. All reactions were prepared in triplicates. The sensitivity and the linear range demonstrated that 10 copies of mutant DNA templates per tube were needed for successful detection (Fig. 2A and B).

Then, we investigated the sensitivity and the linear range for simultaneous detection of mutant and wild-type DNA. The mutant templates were diluted using H_2O to 10^5, 10^4, 10^3, 10^2, and 10 copies/μL, and the wild-type templates were 10^5 copies/μL. By spiking mutant templates into wild-type templates at different copy mixtures, we obtained mutant template to wild template ratios of 1:1, 1:10, 1:100, 1:1000, 1:10000, and 0:1 (the mutation abundance were 50, 10, 1, 0.1, 0.01, and 0%). The sensitivity and the linear range were displayed in Fig. 3A and B.

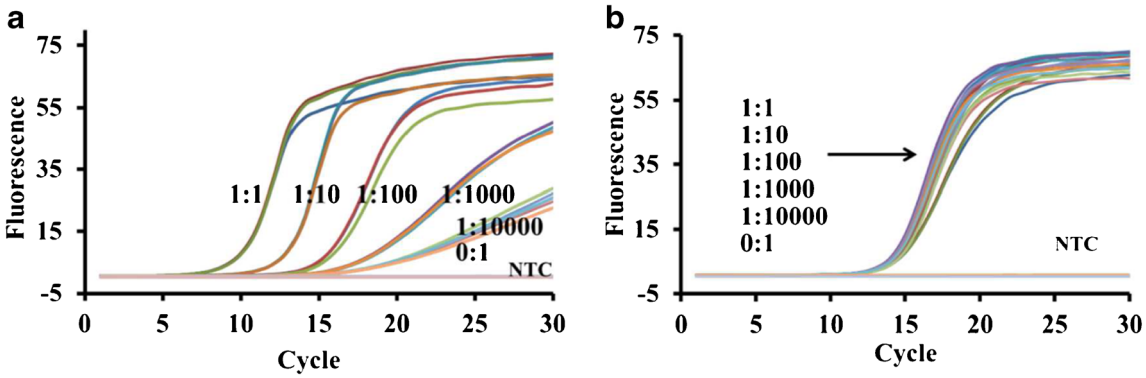

Fig. 3 The detection of targets with different mutation ratios by qPCR-Invader. **A** Amplification results of mutant DNA at different proportion mixture templates. **B** Amplification results of wild-type DNA at different proportion mixture templates. 1:0, 1:1, 1:10, 1:100, 1:1000, 1:10000, 0:1 represented the mixture templates with the proportions of mutant template and wild template were 1:0 (100%), 1:1 (50%), 1:10 (10%), 1:100 (1%), 1:1000 (0.1%), 1:10000 (0.01%), and 0:1 (0%), under the concentrations of the wild templates were 10^5 copies/μL

Results demonstrated that templates containing as low as 0.1% mutant fragments were successfully detected in the presence of wild-type alleles, while also being distinguished by threshold with the non-specific signals caused by wild-type alleles. Although there was a non-specific background signal, the ΔCt values of 0.1% mutant fragments and the ΔCt values of background signal caused by wild templates had significant difference ($P < 0.05$) (see ESM Fig. S4). However, 0.01% mutant fragments could not be distinguished from the non-specific signals caused by wild-type alleles.

Mutation abundance quantification of qPCR-Invader

Since the values of E_x, N_M, and N_W are constant for an optimized reaction condition, the ΔCt is linearly related to the log value of the mutation abundance according to the Formula 3. In order to obtain the linear equation of ΔCt and log ($X_{0(M)}$/$X_{0(W)}$), we detected a serial of artificial templates with different mutation abundance. The mutant DNA were diluted with H_2O to 10^5, 10^4, 10^3, 10^2, and 10 copies/μL, and the wild-type DNA were diluted with H_2O to 10^6, 10^5, and 10^4 copies/μL. By spiking mutant DNA templates into wild-type DNA templates at the different copy numbers, we got mixed templates with the mutation abundance of 0.1, 1, 10, and 50% while the concentration of wild-type DNA templates was 10^6, 10^5, and 10^4 copies/μL, respectively. For each wild-type templates concentration, the mixed templates with the mutation abundance of 0.1, 1, 10, and 50% were detected in triplicates by qPCR-Invader, respectively. The results showed a good linear relationship between the mutation abundance and the ΔCt value ($R^2 = 0.998$) and the linear equation was -log ($X_{0(M)}$/$X_{0(W)}$) = 0.2946 * ΔCt + 1.674 (Formula 4) (Fig. 4A). Therefore, the mutation abundance of a sample can be calculated according to the Formula 4 without the need of making a standard curve every time.

To investigate the quantitative accuracy of the method, analog samples with different mutation abundance were prepared. For each concentration group, we added 2 μL of analog sample into a 20-μL reaction system. The ΔCt was calculated and converted into the measured mutation abundance according to the Formula 4. The detections were carried out in triplicates for each concentration of mixed templates. A box graph with the logarithm of the measured concentration as ordinate and the logarithm of the theoretical concentration as abscissa was plotted (see Fig. 4B). The R^2 is 0.9927, with the slope of 0.9915 and the intercept of 0.0188, indicating a good quantitative accuracy of the method. Thus, the method can be applied to quantify mutation abundance with high sensitivity and accuracy without a standard curve.

Table 2 The detection results of EGFR-L858R mutations in 36 tissue samples by qPCR-Invader and ARMS-PCR, respectively

Detection method	qPCR-Invader			
		Wild	Mutation	Case no.
ARMS-PCR	Wild	22	2	24
	Mutation	0	12	12
	Case no.	22	14	36

Assay validation by detecting clinical samples

In order to verify the accuracy of this method, we performed the qPCR-Invader on clinical tumor samples. First, the method was performed by detecting somatic mutations in tissue samples with L858R (c.2573T>G) mutation. We analyzed 36 tissue specimens detected by ARMS in advance. All ARMS-positive samples were detected as positive, but two ARMS-negative samples were detected as L858R-positive (shown in Table 2) by our method. These two samples were confirmed as L858R-positive by more sensitive method pyrophosphorolysis-activated polymerization (PAP) [24], which can detect as low as 0.01% mutation fractions [25]. Therefore, we presumed that the higher sensitivity of our proposed detection system over the ARMS-PCR led to results achieved.

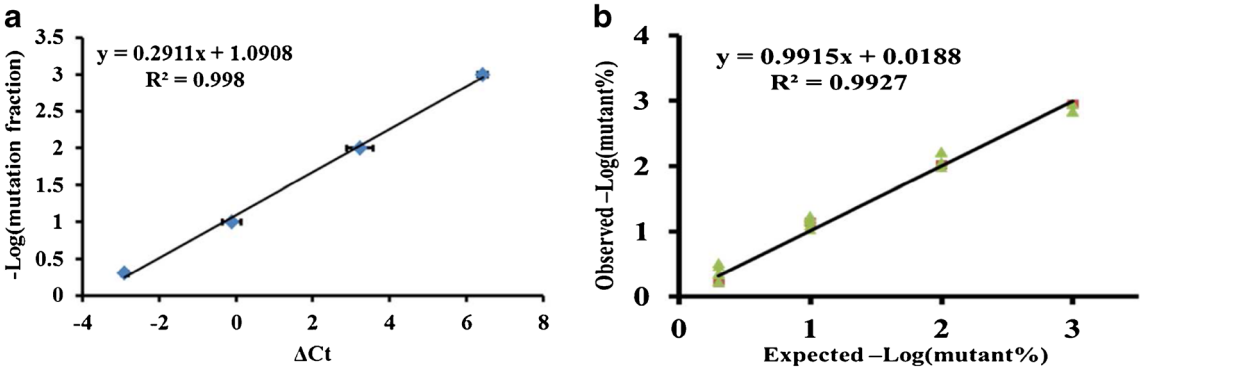

Fig. 4 Mutation abundance quantification of qPCR-Invader. **A** The quantitative relationship between the mutation abundance and the ΔCt value. **B** Quantitative accuracy of L858R mutation concentration. Delta Ct was defined as the difference between the Ct values of mutant and wild-type DNA in a reaction system

Table 3 The detection results of EGFR-L858R mutations in 19 plasma circulating cell-free DNA samples by qPCR-Invader and next-generation sequencing, respectively

Sample	qPCR-Invader (Mutant%)[a]	NGS (Mutant%)[b]
Plasma 1	NA[c]	NA
Plasma 2	3.77%	5.84%
Plasma 3	NA	NA
Plasma 4	NA	NA
Plasma 5	3.25%	5.77%
Plasma 6	86.00%	81.63%
Plasma 7	NA	NA
Plasma 8	11.70%	16.61%
Plasma 9	NA	NA
Plasma 10	NA	NA
Plasma 11	4.56%	6.00%
Plasma 12	NA	NA
Plasma 13	NA	NA
Plasma 14	NA	NA
Plasma 15	NA	NA
Plasma 16	NA	NA
Plasma 17	NA	NA
Plasma 18	NA	NA
Plasma 19	NA	NA

[a] The quantitative detection results of EGFR-L858R mutations in 19 plasma circulating cell-free DNA samples by qPCR-Invader

[b] The quantitative detection results of EGFR-L858R mutation in 19 plasma circulating cell-free DNA samples by high-throughput sequencing

[c] NA represents no mutation detected

In order to verify whether mutation abundance in cfDNA can be quantified using this method, we assayed circulating free DNA of patients with 19 NSCLC patients. The results were shown in Table 3. Five L858R (c.2573T>G) mutation-positive samples were detected among the 19 plasma samples. The mutation abundance of positive samples were almost consistent with the detection results of NGS. These results proved that the method can be used to detect mutation abundance in cfDNA in clinical settings.

Conclusion

In this study, we proposed a novel real-time quantification PCR by introducing serial invasive reaction to each PCR cycle for detecting the abundance of EGFR mutations in cfDNA. The mutant and wild-type targets were amplified with equal amplification efficiency and the mutant and wild-type PCR amplicons were specifically identified by the serial invasive reaction. The amplification curves for mutant and wild-type targets can be obtained by real-time PCR device, and the mutation abundance can be quantified by comparing the Ct values of mutant and wild-type targets without making a standard curve every time. The sensitivity of the method can reach 0.1% mutant targets in total DNA templates corresponding to 10 copies per tube. Thirty-six tissue samples from NSCLC patients were detected by our method and the EGFR L858R mutation was identified in 14/36 tissues. The 36 tissues samples were also detected by ARMS-PCR and just 12/14 L858R-positive samples were identified by ARMS-PCR. Another two samples were confirmed as L858R-positive by PAP method, indicating our method is more sensitive than ARMS-PCR. Nineteen cfDNA samples were detected by our method and the mutation abundance of 5/19 L858R-positive samples detected by our method were close to that from the NGS, indicating that our method can precisely quantify EGFR mutation abundance in cfDNA.

In qPCR-Invader, the specificity depends on the flap endonuclease1 (FEN1) to recognize an invasive structure formed by an upstream probe and a downstream probe, which can be easily designed by using an online software (Universal Invader™ software). For setting up a new assay, only the concentrations of downstream probe and FEN1 should be optimized. Therefore, the experiment set up of our method is relatively easy. We have also detected other EGFR mutations such as T790M, C797S, and insG by using corresponding amplification primers and detection probes (see ESM Fig. S5). Moreover, the fluorescence-labeled probes in our method are universal to any mutation sites leading to a lower cost than conventional methods, whose fluorescence-labeled probes are specific to targeted DNA and should be varied with different target sequences. In addition, qPCR-Invader enables quantifying the EGFR mutations in cfDNA with a common real-time PCR device, and the close-tube reaction effectively reduces the cross-contamination of amplicons. We believe the method enables quantifying EGFR mutation abundance in circulating cell-free DNA much more readily and could be valuable at clinical mutation detection.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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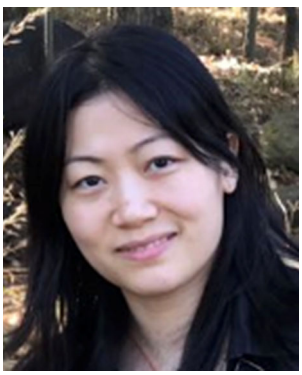

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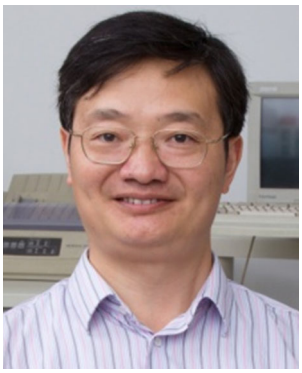

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