ACS APPLIED MATERIALS & INTERFACES

RESEARCH ARTICLE

www.acsami.org

Simple and Sensitive Method for Detecting Point Mutations of Epidermal Growth Factor Receptor Using Cationic Conjugated Polymers

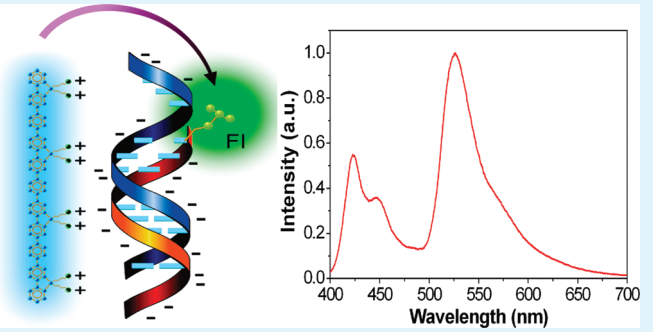
Qiong Yang,[*,†,‡] Tian Qiu,[§,‡] Wei Wu,[†] Chunlei Zhu,[†] Libing Liu,[†] Jianming Ying,[*,§] and Shu Wang[*,†]

[†]Institute of Chemistry, Chinese Academy of Sciences, Beijing 100190, China

[§]Department of Pathology, Cancer Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100021, China

Supporting Information

ABSTRACT: The L858R mutation of epidermal growth factor receptor (EGFR) in nonsmall cell lung cancer is associated with the increased sensitivity to EGFR tyrosine kinase inhibitors. In this paper, a simple and sensitive method for identification of L858R mutation in cell lines and tumor tissues was developed using cationic conjugated polymer-based fluorescence resonance energy transfer technology (CCP-based FRET). The new detection system can detect even as low as 4−8% mutation of the total DNA. Through the detection results for 48 DNA samples from tumor tissues, a sensitivity of 95.24% (20/21) and a specificity of 96.30% (26/27) were demonstrated. Further, the application of this method in clinical molecular diagnosis was validated by detecting T790 M in EGFR of 35 patients. In comparison with DNA sequencing and real-time PCR methods, our new protocol simplifies procedures by eliminating the need for primer labeling, cumbersome workups and sophisticated instruments and improves sensitivity by amplifying fluorescence signals. Our CCP-based FRET technology is particularly attractive because of its higher sensitivity, cost-effective, and simple characteristics. Particularly, this new method could confirm the suspected positive samples arisen by DNA sequencing and real-time PCR methods. Thus, the CCP-based FRET technology opens up an avenue for clinical therapy by guiding medication to lung cancer patients responsive to anti-EGFR therapy.



KEYWORDS: conjugated polymers, EGFR, DNA mutation, FRET, fluorescent sensor, mutation detection

1. INTRODUCTION

The epidermal growth factor receptor (EGFR) mutation status in clinical samples has become an important factor to clinical decisions and guides of anti-lung cancer treatment.[1−4] Three hotspots, which occur in exon 18, 19, and 21 of EGFR gene, respectively, are correlated with the response to gene-targeted kinase inhibitors such as gefitinib and erlotinib. Among them, 44% patients have a L858R point mutation (point substitution T > G) in exon 21, resulting in a leucine (L) to arginine (R) change of codon 858 in EGFR protein. Thus, many techniques have been developed for effective mutation detection.[5−8]

The traditional method for experimental and clinical detection of gene mutations is sequencing of DNA amplified from tumor cells and tissues. However, the sequencing needs sophisticated instrument and is time-consuming, which is not suitable for clinical large scale screening. In addition, the clinical samples for the isolation of genomic DNA often contain not only mutation tissue but also nonmutation cells and normal tissue, which cause mutation not to be found by DNA sequencing. To overcome the problem of lower sensitivity, several methods have been explored for the detection of EGFR mutation, including various PCR-based techniques,[6,8] such as single-strand conformation polymorphism,[1,9] DNA melting curve analysis[7] and enzymatic methods.[10,11] However, because of the requirements of special equipments, complicated procedures and expensive reagents, these methods are still not applied in routine clinical assays. A simple, inexpensive, sensitive, and accurate EGFR-mutation diagnosis method that could detect lower gene mutation of clinical samples in a large scale remains to be explored.

Conjugated polymers (CPs) contain a large number of absorbing units, and the transfer of excitation energy along the whole backbone of the CP to the chromophore reporter results in the amplification of fluorescence signals.[12] Therefore, CPs can be used as the optical platform in biosensors to increase detection sensitivity.[13−18] A series of water-soluble cationic conjugated polymers (CCP) have been designed and synthesized as fluorescent probes for sensitive DNA detections.[19−34] In this paper, we sought to develop a simple and robust molecular diagnosis method

Received: September 14, 2011
Accepted: October 25, 2011
Published: October 25, 2011

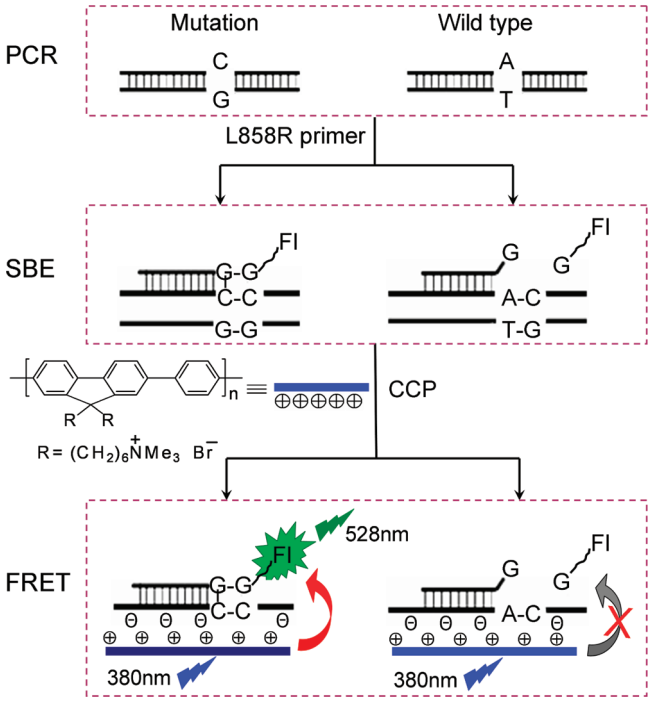


Figure 1. Schematic detection strategy using CCP-based FRET method. L858R primer is perfectly complementary to mutation EGFR gene rather than wild type DNA. As a result, the primer is extended by dGTP-Fl complementary to the base adjacent to mutation base when the extension primer- L858R primer can completely match the PCR product template. SBE indicates single base extension. FRET indicates fluorescence resonance energy transfer.

combining CCP and FRET to facilitate the detection of EGFR L858R mutation in cancer cell lines and clinical samples. Further, the application of this method in clinical molecular diagnosis was validated by detecting T790 M in EGFR of 35 patients. Therefore, the CCP-based detection system has opened up an avenue for routine clinical therapy by guiding medication to lung cancer patients responsive to anti-EGFR therapy.

2. RESULTS

2.1. Construction of CCP-Based FRET Technology for Mutation Detection in EGFR Gene. The overall detection strategy using CCPs is schematically illustrated in Figure 1. Cationic water-soluble poly{(1,4-phenylene)-2,7-[9,9-bis(6′-*N*,*N*,*N*-trimethyl ammonium)-hexyl fluorene] dibromide} (CCP) is selected as the optical probe. GPC analysis shows the molecular weight of CCP is about 15 000 amu. The CCP exhibits an absorption maximum at 380 nm and an emission maximum at 425 and 445 nm with a quantum yield of about 40%.[35] Fluorescein, with an absorption maximum at 488 nm and an emission maximum at 518 nm, was chosen as energy transfer donor because its absorption overlaps with the emission of CCP. Irradiation at 380 nm selectively excites CCP and FRET from CCP (donor) to fluorescein (acceptor) is favored. First, EGFR DNA fragments were amplified by PCR, then these PCR products were used as template of single base extension (SBE) reactions in order to incorporate dGTP-Fl to the 3′ end of L858R or L858 extension primer, and finally, CCP was added into the reaction mixture, which resulting in FRET occurrence from CCP to fluorescein only if the extension primers absolutely matched with the PCR products. In this study, the CCP-based method was established by optimizing the nested PCR for amplifying the targeted EGFR gene, SBE reactions of L858R and L858 primers and FRET experiments. Homozygous mutant and wild type control samples were created artificially by TA cloning (see Figure S1 in the Supporting Information). DNA sequencing results confirmed that the screened pGEM-T L858R and pGEM-T L858 contained pure L858R mutant and wild type EGFR DNA, respectively. Furthermore, EGFR DNA fragments with length of 180 bp (Figure 2a) were amplified by PCR, and then leftover primers and unincorporated dNTPs were removed by using 1 U of shrimp alkaline phosphatase (SAP), 1 U of *E. coli* exonuclease I and 0.5 U of pyrophosphatase (PPS). Finally, CCP was added as FRET donor after SBE reaction. FRET ratio was calculated according to fluorescence intensity of fluorescein at 528 nm to that of CCP at 440 nm with the selective excitation of CCP by light of 380 nm. The new CCP-based FRET technology can distinguish the mutation from wild type EGFR gene amplified from pEGM plasmids (Figure 2a). When L858R primers that correspond to mutant EGFR gene were used to SBE, FRET ratio of L858R mutation group was significantly higher than that of blank control, whereas FRET ratio of wild type group had not significant difference from control. Likewise, when L858 primers differing by a single base corresponding to the wild type rather than mutation site were used in this detection system, wild type gene PCR products from pGEM-T L858 showed higher FRET ratio; conversely, the mutation PCR products yielded background FRET ratio. Also, we recorded the emission fluorescence spectra of the single-base extension (SBE) reaction mixture in the presence of CCP (Figure 2b) to confirm the FRET process. When L858R extension primers were used, spectra of fluorescence emission yielded a characteristic sharp FRET peak -the emission peak of Fl (525 nm) for mutation products. In contrast, wild type PCR products remained at approximately background fluorescence, and no distinct FRET peak was observed when L858R extension primers were used. The efficiency of CCP-based FRET was also confirmed by L858 primer. When L858 primers were used, spectra of fluorescence emission yielded a sharp emission peak of Fl for wild type PCR products over mutation PCR products.

2.2. Sensitivity Analysis of CCP-Based FRET Technology for Mutation Detection in Mixed EGFR Gene Samples. Because tumor tissue samples frequently contain numerous normal tissue cells, a useful diagnostic method for mutation detection must be able to detect mutations in the background of wild type genomic DNA samples. To test the CCP-based FRET technology for this capability, we conducted serial mutation percentage experiments to determine detection limits using pGEM-T plasmids carrying wild type or L858R mutation EGFR DNA. The agarose-gel electrophoresis revealed that higher quantity PCR products were obtained (Figure 3a). Following the SBE reaction, this new method could detect mutations that only represented 4−8% of the total pure EGFR gene (Figure 3a). These data also generated a standard curve of FRET ratio to mutation percentage. The R^2 value is 0.9855 that confirms the quantitative nature (Figure 3b). The standard curves can be used to estimate the percentage of the mutation in the sample.

Two known cell lines were further used to investigate the sensitivity of the CCP-based FRET technology for the detection of gene mutation. The genomic DNAs from NCI-H1975, one lung cancer cell line with heterozygous TG mutation and A549,

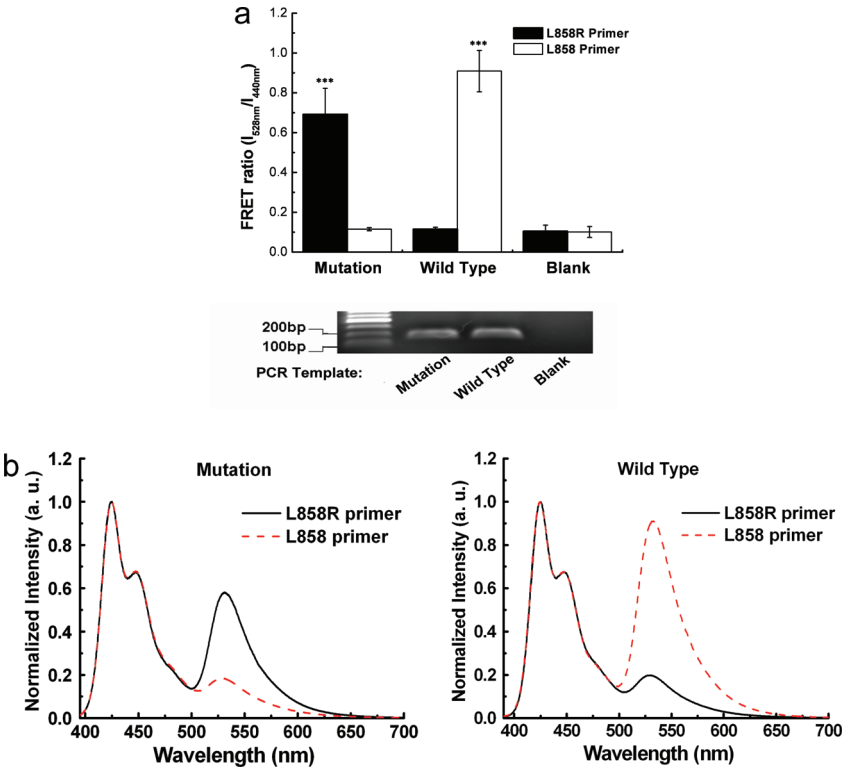


Figure 2. Construction of CCP-based FRET method for the detection of L858R mutation in EGFR gene. (a) Detection for L858R mutation using CCP-based FRET method. Top plot indicates FRET ratios of EGFR genes from pGEM-T L858R and pGEM-T L858 samples; the agarose gel electrophoresis presents the PCR-amplified products used to SBE reaction and FRET ratio experiments. The two-tail *t* test: ***, $p < 0.001$ compared with the blank control. For mean, error bars indicate ± s.d. (n = 5−6). (b) Emission spectra of the extension products of L858R mutation and wild type pGEM-T L858 samples. All the experiments were performed in Hepes buffer solution (25 mM, pH 7.5). The excitation wavelength is 380 nm. [dGTP-Fl] = 5×10^{-8} M; and [CCP] = 1.5×10^{-6} M.

another lung cancer cell line with wild type EGFR gene, were isolated and then serial percentage experiments were also performed. By comparing the FRET ratio values obtained from cell line sample (see Figure S2 in the Supporting Information), the mutation was detectable even when NCI-H1975 cells represented 8 to 16% of the total. This result was in agreement with the sensitivity of 4 to 8% from pGEM-T plasmids, since the exon 21 point mutation of EGFR in NCI-H1975 cell is heterozygous.

2.3. Detection of L858R Mutation by CCP-Based FRET in Cell Lines. Since the established CCP-based FRET can detect the L858R mutation with higher sensitivity and specificity, we employed this new method to distinguish different cell lines. When using L858R primer, the FRET ratio of NCI-H1975 cell line sample was significantly higher than that of control (Figure.4), which demonstrated that NCI-H1975 cell line contained mutation EGFR gene. By contrast, when using wild type extension primer L858, all the cell lines including Jurkat T, A549, A498, U251, MCF-7 and NCI-H1975 gave higher FRET ratios than that of blank control, which confirmed that these cells contained wild type EGFR gene and NCI-H1975 cell line was heterozygous for the L858R mutation.

2.4. Comparative Analysis of the CCP-Based FRET Method with Real-Time PCR and DNA Sequencing for Detection of L858R Mutation in Tumor Samples. On the basis of mutation detection in cell lines, this new method could be developed to detect L858R gene mutation in clinical samples. Therefore, 48 lung cancer samples were collected to evaluate the efficiency of our CCP- based FRET method, including 19 samples with L858R EGFR mutant and 29 samples with EGFR wild type, based on DNA sequencing results. We also used a higher sensitive real-time PCR assay to confirm the L858R mutation status for all tumor samples. The results showed that all 19 samples with L858R mutant detected by DNA sequencing were also positive for L858R mutation by real-time PCR; but two of 29 wild type specimens were positive for L858R mutation by real-time PCR. Combined with the real-time PCR results, these samples were divided into L858R mutation group (n = 21) and wild type group (n = 27). Using our CCP-based FRET method, the FRET ratio of L858R mutation group was significantly higher than that of wild type group when using the L858R primer (two-tail *t* test: $p < 0.001$). The FRET ratio was 0.25 ± 0.08 (mean ± s.d.; n = 21) for mutation group and 0.13 ± 0.02 for wild type group (mean ± s.d.; n = 27) (Figure 5a), indicating that the method could diagnose the specific mutation from clinical samples. A threshold value (0.174) was established as the mean + 2 s.d. with the FRET ratio values of 27 wild type samples, where the sample was deemed as positive if FRET ratio exceeded this value. Using the threshold value, all samples except for the one with the L858R mutation revealed by DNA sequencing and/or real time PCR were also verified by CCP-based FRET method and the sensitivity of this new method reached to 95.24%. Comparison of the data obtained from these methods (Table 1) revealed that the positive rate of CCP-based FRET technology is comparable to those of DNA sequencing and real-time PCR methods (41.7% vs 39.6% and 43.8%). Noted that one sample in the analyzed samples by CCP-based method was a false

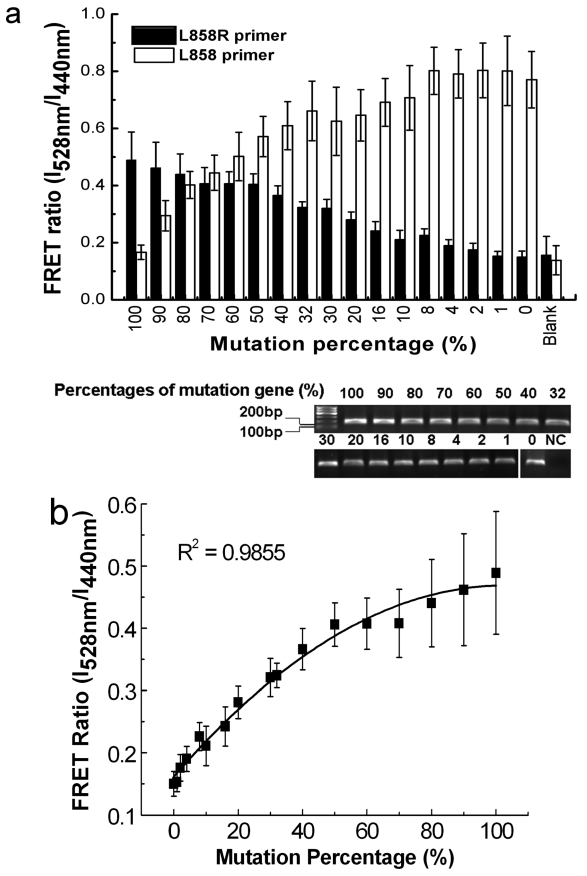


Figure 3. Sensitivity analysis of CCP-based FRET method using the EGFR gene from pGEM-T plasmids and cell lines. (a) The FRET ratio ($I_{528\ nm}/I_{440\ nm}$) from solutions containing CCP and extension products of various percentages of mutant EGFR gene fragments obtained by PCR amplifying recombinant pGEM-T plasmids. The agarose gels show the PCR products amplified from pGEM-T L858R and pGEM-T L858 plasmids. (b) Standard curve for mutation percentages generated by various ratios of pGEM-L858R to pGEM-L858 plasmids. NC indicates negative control. For the mean, error bars indicate ± s.d. (n = 3). All the experiments were performed in Hepes buffer solution (25 mM, pH 7.5). The excitation wavelength is 380 nm. [dGTP-Fl] = 5×10^{-8} M; and [CCP] = 1.5×10^{-6} M.

negative in comparison to real-time PCR (20/48 vs 21/48). For this false-negative sample, three repeated experiments all show that its PCR amplified products is weaker band on Gold view stained agarose-gel than other samples, which maybe results from the low-quality sample. Of course, if the PCR product was reamplified by second PCR to obtain sufficient templates for SBE reaction, this false-negative sample was detected as positive signal.

Among L858R mutation samples, there was one suspected positive sample whose mutation signal was weak in both DNA sequencing and real-time PCR analysis. We selected this suspected positive sample and compared the data from CCP-based FRET (Figure 5b), DNA sequencing, and real-time PCR. The CCP-based FRET system could clearly distinguish this low-level mutation (see Figure S3 in the Supporting Information).

2.5. Detection of L858R and T790 M Mutations by CCP-Based FRET. To validate the application of CCP-based FRET in clinical molecular diagnosis, we detected the L858R and T790 M mutations in EGFR of 35 patients that confer different responses

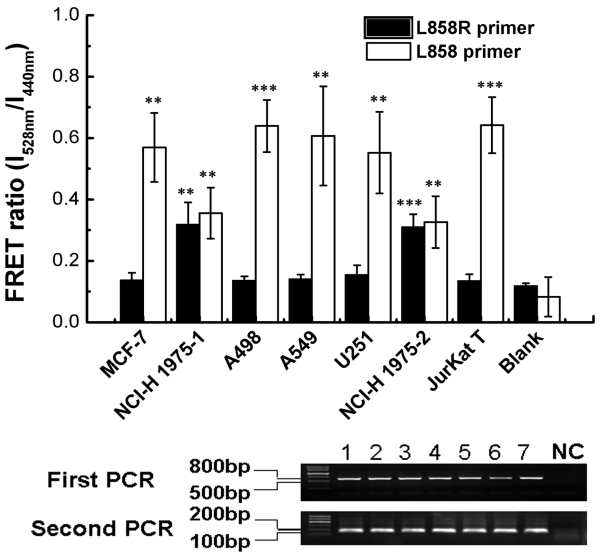


Figure 4. EGFR L858R mutation detection of cell lines by CCP-based FRET method. The top plot indicates the FRET ratio from seven cell samples (from 1 to 7). The agarose gel electrophoresis shows the PCR amplification bands from different cell lines that were used to SEB reaction and FRET experiments. The two-tail t test: ** $p < 0.01$, *** $p < 0.001$ compared with the blank control. For the mean, error bars indicate ± s.d. (n = 21 and 27). NC indicates negative control. All the experiments were performed in Hepes buffer solution (25 mM, pH 7.5). The excitation wavelength is 380 nm. [dGTP-Fl] = 5×10^{-8} M; and [CCP] = 1.5×10^{-6} M.

to either gefitinib or erlotinib (see Figure S4 and Table S2 in the Supporting Information). As expected, the two mutations in lung tumors can be detected, which is consistent with the results of DNA sequencing. These results indicate that CCP-based FRET method is suitable for point mutation detection in clinical samples.

3. DISCUSSION

In this study, we determined the detection limit of new CCP-based FRET method for EGFR L858R mutation. By using various percentages of L858R mutation in PCR amplified-DNA samples derived from two pGEM-T plasmids and two lung cancer cell lines, the point mutation could be detected even when it presents at 4% in samples. It is very important to improve the sensitivity of mutation detection because of the presence of normal cells in tumor sample and/or low mutation frequency of tumor cells.

Moreover, as the standard curves of two serial percentage experiments showed a good quantitative property, mutation percentage could be accessed. Thus, compared with DNA sequencing, our method not only has higher sensitivity but also can provide a quantitative assay for point mutation detection. The quantitative assay for a single base change is useful to investigate the relationship between gene mutations and diseases, although in this study, we mainly focused on the gene mutation detection.

We also validated and compared the detection efficacy of CCP-based FRET method for mutation of cell lines and tumor tissue samples that contained L858R mutation. In CCP-based FRET detection system, the optical amplification effect of conjugated polymer imparts our method higher sensitivity and the mutation as low as 4% in sample can be detected (Figure 3 and 4). Our data from clinical samples showed that the accuracy

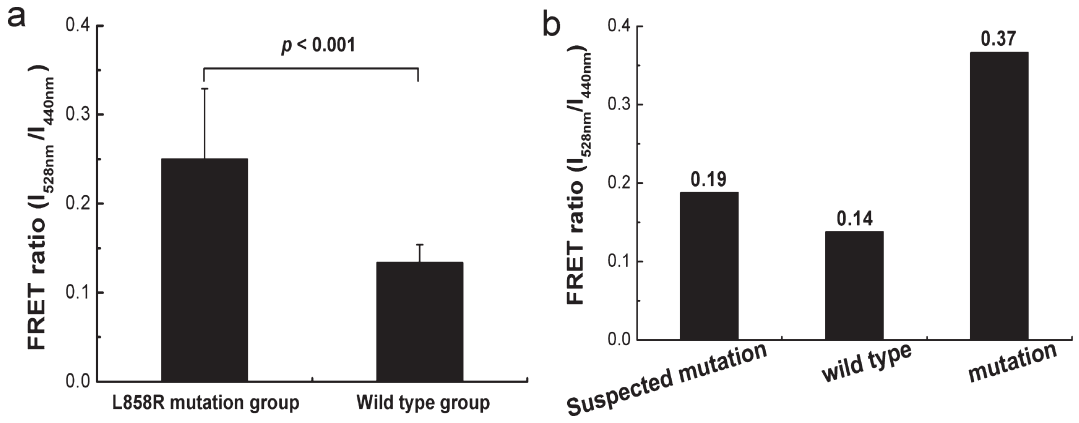


Figure 5. (a) FRET ratio as functions of mutation and wild type groups in tumor samples using L858R primer. (b) Analysis of one suspected mutation sample by CCP-based FRET method. All the experiments were performed in Hepes buffer solution (25 mM, pH 7.5). The excitation wavelength is 380 nm. [dGTP-Fl] = 5×10^{-8} M; and [CCP] = 1.5×10^{-6} M.

Table 1. Summary of CCP-Based FRET, Real Time PCR, and DNA Sequencing Results

methods	no. of L858R +	no. of L858R −	positive rate
CCP-based FRET	20	28	41.7% (20/48)
DNA sequencing	19	29	39.6% (19/48)
Real time PCR	21	27	43.8% (21/48)

of CCP-based FRET method for mutation detection was similar to that of real time PCR, which was considered to be the most effective method at the present time (Table 1). Another advantage of our CCP-based FRET method is that no primer labeling is required in comparison with real-time PCR technique to make it cost-effective. Because most of anticancer medicines have a strong cytotoxic effect on normal cells and tissues, it is very important to develop new method for mutation detection with high specificity. For CCP-based FRET method, only when dGTP-Fl was incorporated into DNA fragments, the FRET signal could be detected, which ensured its high specificity. Recently, although other FRET technology has been explored for various gene mutation detections, it is still difficult to site-specifically label a tested site with two fluorophores on the nanometer scale. In our CCP-based FRET method, CCP could uncovalently bind to the DNA molecules by electrostatic interactions in solution without any labeling procedure. Therefore, CCP-based FRET technology is a powerful tool for disease diagnosis at molecular level since it allows for mutation detection with higher sensitivity and specificity. As expected, our data showed that the mutation samples could yield higher FRET signal than wild type samples when using mutation extension primer -L858R primer (Figure 5a). The FRET ratio of mutation EGFR gene was significantly higher than that of wild type gene, indicating that our CCP-based method described in the current work could detect this known EGFR point mutation in practical samples. Particularly, this new method could confirm the suspected positive samples (Figure 5b−d).

4. CONCLUSION

In summary, we developed a cost-effective and simple methodology for the detection of clinically relevant hot point mutations using CCP-based FRET technology. For its clinical application, we tested 48 DNA samples from tumor tissues embedded in paraffin. The assay demonstrated a sensitivity of 95.24% (20/21) and a specificity of 96.30% (26/27). In comparison with DNA sequencing and real-time PCR methods, our new protocol simplifies procedures by eliminating the need for primer labeling, cumbersome workups and sophisticated instruments and improves sensitivity by amplifying fluorescence signals. Our CCP-based FRET technology is particularly attractive because of its higher sensitivity, cost-effective, and simple characteristics. Particularly, this new method could confirm the suspected positive samples by other methods. It opens up an avenue for routine clinical therapy by guiding medication to lung cancer patients responsive to anti-EGFR therapy. It can also be expected to be used for detecting other mutations instead of EGFR L858R mutation as a routinely clinical molecular diagnosis method.

5. EXPERIMENTAL SECTION

Reagents. Cationic conjugated polymer (CCP) was synthesized using the procedures described in our previous paper.[20] r-Taq polymerase, shrimp alkaline phosphatase, *E. coli* exonuclease, dNTPs were purchased from Takara (Dalian, China). The GoTaq polymerase mix, pfu GoTaq polymerase were provided by Promega. dGTP-Fl was from Perkin company. Ex-EGFR-21F, Ex-EGFR-21R, EGFR-21F, EGFR-21R, L858, and L858R primers were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co.;Ltd. (Shanghai, China). Water was deionized through a Milli-Q water purification system (Millipore).

Cell Lines. The human cancer cell lines NCI-H1975, MCF-7, A549, A498, U251 and Jurkat T were from Cell Culture Center, Chinese Academy of Medical Sciences and Peking Union Medical College (Peijing, China). All of them were maintained in DMEM (Gibco) supplemented with 10% fetal bovine serum (Hyclone) at 37 °C air containing 5% CO_2.

Clinical Samples. Clinical samples were excised from 48 formalin-fixed, paraffin embedded (FFPE) tumor blocks collected from lung cancer patients. The study was approved by the medical ethical committee of Cancer Institute and Hospital Chinese Academy of Medical Sciences. Also, informed consent was obtained from each subject. To identify the L858 gene status of these samples, we sequenced their PCR amplified products in both directions (ABI Prism 3730 Genetic Analyzer, Applied Biosystem).

DNA Extraction. Genomic DNA of cancer cell lines and FFPE tissue sections were extracted by using a QIAamp DNA blood mini kit (QIAGEN) according to the manufacturer's instructions. Briefly, the cell or tissue samples were lysed thoroughly with lysis buffer containing proteinase K, followed by incubating 56 °C for 10 min or overnight, and equal volume of 100% ethanol were then added. After passing through the QIAamp spin mini column, the genomic DNAs bound to the column. Pure genomic DNAs were collected by washing twice and then eluting. To extract the DNA from FFPE tissues, we cut eight 10 μm thick sections from the samples and cleared of paraffin by treatment with xylene, and rinsed them with 100% ethanol. DNA concentration and quality were measured by absorbance at 260 and 280 nm using Multi-Mode Microplate Reader (Bio-Tek, Synergy 2).

PCR Amplification of EGFR Gene Fragment. To amplify EGFR gene from cell lines and tumor tissue samples, nested PCR was performed. The two pair primers were as following: Ex-EGFR-21F: TCA GCC TGG CAA GTC CAG TAA G; Ex-EGFR-21R: AgC TCT ggC TCA CAC TAC CAg; EGFR-21F: CCT CAC AGC AGG GTC TTC TC; EGFR-21R: TGC CTC CTT CTG CAT GGT A. 100 ng of DNA was added to 25 μL of a reaction mixture comprising of 12.5 μL of 2 × GoTaq PCR mix (Promega) and 1.0 μL of 10.0 μM of each primer. The amplification program consisted of initial denaturation at 95 °C for 4 min, followed by 40 cycles of 95 °C for 1 min 30 s, 60 °C for 30 s, 72 °C for 30 s; and final extension at 72 °C for 10 min. PCR products were then analyzed utilizing 2% agarose gel electrophoresis.

Construction of pGEM-T Plasmids Containing L858R Point Mutation or Wild Type EGFR gene fragment. To establish and analyze the new CCP-based FRET method, we constructed pGEM-T plasmids containing L858R point mutation (pGEM-TL858R) or wild type EGFR (pGEM-T L858). EGFR PCR products from genomic DNA samples of A549 and NCI-H1975 cell lines were purified and then cloned into pGEM-T vectors by using TA cloning kit (Tiangen). The nucleotide sequences of plasmids were confirmed by using an automated DNA sequencer (ABI Prism 3730 Genetic Analyzer).

Single Base Extension Reaction. The unincorporated dNTP and excessive primers in PCR production required to be inactivated prior to SBE experiments. Eight μL of enzymatic mixture containing 2 units of shrimp alkaline phosphatase, 1 unit of *E. coli* exonuclease I in shrimp alkaline phosphatase buffer (20 mM Tris-HCl, pH 8.0/10 mM $MgCl_2$) was added to the PCR products. Following incubation at 37 °C for 30 min, the mixture was subjected to heat inactivation at 80 °C for 15 min. SBE reactions were conducted in a total volume of 15 μL containing 1.5 μL of degraded PCR products described above, 1.2 U of rTaq polymerase, 1.5 μL of PCR buffer (from a 10 × stock solution), 2 μM of dGTP-Fl, and 1 μM of extension primer-L858R (5′GAT CAC AGA TTT TGG GCG3′) or L858 primer (5′GA TCA CAG ATT TTG GGC T3′). In these two extension primers, a nucleotide difference (G or T) located at the 3′ terminal base was used to discriminate mutant and wild type alleles. The extension reaction was ran in an Mycycler thermal cycler (Biorad) and the conditions were 95 °C for 1 min, followed by 40 cycles of 95 °C for 30s and 57 °C for 30s, ending with a 10 min incubation at 4 °C. To digest the unincorporated dGTP-Fl, 15 μL of SBE mixture system was treated with 0.5U of SAP before detecting the FRET.

Detection of the EGFR L858R Gene Mutation by FRET Based on CCP. When the extension primer and complementary dGTP-Fl are added to the SBE reaction, the primers are extended by labeled dGTP. In our system, CCP was a FRET donor to Fl dye. Fluorescence signals were directly recorded in a 96-well plate on a spectrofluorimeter after 24 μL of CCP reaction buffer (final concentration 15 μM) was added into the SEB reaction system from method 8. Fl shows emission at 528 nm when CCP was excited at 380 nm, and the FRET ratio was calculated using the following equation

$$\text{FRETratio} = \frac{I_{528\ \text{nm}}}{I_{440\ \text{nm}}}$$

where $I_{528\ \text{nm}}$ and $I_{440\ \text{nm}}$ are the emission intensity of Fl at 528 nm and that of CCP at 440 nm with an excitation wavelength of 380 nm, respectively. The emission spectra in reaction buffer were also recorded on a Hitachi F-4500 spectrofluorimetry (Hitachi, Japan).

Sensitivity Analysis of the CCP-Based FRET Method. For sensitivity assay, the 180-bp EGFR gene fragment template used in the SBE extension reaction was generated from the pGEM-T L858R and pGEM-T L858 as described in "method" section. The two different pGEM-T plasmids were mixed with the mutation percentages from 0, 1, 2, 4, 6, 8, 10, 20, 30, 32, 40, 50, 60, 70, 80, 90, up to 100%. Wild type primer- L858 primer was also used to SBE. Further, we prepared various ratios of PCR templates comprised of wild type and mutation genomic DNAs from A549 cell line and NCI-H1975 cell line, respectively. Then the PCR amplification, SBE, FRET experiments were done and curve of FRET efficiencies were drafted.

Amplification and Analysis of Real-Time PCR. Real-time fluorescent PCR was conducted using Human EGFR Mutation Detection Kit (Beijing ACCB Biotech Ltd.) .The Agilent-Stratagene Mx3000P Q-PCR System was used to monitor PCR amplification by specific primers and fluoresce probe. The amplification program consisted of 1 cycle of 95 °C with 120-s hold followed by 35 cycles of 95 °C with 15-s hold, specified annealing temperature of 65 °C with 60-s hold. A negative control without DNA template was conducted with every assay to assess the overall specificity. A threshold cycle (C_t) was determined for each sample using the exponential growth phase and the baseline signal from fluorescence versus cycle number plots. A sample was deemed positive if fluorescence exceeded the threshold. Threshold fluorescence level was automatically set by the Mx3000P software. To confirm the efficiency of the CCP-based FRET method, the amplification of exon 20 was performed using the primers flanking the genomic region containing the T790 M mutation of 36 patients. The PCR primers are demonstrated in Supporting Information, Table S1.

Statistics Analysis. Results were expressed as average ± s.d. The two-tail t test was used to compare the FRET ratio between the experiment and blank control. Differences between groups were considered significant for $p < 0.05$.

■ ASSOCIATED CONTENT

Supporting Information. Primers for PCR amplification and SBE (Table S1); maps of plasmids and DNA sequencing analysis of pGEM-T L858R and pGEM-T L858 (Figure S1); FRET ratio values obtained from cell line sample (Figure S2); analysis of mutation sample by DNA sequencing and real-time PCR (Figure S3 and Table S2) and L858R and T790 M mutation detections in 35 samples from lung cancer patients by CCP-based FRET method (Figure S4). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Email: yangqiong@iccas.ac.cn (Q.Y.); wangshu@iccas.ac.cn (S.W.); jmying@hotmail.com (J.Y.).

Author Contributions

‡These authors made equal contributions to this work.

■ ACKNOWLEDGMENT

The authors are grateful to the National Natural Science Foundation of China (20725308, 21033010, 90913014, and TRR61) and the Major Research Plan of China (2011CB932302,

2011CB935800) for financial support. The authors thank all patients who donated DNA for this research.

REFERENCES

(1) Lynch, T. J.; Bell, D. W.; Sordella, R.; Gurubhagavatula, S.; Okimoto, R. A.; Brannigan, B. W.; Harris, P. L.; Haserlat, S. M.; Supko, J. G.; Haluska, F. G.; Louis, D. N.; Christiani, D. C.; Settleman, J.; Haber, D. A. *N. Engl. J. Med.* **2004**, *350*, 2129–2139.

(2) Marchetti, A.; Martella, C.; Felicioni, L.; Barassi, F.; Salvatore, S.; Chella, A.; Camplese, P. P.; Iarussi, T.; Mucilli, F.; Mezzetti, A.; Cuccurullo, F.; Sacco, R.; Buttitta, F. *J. Clin. Oncol.* **2005**, *23*, 857–865.

(3) Paez, J. G.; Jänne, P. A.; Lee, J. C.; Tracy, S.; Greulich, H.; Gabriel, S.; Herman, P.; Kaye, F. J.; Lindeman, N.; Boggon, T. J.; Naoki, K.; Sasaki, H.; Fujii, Y.; Eck, M. J.; Sellers, W. R.; Johnson, B. E.; Meyerson, M. *Science* **2004**, *304*, 1497–1500.

(4) Pao, W.; Miller, V.; Zakowski, M.; Doherty, J.; Politi, K.; Sarkaria, I.; Singh, B.; Heelan, R.; Rusch, V.; Fulton, L.; Mardis, E.; Kupfer, D.; Wilson, R.; Kris, M.; Varmus, H. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 13306–13311.

(5) Matsukuma, S.; Yoshihara, M.; Kasai, F.; Kato, A.; Yoshida, A.; Akaike, M.; Kobayashi, O.; Nakayama, H; Sakuma, Y.; Yoshida, T.; Kameda, Y.; Ysuchiya, E.; Miyagi, Y. *J. Mol. Diagn.* **2006**, *8*, 504–512.

(6) Sasaki, H.; Endo, K.; Konishi, A.; Takada, M.; Kawahara, M.; Iuchi, K.; Matsumura, A.; Okumura, M.; Tanaka, H.; Kawaguchi, T.; Shimizu, T.; Takeuchi, H.; Yano, M.; Fukai, I.; Fujii, Y. *Clin. Cancer Res.* **2005**, *11*, 2924–2929.

(7) Nomoto, K.; Tsuta, K.; Takano, T.; Fukui, T.; Fukui, T.; Yokozawa, K.; Sakamoto, H.; Yoshida, T.; Maeshima, A. M.; Shibata, T.; Furuta, K.; Ohe, Y.; Matsuno, Y. *Am. J. Clin. Pathol.* **2006**, *126*, 608–615.

(8) Hiroaki, O.; Kouki, O.; Akiko, O.; Satsuki, M.; Tomoyuki, G.; Takashi, W. *Diagn. Mol. Pathol.* **2006**, *15*, 101–108.

(9) Orita, M.; Iwahana, H.; Kanazawa, H.; Hayashi, K.; Sekiya, T. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *86*, 2766–2770.

(10) Felley-Bosco, E.; Pourzand, C.; Zijlstra, J.; Amstad, P.; Cerutti, P. *Nucleic Acids Res.* **1991**, *19*, 2913–2919.

(11) Pan, Q.; Pao, W.; Ladanyi, M. *J. Mol. Diagn.* **2005**, *7*, 396–403.

(12) Feng, X.; Liu, L.; Wang, S.; Zhu, D. *Chem. Soc. Rev.* **2010**, *39*, 2411–2419.

(13) Duan, X.; Liu, L.; Feng, F.; Wang, S. *Acc. Chem. Res.* **2010**, *43*, 260–270.

(14) Thomas, S. W.; Joly, G. D.; Swager, T. M. *Chem. Rev.* **2007**, *107*, 1339–1386.

(15) Ho, H. A.; Najari, A.; Leclerc, M. *Acc. Chem. Res.* **2008**, *41*, 168–178.

(16) Liu, B.; Bazan, G. C. *Chem. Mater.* **2004**, *16*, 4467–4476.

(17) Jiang, H.; Taranekar, P.; Reynolds, J. R.; Schanze, K. S. *Angew. Chem., Int. Ed.* **2009**, *48*, 4300–4316.

(18) Bunz, U. H. F.; Rotello, V. M. *Angew. Chem., Int. Ed.* **2010**, *49*, 3268–3279.

(19) Duan, X.; Li, Z.; He, F.; Wang, S. *J. Am. Chem. Soc.* **2007**, *129*, 4154–1455.

(20) Duan, X.; Yue, W.; Liu, L.; Li, Z.; Li, Y.; He, F.; Zhu, D.; Zhou, G.; Wang, S. *Nat. Protoc.* **2009**, *4*, 984–991.

(21) Gaylord, B. S.; Heeger, A. J.; Bazan, G. C. *Proc. Natl. Acid. Sci. U.S.A* **2002**, *99*, 10954–10957.

(22) Lee, K.; Rouillard, J.-M.; Pham, T.; Gulari, E.; Kim *J. Angew. Chem. Int. Ed.* **2007**, *46*, 4667–4676.

(23) Nilsson, K. P. R.; Inganäs, O. *Nat. Mater.* **2003**, *2*, 419–424.

(24) Ho, H. A.; Dore, K.; Boissinot, M.; Bergeron, M. G.; Tanguay, R. M.; Boudreau, D.; Leclerc, M. *J. Am. Chem. Soc.* **2005**, *127*, 12673–12676.

(25) He, F.; Tang, Y. L; Yu, M. H; Feng, F. D; An, L. L; Sun, H.; Wang, S.; Li, Y. L; Zhu, D. B; Bazan, G. C. *J. Am. Chem. Soc.* **2006**, *128*, 6764–6765.

(26) Feng, X. L; Duan, X. R; Liu, L. B; An, L. L; Feng, F. D; Wang, S. *Langmuir* **2008**, *24*, 12138–12141.

(27) Liu, B.; Wang, S.; Bazan, G. C.; Mikhailovsky, A. *J. Am. Chem. Soc.* **2003**, *125*, 13306–13307.

(28) Achyuthan, K. E.; Bergstedt, T. S.; Chen, L.; Jones, R. M.; Kumaraswamy, S.; Kushon, S. A.; Ley, K. D.; Lu, L.; McBranch, D.; Mukundan, H.; Rininsland, F.; Shi, X.; Xia, W.; Whitten, D. G. *J. Mater. Chem.* **2005**, *15*, 2648–2656.

(29) Ren, X.; Xu, Q.-H. *Langmuir* **2009**, *25*, 43–47.

(30) Baker, E. S.; Hong, J. W.; Gaylord, B. S.; Bazan, G. C.; Bowers, M. T. *J. Am. Chem. Soc.* **2006**, *128*, 8484–8492.

(31) Ho, H. A.; Boissinot, M.; Bergeron, M. G.; Corbeil, G.; Doré, K.; Boudreau, D.; Leclerc, M. *Angew. Chem., Int. Ed.* **2002**, *41*, 1548–1551.

(32) Zheng, W.; He, L. *J. Am. Chem. Soc.* **2009**, *131*, 3432–3433.

(33) Zhang, Y.; Li, Z.; Cheng, Y.; Wang, Y. *Talanta* **2009**, *79*, 27–31.

(34) Xu, H.; Wu, H.; Huang, F.; Song, S.; Li, W.; Cao, Y.; Fan, C. *Nucleic Acids Res.* **2005**, *33*, e83.

(35) Liu, B.; Gaylord, B. S.; Wang, S.; Bazan, G. C. *J. Am. Chem. Soc.* **2003**, *125*, 6705–6714.