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Imaging analysis of EGFR mutated cancer cells using peptide nucleic acid (PNA) – DNA probes

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Abstract

Lung-cancer cells harbor the various gene mutations in the mRNA sequence of the Epidermal Growth Factor Receptor (EGFR), especially mutations of exon19del E746-A750, T790M, and L858R. This results in cancer progression and resistance to anticancer drugs (tyrosine kinase inhibitor; TKI). Therefore, the imaging analysis of EGFR mutations is required for the treatment planning for non-small cell lung-cancers. This study focused on the imaging analysis of a single nucleotide substitute in EGFR mutated cancer cells. We developed three novel peptide nucleic acid (PNA)-DNA probes for recognizing and detecting the following three gene mutations in EGFR gene mutations. The PNA-DNA probes consist of fluorescein isothiocyanate (FITC) conjugated PNA as a detection probe and Dabcyl conjugated DNA as a quencher probe. The PNA-DNA probes were used to validate the feasibility for detecting three EGFR mutated sequences: exon19del E746-A750, T790M, and L858R. The three probes emitted fluorescent dose-dependent signals against three target DNA and RNA. Using the three PNA-DNA probes, we succeeded in discriminating three kinds of lung-cancer cell lines (H1975, PC-9, and A549) which have different EGFR mutation distinguished using the by fluorescence in situ hybridize (FISH) method.

1. Introduction

The tyrosine kinase inhibitor (TKI), represented by Gefitinib, is a molecular targeting anticancer drug that binds to the tyrosine kinase domain of the Epidermal Growth Factor Receptor (EGFR) protein[1,2]. Gefitinib inhibits the signal transduction of the EGF signal and induces cell death[3]. It is reported that the cancer cells that have the EGFR gene mutation (in particular, exon19del E746-A750 and L858R) respond to the Gefitinib[3–6]. However, long-term administration of Gefitinib induces TKI resistance cells. These TKI resistant cells frequently possess the T790M gene mutation (more than 50 %)[7–9]. T790M mutated EGFR protein loses binding affinity to Gefitinib and gains recalcitrance against TKI[10]. Therefore, the analysis of the DNA or RNA sequences of mutated EGFR is necessary for diagnosis and efficient treatment of the lung-cancer[11]. In recent, next-generation sequencers have been intensively developed and provide accurate whole-genome sequencing of cancer cells[12]. However, to sequence the genome accurately, it is necessary to obtain more than few thousand target cells (50 ng~ DNA). Therefore, it is difficult to preemptively diagnose EGFR mutated cells with next-generation sequencers using only a few gene-mutated cells that have been collected from the small amount of lung-cancerous tissues. For early diagnosis of EGFR gene-mutated cancer cells, a new detection system needs to be developed at the single cell level. In order to analyze single cell gene mutations, we have developed novel probes for the imaging analysis of three EGFR mutated genes (exon19del E746-A750, L858R, and T790M). Various imaging probes using DNA, like Molecular Beacon et al.[13–

[21], were reported to work for the imaging analysis of target DNA or target RNA. However, some DNA-based probes possess large molecular weight, therefore present difficulty when attempting to introduce these probes into target cells. Furthermore, it is quite difficult to specifically detect single nucleotide substitution for mutated genes such as T790M and L858R. Other methods for conducting single nucleotide polymorphism (SNP) analysis were also reported to use DNA-based probes[22–24]. However, these methods basically need the extracted DNA from dissolved cells, making it quite difficult to conduct in situ analysis at the single cell level. In addition, classical DNA probes have not enough affinity and specificity for high sensitive SNP detection[25].

In present study, we have developed peptide nucleic acid (PNA)[26–29]- DNA probes (Fig. 1A) in effort to conduct imaging analysis of single nucleotide substitution in EGFR mutated cells. We considered that the PNA based probe is suited in SNP detections using fixed cells and even living cells, which is compatible with target recognition ability and small molecular weight. Because PNA has high target binding ability due to PNA's lack of electronic charge in the backbone and has the high specificity[30,31]. Small DNA probes make us easy to induce cells, while it normally reduces the target's recognition specificity caused by non-specific binding by lower melting temperature. In fact, some PNA based beacons were reported for target bacterial DNA detection[32–34]. Also Wang's group reported[35] that even inducible nitric oxide synthase (iNOS) mRNA in mouse macrophage cells is detected by PNA probes. These PNA probes are expected to be more suitable than DNA probes when

it comes to gene mutation analysis of single nucleotide substitution. However, to further facilitate introduction into cells, PNA probes with lower molecular weight are required.

In this study, we developed novel small molecular FITC-PNA and quencher-DNA hybridized imaging probes (PNA-DNA probes; Fig. 1A) to be compatible with high specificity and easy to induce cells for gene mutation analysis of single nucleotide substitution. We designed and constructed the novel three PNA-DNA probes for recognizing and detecting the three following EGFR mutated genes; exon19del E746-A750, T790M, and L858R (Fig. 1B). Using the three PNA-DNA probes, we examined the feasibility for detecting different types of EGFR gene mutations. These mutated cell act as models of anticancer drug resistant cells through the use of lung-cancer cell lines: H1975[36] and PC-9[37,38].

2. Experimental

Design and construction of PNA-DNA probes

Table 1 shows the sequences of synthetic FITC conjugated PNA (FITC-PNA; Panagene, Daejeon, South Korea), quencher conjugated DNA (Q-DNA; Integrated DNA Technologies, Coralville, IA, USA). And Table 2 shows the sequences of synthetic DNA for model target (Eurofins Genomics, Brussels, Belgium) used in this study. Three PNA-DNA probes were constructed by hybridization of the synthesized FITC-PNA and Q-DNA. FITC-PNA and Q-DNA (5 μM final) were mixed in a PBS solution and self-assembled with annealing program as follow. The mixed reagent was heated at 95°C

for 5 minute for denature. To hybridize the FITC-PNA and Q-DNA, the temperature was gradually decreased at rate of -1.38°C / min to 30°C. Finally, the mixture was incubated at 30°C for 10 min, and kept at 10°C. The product was directly used in further experiments.

Production of model EGFR target mRNA

EGFR mRNA was cloned from human lung-cancer cell lines; H1975, PC-9, A549 (ATCC, Manassas, VA, USA). Total RNA was extracted using a RNeasy Mini Kit (Qiagen, Venlo, Netherlands) by following manufacturer's instructions. A cDNA library was then constructed using a cDNA Synthesis Kit (Takara Bio, Shiga, Japan) according to the manufacturer's protocol. This library was subjected to PCR amplification using the primers "forward; 5'- GGG CTC GAG ATG CGA CCC TCC GGG ACG GCC GGG G -3'" and "reverse; 5'- GGG TCT AGA TCA TGC TCC AAT AAA TTC ACT GCT T -3'", in addition to KOD-Plus-Ver.2 polymerase (Toyobo, Osaka, Japan). The amplified gene was cloned into XhoI and XbaI restriction sites of the pTNT vector (Promega, Madison, WI, USA) after restriction enzyme digestions. The transcripts of EGFR mRNA were produced with a vector using ScriptMAX Thermo T7 Transcription Kit (Toyobo) according to the manufacturer's instructions. The purity of the translated RNA product was confirmed by denaturing gel electrophoresis (Data not shown).

For the sequencing analysis involving EGFR mRNA of H1975, PC-9, and A549, the synthesized

cDNA library was subjected to PCR amplification using the primers "forward; 5'- CCA ACC AAG CTC TCT TGA GG -3'" and "reverse; 5'- TTC TTT CAT CCC CCT GAA TG -3'", in addition to KOD-Plus-Ver.2 polymerase. The amplified partial sequence of EGFR containing exon19del E746-A750, T790M, and L858R were directly adopted into sequence analysis (DNA sequencing Service; Eurofins Genomics).

Fluorescence measurement for PNA-DNA probes reacting with target DNA and RNA

PNA-DNA probes (500 nM final) were either mixed with each concentration of model EGFR target DNA (Table 2) or transcribed EGFR RNA in vitro in a PBS solution for 2 h at 37°C. FITC fluorescence intensity at 518 nm was measured using an infinite M200 multiple reader (TECAN, Männedorf, Switzerland) in a 96-well format with a 50 μL mixture by 460 nm excitation. The fluorescence of PNA-DNA probes with each concentration of target DNA or RNA were divided by the fluorescence with the target omitted.

Fluorescence in situ hybridization (FISH) analysis for EGFR mutated cancer cells

Human lung-cancer cell lines (H1975, PC-9, A549) were maintained in a RPMI1640 culture medium (Nacalai Tesque, Kyoto, Japan) with a 10% (v/v) heat-inactivated fetal bovine serum (Sigma–Aldrich, St. Louis, MO, USA), 100 units/mL of penicillin, and 100 μg/mL of streptomycin (Thermo Fisher

Scientific, Waltham, MA, USA) in a 5% CO_2 atmosphere at 37°C. The cells cultured on Nunc Lab-Tek Chamber Slide Systems (Thermo Fisher Scientific) were first washed with a PBS buffer and fixed with 3.7% formaldehyde for 10 min. The slides were washed twice with PBS buffer, followed by a 70% ethanol treatment for 2 h at 4°C to permeabilize the cellular membrane. The PNA-DNA probes (60 nM final) in Hybridization buffer [10% dextran sulfate (Nacalai Tesque), 0.02% RNase free BSA (Promega), 2 mM vanadyl ribonucleoside complex (New England Biolabs, Ipswich, MA, USA), 10% formamide (Nacalai Tesque), 1.25 U ribonucleic acid, transfer from Escherichia coli (Sigma–Aldrich), 2× SSC Buffer (Nacalai Tesque)] was added to the cells and left overnight at 30°C, after which they were washed with 10% formamide. The cells were further washed with 10% formamide, 15% formamide, and 20% formamide, for 30 min at 30°C each. Finally, the cells were washed three times with a 2 × SSC buffer. The anti-fade solution, ProLong Diamond Anti-fade Mountant with nuclear staining dye; DAPI (Thermo Fisher Scientific)—was added to the sample. Fluorescent images were then taken using an Olympus FV3000 confocal laser microscope (Olympus, Tokyo, Japan). The FITC image was taken with a 488 nm laser and a variable barrier filter set at 500-600 nm. The DAPI image was obtained with a 405-nm laser and a variable barrier filter set at 430-470 nm.

3. Results and Discussion

Construction and characterization of PNA-DNA probes

To attain a proper diagnosis of anticancer drug (e.g., TKI) resistance, it is necessary to develop sensitive and accurate detection methods for single nucleotide mutations in EGFR mRNA. Furthermore, detection probes which have small molecular weight are desired for efficient delivery into target cancer cells at the single cell level. Therefore, this study sought to design and develop novel PNA-DNA probes that have high specificity and small molecular weight (for exon19del E746-A750; 6.81 kDa, T790M; 6.84 kDa, and L858R; 6.79 kDa) for EGFR gene mutation analysis of lung-cancer related mutations, exon19del E746-A750 and L858R, and TKI resistant related mutation, T790M. The most important detection points of target mutations were set in the middle of PNA sequences as a first prove design. The developed PNA-DNA probes are the hybrid of FITC-PNA and Q-DNA (Fig.1 A, B). PNA has complementary sequences of target EGFR mutated mRNA sequences (Table 1). Fluorescence intensity of the PNA-DNA probe was assumed to increase due to recognition of the target mRNA, which accompanies dissociation of Q-DNA from FITC-PNA. Therefore, by introducing the PNA-DNA probes into cancer cells, EGFR gene mutations can be detected by FITC fluorescence imaging analysis. The melting temperatures were calculated as 47.5°C (exon19del E746-A750), 58.2°C (T790M), 66.3°C (L858R) in PNA-DNA full-complementary-duplexes. In the other hands, melting temperatures were calculated as 24.0°C (exon19del E746-A750), 30.0°C (T790M), 38.0°C (L858R) in DNA-DNA full-complementary-duplexes. Therefore, since PNA-DNA has the higher

melting temperature than that of DNA-DNA, PNA probes appear to be suitable to detect target genes and SNPs. Using human lung-cancer cell lines—H1975, PC-9, and A549—we discuss the feasibility of three PNA-DNA probes for the imaging analysis of EGFR mutated DNA, mRNA, and cancer cells. First, we examined the ability of PNA-DNA probes to recognize target mutated sequences. The fluorescent intensities of FITC-PNA were measured with either a wild type or mutated DNA sequence (Table 2) of exon19del E746-A750, T790M, and L858R acting as model target genes. The fluorescence intensities were dose-dependently increased using all three probes following the addition of the target DNA with three mutated sequences (Fig. 2 A-C). These results indicate that all the three PNA-DNA probes recognize the target sequences, respectively. However, in the case of using PNA-DNA probes for L858R (Fig. 2C), fluorescent intensities were slightly increased using wild type target DNA. This is speculated to be caused by a GC-rich sequence in L858R. Although few FITC-PNA probes slightly bound to wild type sequence, the differences in L858R fluorescent intensity between the wild type sequence and mutated target sequence were significant enough in order to discriminate from each other (Fig. 2C). Although the increases in fluorescence were observed to be unstable in the PNA-DNA probe, the fluorescence intensities were clearly increased dose-dependently when compared with the wild type sequence in every cases (Fig. 2A-C). We will continue to improve the probe design in future to strengthen detection stability. Thus, we concluded that the three different mutated EGFR sequences can be easily and accurately detected by using the three PNA-DNA probes.

Identification of EGFR mutated cells using PNA-DNA probes

We evaluated the feasibility of using PNA-DNA probes to detect three different mutated EGFR mRNA sequences (exon19del E746-A750, T790M, L858R). In this study, lung-cancer cell lines—H1975 and PC-9—were employed as the models of EGFR mutated cancer cells. The A549 lung-cancer cell line which has no EGFR mutation gene, was also used as the negative control cell. First, we confirmed the EGFR mRNA sequences of H1975 and PC-9. The EGFR mRNA sequences were analyzed by using the reverse transcriptional-based PCR method. The results showed that H1975 has two T790M and L858R mutations, while PC-9 has one exon19del E746-A750 mutation (Fig. 3). The A549 control cell has no mutation. These results are consistent with previous reports[37,39]. For the examination of target recognition ability of PNA-DNA probes against target EGFR mRNA, target RNA were synthesized by in vitro transcription using extracted EGFR sequences from H1975, PC-9, or A549. PNA-DNA probes for exon19del E746-A750 showed the dose-dependent fluorescence increases against RNA of the PC-9 cell (Fig. 4 A). However, in the case of the EGFR RNA of either H1975 or A549 cells, the fluorescent intensities did not increase. Whereas, PNA-DNA probes for T790M and L858R showed increases in fluorescent intensities against the EGFR RNA of H1975 cells only (Fig. 4 B, C). All PNA-DNA probes did not show increase fluorescent intensities against the EGFR RNA of A549 cells (control) (Fig. 4A-C). These results indicate that three PNA-DNA probes

can be used that recognize target mutated RNA sequences. We have concluded that the result of the target recognition analysis of the developed probes against DNA and RNA (Fig. 2, Fig. 4) demonstrate that the three PNA-DNA probes can be used to discern the three specific target sequences.

Next, we confirmed the affinity of PNA-DNA probes to recognize native mRNA inside different types of cancer cells by using fluorescence in situ hybridization (FISH) analysis. H1975, PC-9 cells, and A549 cells were inoculated onto a chamber slide glass and the three PNA-DNA probes reacted following the fixation of the cells. The fluorescent images were obtained using a lase-confocal microscope (Fig. 5A). Using a PNA-DNA probe for exon19del E746-A750 detection, only the PC-9 cells showed strong fluorescence of FITC, while the H1975 cells showed only slight fluorescence (Fig. 5 A, B). The fluorescence intensity of PC-9 was more than three times the fluorescence intensity of the control A549 cell (Fig. 5B). Whereas, in the cases of using the PNA-DNA probes for T790M or L858R, only H1975 cells showed strong fluorescence of FITC, while PC-9 cells showed only slight fluorescence intensity (Fig. 5A, B). The fluorescence intensity of H1975 was more than 2-4 times higher than that of the control cell A549 (Fig. 5B). Whereas, the fluorescent intensities of PC-9 against T790M and L858R showed nearly the same intensity as those of A549 (Fig. 5B). It was reported that PC-9 has the amplified the EGFR gene[40] and have the higher expression level of EGFR than those of the H1975. However, the fluorescence intensity of PC-9 induced the PNA-DNA probe for exon19del E746-A750 was lower than the case of H1975 induced the PNA-DNA probes for T790M or L858R.

This might be caused by the efficiency of the probe induction into the cancer cells or binding affinities against each target sequences. A549 cells used as the negative control showed only slight fluorescence intensity when using all types of PNA-DNA probes (Fig. 5A, B). In this stage, we succeeded to distinguish the three gene mutant cancer cells using developed PNA-DNA probes. The results of the mRNA sequence analyses displayed in Fig. 3 show that the H1975 cell has T790M and L858R mutated EGFR mRNA, while the PC-9 cell has the exon19del E746-A750 mutated EGFR mRNA. The results of sequencing and FISH imaging are not contradictory. In the FISH imaging analysis, the fluorescence signals were stably observed (Fig. 5B). However in vitro analysis (Fig. 2B), especially results of T790M probe showed more unstable fluorescence signal than those of other probes. These unstable results are considered to be caused by the weak binding with PNA and target DNA, induced by the binding formation between adenine and thymine (change from C-G to T-A) as a result of SNP mutation. On the other hands, in the FISH analysis, fluorescence images were clearly observed due to the optimization of hybridization temperature and washing procedures after hybridization step. These operations are also considered to be contribute to the stability in FISH analysis. We will further improve the probe design for stable analysis in homogeneous assay conditions. These imaging results clearly indicate that the FITC-PNA probe dissociated from the Q-DNA probes and hybridized with target mutated EGFR mRNA within the cancer cells. On the other hands, FITC-PNA and Q-DNA were not dissociated in the cells whose EGFR mRNA do not have the target mutations. It means that

the only PNA-DNA probes which has the fill-complementary sequences showed the significant fluorescent intensities. Therefore, we concluded that different types of EGFR mRNA mutated cells are able to be detected and discriminated by executing imaging analysis techniques that use the three specified PNA-DNA probes. One of major previous mutation analysis methods, full genome sequencing via next-generation sequencers required the destruction of more than few thousand cells and 10-24 hours of analysis time. In contrast, the FISH-based mutation analysis with PNA-DNA probes developed in this study is able to detect the small number of EGFR mutated cells more rapidly and easily while using smaller amount of cell samples. Furthermore, the developed method could be applied as a new diagnostic method since the mutation types or existing ratio of EGFR mutated cells can be analyzed in tissue section samples.

In this study, we showed the potential for simple and rapid detection method of EGFR gene-mutated lung-cancer cells, which can lead toward improving anticancer drug resistance diagnosis by using novel PNA-DNA probes at the cellular level without the need for DNA sequencing. Although more functional improvements of the PNA-DNA probes are necessary in order for detection at the single living cell level, these methods overall will be an effective tool for providing quick and accurate analysis; also to be used for diagnosis of EGFR mutations in tissue section collected from patients in near future.

4. Conclusions

In this study, we developed three novel PNA-DNA probes for as an easy and rapid detection system for three EGFR gene mutations, such as exon19del E746-A750, T790M, and L858R. The three PNA-DNA probes showed increases in fluorescence intensity against dose-dependent, in vitro target EGFR mutated sequences, which indicates that FITC-PNA probes are separated from Q-DNA probes, and specifically bind to the target sequence of mutated EGFR mRNA. Furthermore, three different mutant cancer cells were successfully identified using three types of PNA-DNA probes during FISH analysis. Thus, in the future it is strongly suggested that developed PNA-DNA probes could be adopted in the rapid detection of EGFR mutations in cancer tissue samples from cancer patients.

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Conflicts of interest

The authors declare no competing financial interests.

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Fig. legends

Fig.1 Principle of target mutated EGFR mRNA detection with three new PNA-DNA probes. (A) When the FITC-PNA probes recognize their target RNA, the hybridized Q-DNA is detached from the PNA-DNA probes. Far from the Q-DNA, the distance between the FITC and Dabcyl changes, and this produces a fluorescence signal. (B) The anticancer drug (TKI)-resistance lung-cancer cells have the high frequently gene mutation of exon19del E746-A750, T790M, and L858R. The three PNA-DNA probes developed bind to three mutated sequences of EGFR mRNA and showed fluorescent signals.

Table_1 The sequences of FITC-PNA and Q-DNA probes.

Table_2 The sequences of model target DNA used in this study.

Fig. 2 Dose-response of three PNA-DNA probes against model target mutated DNA. (A) The fluorescence intensities of PNA-DNA probe for detect exon19del E746-A750 mutation. (B) The fluorescence intensities of PNA-DNA probe for detect T790M mutation. (C) The fluorescence intensities of PNA-DNA probe for detect L858R mutation. The blue dotted lines indicate the wild type DNA sequences. The red solid lines indicate the mutated DNA sequences. The results of the figures are shown as means ± standard error of three replicates.

Fig. 3 Partial sequencing of exon19del E746-A750, T790M, and L858R in EGFR mRNA of the lung-cancer cell line, H1975, PC-9. (A) The sequences without mutation. (B) The sequences of H1975 cell line. (C) The sequences of PC-9 cell line.

Fig. 4 Dose-response of three PNA-DNA probes against in vitro transcribed target EGFR RNA from three types of lung-cancer cells. (A) The fluorescence intensities of PNA-DNA probe for detect exon19del E746-A750 mutation. (B) The fluorescence intensities of PNA-DNA probe for detect T790M mutation. (C) The fluorescence intensities of PNA-DNA probe for detect L858R mutation. The blue lines (closed circle) indicate the EGFR mRNA transcribed from H1975. The red lines (closed square) indicate the EGFR mRNA transcribed from PC-9. The green lines (closed triangle) indicate the EGFR mRNA transcribed from A549. The results of the figure are shown as means ± standard error of three replicates.

Fig. 5 (A) FISH images of H1975, PC-9, and A549 cells stained with three PNA-DNA probes after fixation. H1975 cells (left images), PC-9 cells (middle images) and A549 cells (right images) stained with DAPI or PNA-probes for exon19del E746-A750, T790M, or L858R. (B) The analysis of fluorescence intensities in EGFR mutated cancer cells; H1975 (blue), PC-9 (red), A549 (green). The

fluorescence intensities were divided by cell area. The results of the figure are shown as means ± standard error of cells.

Graphical abstract

The PNA-DNA probes for detecting different types of EGFR mRNA harboring single nucleotide gene mutation were developed. (17 words)

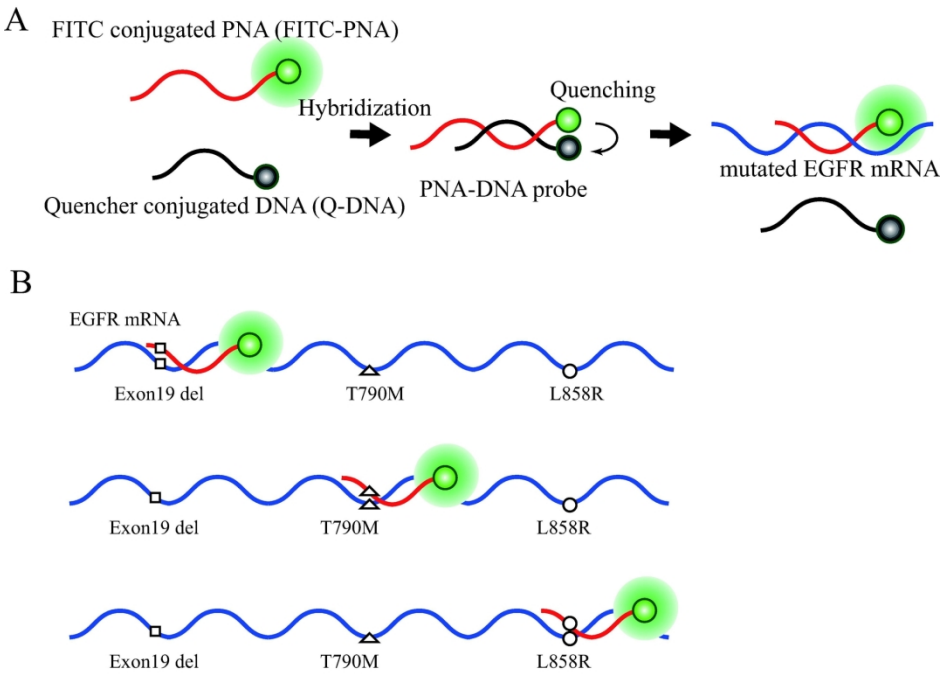

Figure1

Fig.1 Principle of target mutated EGFR mRNA detection with three new PNA-DNA probes. (A) When the FITC-PNA probes recognize their target RNA, the hybridized Q-DNA is detached from the PNA-DNA probes. Far from the Q-DNA, the distance between the FITC and Dabcyl changes, and this produces a fluorescence signal. (B) The anticancer drug (TKI)-resistance lung-cancer cells have the high frequently gene mutation of exon19del E746-A750, T790M, and L858R. The three PNA-DNA probes developed bind to three mutated sequences of EGFR mRNA and showed fluorescent signals.

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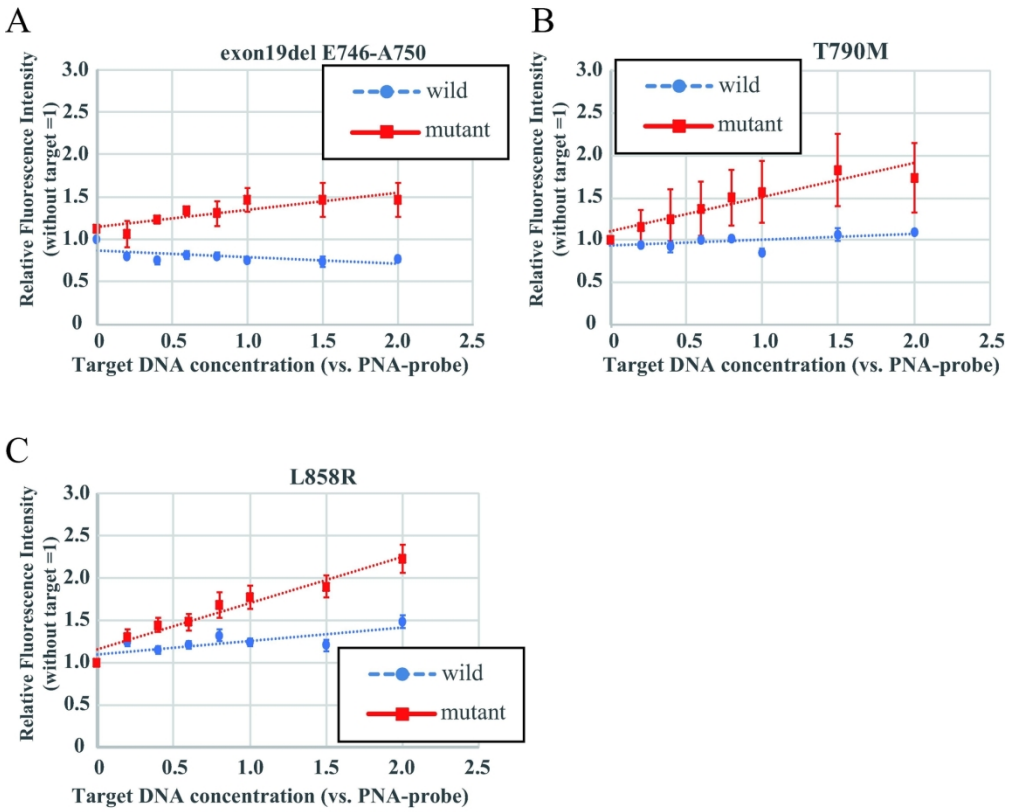

Figure2

Fig. 2 Dose-response of three PNA-DNA probes against model target mutated DNA. (A) The fluorescence intensities of PNA-DNA probe for detect exon19del E746-A750 mutation. (B) The fluorescence intensities of PNA-DNA probe for detect T790M mutation. (C) The fluorescence intensities of PNA-DNA probe for detect L858R mutation. The blue dotted lines indicate the wild type DNA sequences. The red solid lines indicate the mutated DNA sequences. The results of the figures are shown as means ± standard error of three replicates.

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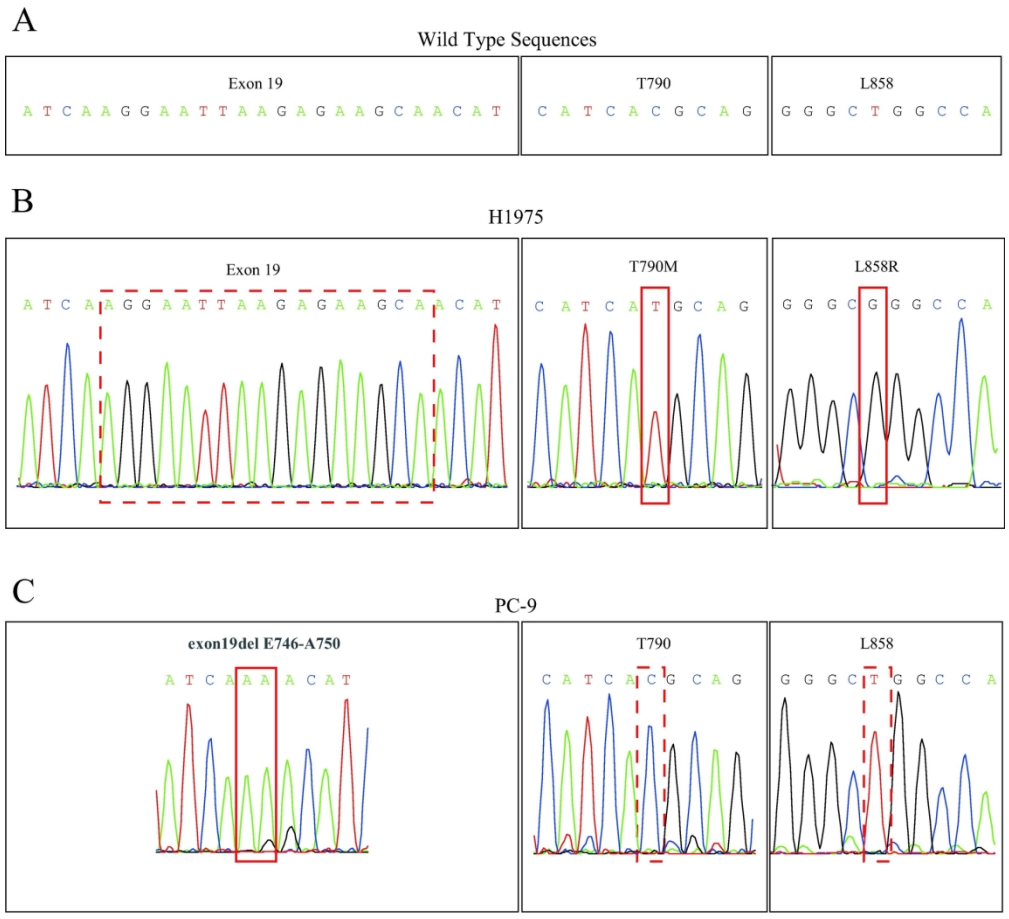

Figure3

Fig. 3 Partial sequencing of exon19del E746-A750, T790M, and L858R in EGFR mRNA of the lung-cancer cell line, H1975, PC-9. (A) The sequences without mutation. (B) The sequences of H1975 cell line. (C) The sequences of PC-9 cell line.

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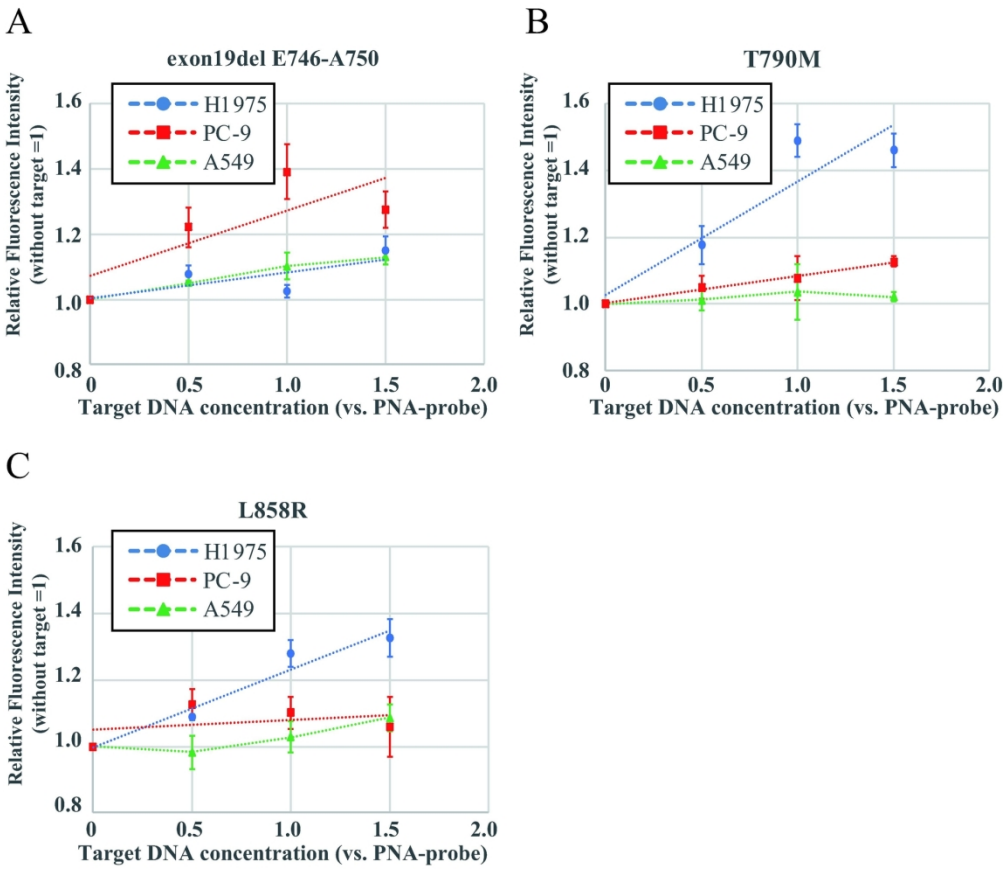


Figure4

Fig. 4 Dose-response of three PNA-DNA probes against in vitro transcribed target EGFR RNA from three types of lung-cancer cells. (A) The fluorescence intensities of PNA-DNA probe for detect exon19del E746-A750 mutation. (B) The fluorescence intensities of PNA-DNA probe for detect T790M mutation. (C) The fluorescence intensities of PNA-DNA probe for detect L858R mutation. The blue lines (closed circle) indicate the EGFR mRNA transcribed from H1975. The red lines (closed square) indicate the EGFR mRNA transcribed from PC-9. The green lines (closed triangle) indicate the EGFR mRNA transcribed from A549. The results of the figure are shown as means ± standard error of three replicates.

167x170mm (300 x 300 DPI)

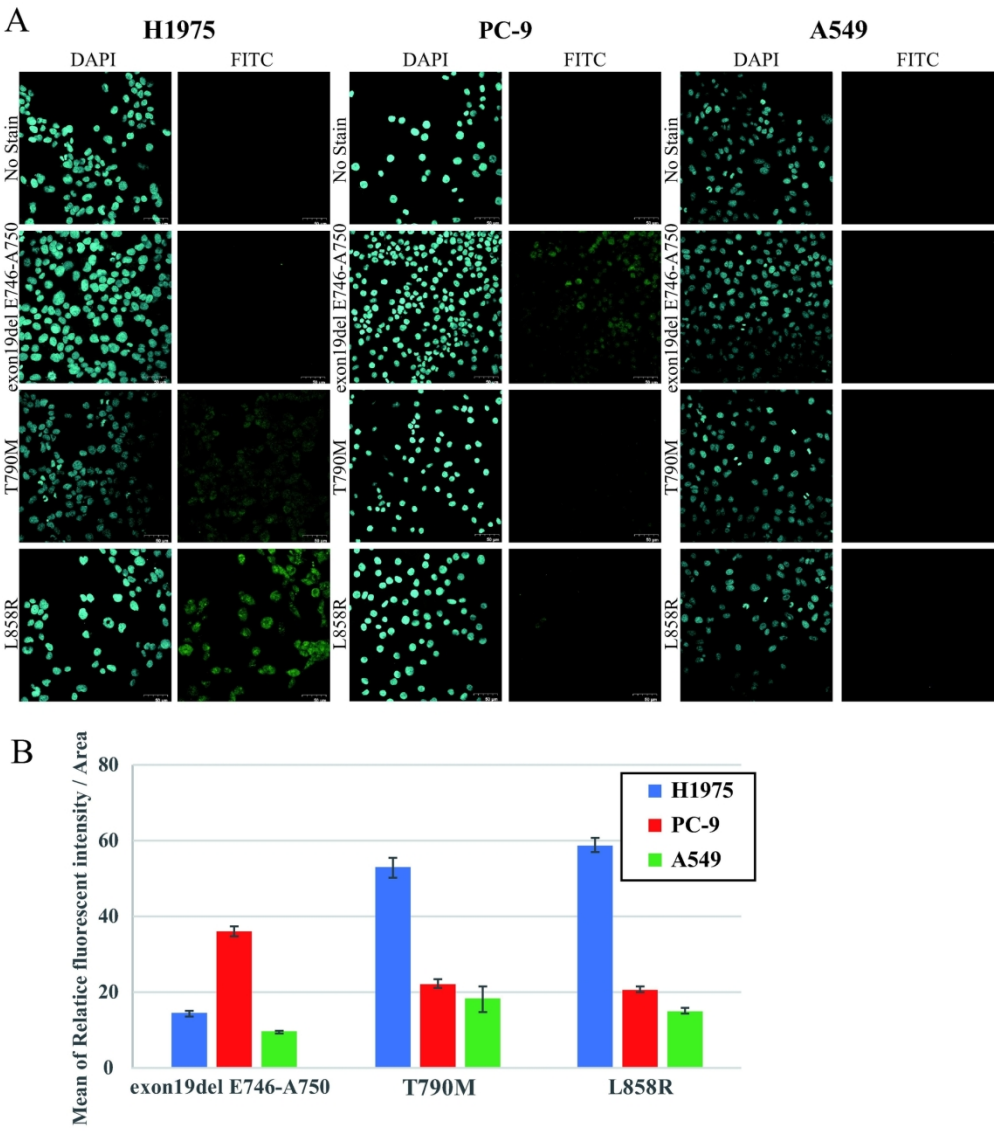


Figure5

Fig. 5 (A) FISH images of H1975, PC-9, and A549 cells stained with three PNA-DNA probes after fixation. H1975 cells (left images), PC-9 cells (middle images) and A549 cells (right images) stained with DAPI or PNA-probes for exon19del E746-A750, T790M, or L858R. (B) The analysis of fluorescence intensities in EGFR mutated cancer cells; H1975 (blue), PC-9 (red), A549 (green). The fluorescence intensities were divided by cell area. The results of the figure are shown as means ± standard error of cells.

168x203mm (300 x 300 DPI)

	Sequence Name
FITC-PNA	exon19del E746-A750
	T790M
	L858R
Q-DNA	exon19del E746-A750
	T790M
	L858R

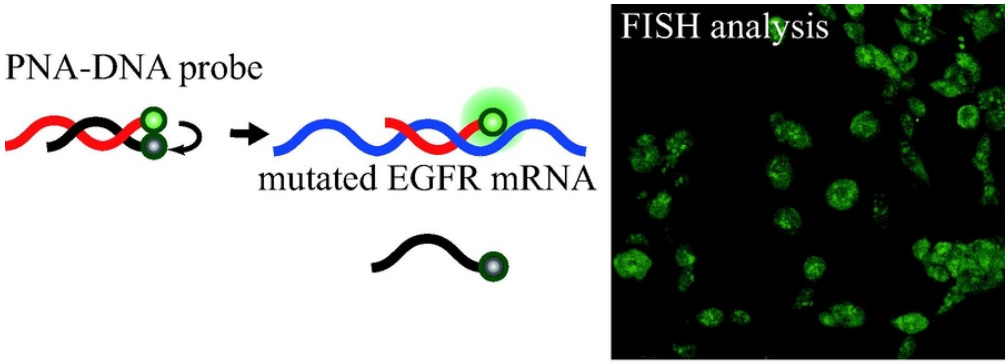
Table_1

Sequece (N'→C' or 5'→3')
fluorescein-O-Linker-PNA(ATGTTTTGAT)-k-k
fluorescein-O-Linker-PNA(CTGCATGATG)-k-k
fluorescein-O-Linker-PNA(TGGCCCGCCC)-k-k
CAAAACAT-Dabcyl
TCATGCAG-Dabcyl
GCGGGCCA-Dabcyl

	Sequence Name
Target DNA	exon19del E746-A750 wild
	exon19del E746-A750 mutant
	T790M wild
	T790M mutant
	L858R wild
	L858R mutant

Table_2

Sequece (5'→3')
ATCAAGGAATTAAGAGAAGCAACAT
ATCAAAACAT
CATCACGCAG
CATCATGCAG
GGGCTGGCCA
GGGCGGGCCA



Graphical abstract

76x47mm (300 x 300 DPI)