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Label/Quencher-Free Detection of Exon Deletion Mutation in Epidermal Growth Factor Receptor Gene Using G-Quadruplex-Inducing DNA Probe

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Detection of exon 19 deletion mutation in the epidermal growth factor receptor (EGFR) gene, which results in increased and sustained phosphorylation of EGFR, is important for diagnosis and treatment guidelines in non-small-cell lung cancer. Here, we have developed a simple and convenient detection system using the interaction between G-quadruplex and fluorophore thioflavin T (ThT) for discriminating EGFR exon 19 deletion mutant DNA from wild type without a label and quencher. In the presence of exon 19 deletion mutant DNA, the probe DNAs annealed to the target sequences were transformed into G-quadruplex structure. Subsequent intercalation of ThT into the G-quadruplex resulted in a light-up fluorescence signal, which reflects the amount of mutant DNA. Due to stark differences in fluorescence intensity between mutant and wild-type DNA, we suggest that the induced G-quadruplex structure in the probe DNA can report the presence of cancer-causing deletion mutant DNAs with high sensitivity.

Keywords: EGFR exon 19 deletion, thioflavin T, G-quadruplex, hairpin DNA

Introduction

Lung cancer is one of the major causes of cancer deaths, accounting for about 1.3 million deaths annually throughout the world [1]. Lung cancer has two types: non-small-cell lung cancer (NSCLC) (87% of cases) and small-cell lung cancer [2]. It has been reported that NSCLC is caused by mutations in the epidermal growth factor receptor (EGFR) gene, which is a receptor tyrosine kinase (TK) located in epithelial cell surface [3]. EGFR mutations increase the kinase activity of EGFR, which induces uncontrolled proliferation of cancer cell [4, 5]. One of the most commonly found EGFR mutations is the in-frame deletions in exon 19 that account for 48% of all EGFR-mutated NSCLC [3, 6]. The EGFR exon 19 deletion is associated with sensitivity to small molecule TK inhibitors (TKIs), including erlotnib and gefitnib, which have been preferably prescribed for EGFR-mutated NSCLC [7]. Thus, precise detection of the EGFR exon 19 mutation is clinically important to determine suitable treatment guidelines in NSCLC.

To date, several molecular detection methods have been introduced to discriminate EGFR exon 19 deletion, including Scorpion-ARMS analysis, PCR invader, and PNA-clamping PCR [8-11]. However, an efficient and accurate detection of EGFR exon 19 deletion still has a challenge because of various types of deletion mutations and low specificity [6]. Analytical platforms using the G-quadruplex DNA structure have obtained meaningful consideration due to the sensitive structural change of the G-quadruplex sequence [12]. The G-quadruplex is a four-stranded DNA secondary structure containing square-planar guanines stabilized by Hoogsteen base-pairing [13]. Various groups have observed that selective detection of the target sequence can be accomplished by the inducement of G-quadruplex structures upon hybridization of the G-quadruplex flanking sites [14, 15]. The intercalating benzothiazole dye, thioflavin T (ThT), has been reported as a specific light-up fluorescence probe for the G-quadruplex structures [16].

In this study, we took advantage of the structural specificity for G-quadruplexes that makes ThT as a useful

fluorophore. Two probe DNAs were designed for detection of the exon 19 deletion of the EGFR gene, in which the presence of the mutant DNA results in inducing G-quadruplexes in the probe DNA due to hybridization with target DNA. Subsequently, the ThT fluorophore can light up fluorescence by forming the G-quadruplex/ThT complex, which reflects the amount of the mutant DNA with high sensitivity and specificity.

Materials and Methods

DNA Oligonucleotides and Reagents

DNA oligonucleotides (probe 1, probe 2, wild-type DNA, and mutant DNA) were chemically synthesized and purified by the high affinity purification method and polyacrylamide gel electrophoresis (Bionics, Republic of Korea). The sequences of DNA oligonucleotides are listed in Fig. 1C. Thioflavin T (ThT) for fluorescence detection was purchased from Sigma-Aldrich Korea (Korea).

Intercalation of Thioflavin T and Fluorescence Detection

For fluorescence detection of EGFR exon 19 deletion mutant DNA, 5 μl of 100 nM mutant DNA was added to 45 μl of fluorescence detection mixture, which contained 3 μM of thioflavin T , probe 1 or probe 2 (100 nM), and 20 mM Tris-HCl (pH 7.2). For fluorescence measurements, the incubated mixture was transferred to a quartz cuvette (Hellma Analytics, Singapore), and the fluorescence was scanned using a Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies, USA). The excitation wavelength was 425 nm, and emission spectra were scanned from 450 to 650 nm.

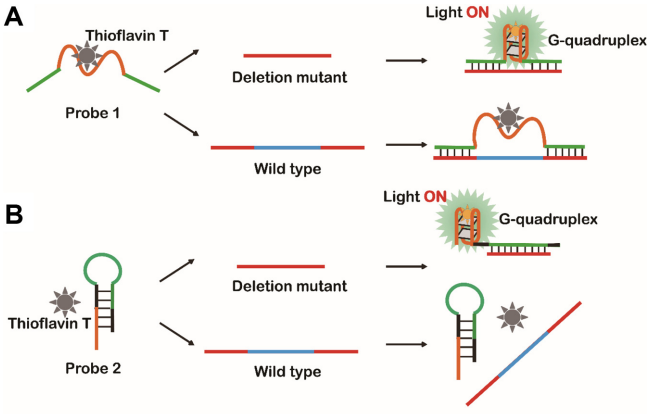
Target Sensitivity Assessment

The target sensitivity assay was performed in 50 μl of reaction mixture, containing probe 1 DNA or probe 2 DNA (100 nM), and various concentrations (200, 100, 50, 20, 10, 5, 2, and 1 nM) of mutant DNA oligonucleotides in 20 mM Tris-HCl (pH 7.2). Fluorescence measurement for the ThT light-up signal was performed as described above.

Results and Discussion

Probe DNAs Containing G-Quadruplex Sequence for Detection of EGFR Exon 19 Deletion

We designed two types of probe DNA for EGFR exon 19 deletion containing the G-quadruplex sequence (Fig. 1). First, probe 1 DNA was designed to induce the G-quadruplex structure upon hybridizing to the mutant DNA (Fig. 1A). In the presence of mutant DNA, the duplex formed between the single-stranded site (green line) in probe DNA and the target mutant (red line) assembles the ends of the G-quadruplex tightly and makes a robust G-quadruplex



C

Name	Sequence
Probe 1	5'- GCT ATC AAG TTT A GGG TTA GGG TTA GGG TTA GGG TTT ACA TCT CCG -3'
Probe 2	5'- A GGG TTA GGG TTA GGG TTA GGG CGG AGA GCT ATC AAG ACA TCT CCG CCC TAA C -3'
Wild type	5'- GGC TTT CGG AGA TGT TGC TTC TCT TAA TTC CTT GAT AGC GAC GGG -3'
Mutant	5'- GGC TTT CGG AGA TGT CTT GAT AGC GAC GGG -3'

Fig. 1. Schematic illustration of the detection process of the exon 19 deletion mutant EGFR gene using probe DNAs inducing G-quadruplex structure and fluorogenic thioflavin T. Detection system using (**A**) Probe DNA 1 and (**B**) Probe DNA 2. (**C**) Table of oligodeoxynucleotide sequences used in this study.

structure. Next, the fluorescence enhancement of the ThT upon binding to the G-quadruplex reports the presence of the mutant DNA. On the other hand, the duplex formed between the single-stranded site (green line) and the undeleted wild-type DNA (red and blue line) puts some distance between the ends of the G-quadruplex. This hybridized DNA form is likely to form a weak G-quadruplex structure, which results in the quiescent fluorescence signal of ThT.

Second, probe 2 DNA was designed by extending the G-quadruplex sequence to form a hairpin structure, in which a G-quadruplex transformation and subsequent intercalation of ThT into the G-quadruplex is not possible without resolving the hairpin structure (Fig. 1B). Resolving the stem structure by target (mutant) DNA binding can release the G-quadruplex sequence and allow intercalation of ThT into the G-quadruplex. However, wild-type DNA cannot resolve the stem of the hairpin structure, and thereby the G-quadruplex sequence is not released. Light-up fluorescence of ThT occurs only with the G-quadruplex structure, not with single-stranded DNA or double-stranded DNA, by which significant fluorescence signal difference is dictated

between target mutant DNA and wild-type DNA.

Fluorometric Detection of Target Mutant DNA (EGFR Exon 19 Deletion)

To validate our G-quadruplex and ThT platform for detection of EGFR exon 19 deletion in DNA, we investigated the fluorescence emission spectrum of ThT in the presence of mutant DNA or wild-type DNA (Fig. 2A). As shown in Fig. 2A, a weak fluorescent emission was observed in the absence of mutant DNA and wild type. The addition of wild-type DNA into the probe 1 DNA mixture caused a slight increase of ThT fluorescence. In contrast, when the mutant DNA was added, the ThT fluorescence was significantly enhanced, and the signal to background ratio (S/B) reached to about 4.0. This result indicated that the G-quadruplex of probe 1 was formed only in the presence of EGFR exon 19 deletion mutant DNA due to the duplex formed between probe 1 and the target mutant sequence. We suggest that the duplex formation between probe 1 DNA and the target mutant DNA assembles G-quadruplex tightly and makes a robust G-quadruplex structure.

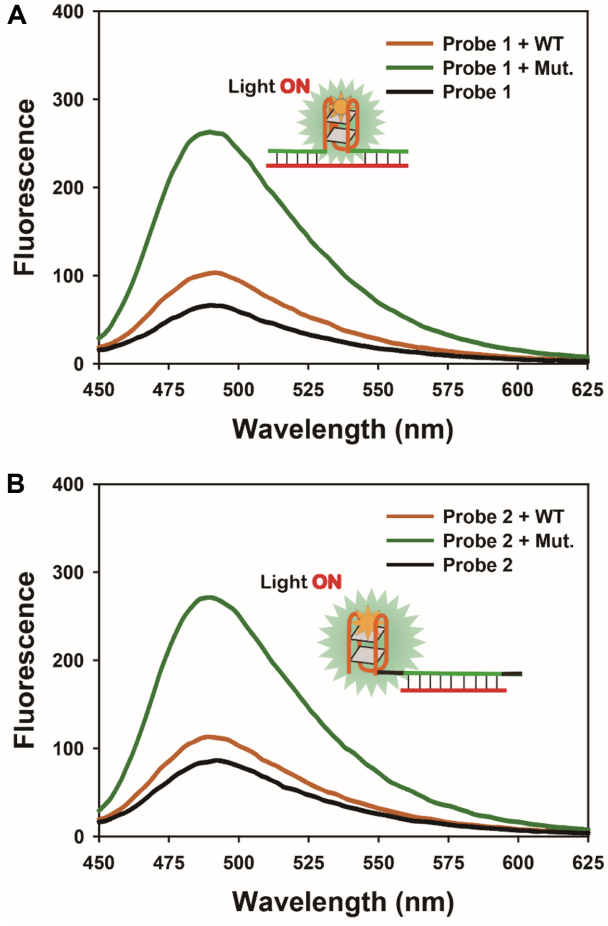


Fig. 2. Fluorescence emission spectra (λ_{ex} = 425 nm) of thioflavin T in the presence of wild-type DNA (WT) or deletion mutant-type DNA (MT) using the system of (**A**) probe DNA 1 containing G-quadruplexes, and (**B**) hairpin-type probe DNA 2 containing G-quadruplexes.

We next monitored the fluorescence emission spectrum of ThT in the probe 2 DNA system, which has a hairpin structure (Fig. 2B). When wild-type DNA was added to a solution containing the probe 2 DNA, fluorescence was slightly enhanced similar to the background level, which was measured in the presence of only probe 2 DNA. In contrast, fluorescence was starkly increased in the presence of the mutant DNA in a solution containing probe 2 DNA, reaching the S/B of about 3.2. Thus, the hairpin structure probe 2 DNA maintains its stem form in the presence of wild-type DNA, whereas in the presence of mutant DNA, the hairpin structure is resolved and induced to the G-quadruplex structure owing to annealing of the probe DNA to mutant DNA.

Evaluation of Sensitivity for Target Mutant DNA (EGFR Exon 19 Deletion)

To evaluate the sensitivity of the EGFR exon 19 deletion mutant detection system using probe 1 DNA, we carried out fluorescence emission titration experiments as the concentration of mutant DNA was decreased (Fig. 3). As expected, the fluorescence intensity of G-quadruplex was enhanced with increasing concentrations of mutant DNA (Fig. 3A), indicating that probe 1 formed the G-quadruplex after hybridization with the target mutant DNA in a quantitative manner. Maximum fluorescence emission at each mutant DNA concentration provided a linear range of detection for mutant type DNA from 1 to 200 nM (Fig. 3B). The lower limit of detection (LLOD) was calculated with a 1% confidence level, as obtained by the equation LLOD = 3.3 × (SD/S), where SD is the standard deviation of the response, and S is the slope of the linear curve. According to the equation, the LLOD of the ThT-based EGFR exon 19 detection system was calculated to be 2.7 nM, which suggests that the probe 1 DNA system containing G-quadruplex/ThT fluorescence can detect mutant DNA as low as 2.7 nM without any quencher or label.

To assess the quantitative sensitivity of the EGFR exon 19 mutant detection with the hairpin-type probe 2 DNA, fluorescence emission spectra were monitored at decreasing concentrations of mutant DNA (Fig. 4A). The fluorescence intensity gradually increased as the concentration of the target mutant DNA increased. The linear relationship was

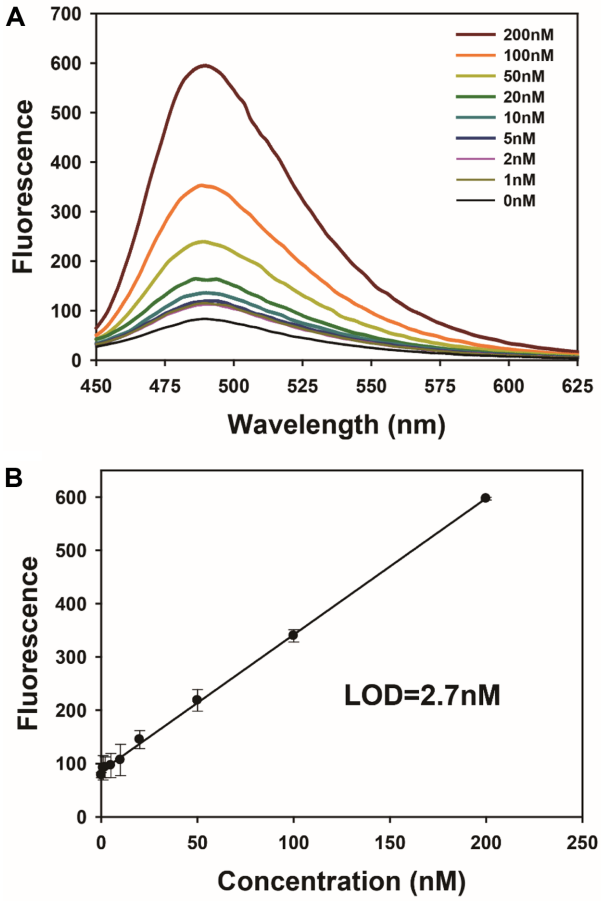


Fig. 3. Sensitivity of the G-quadruplex/ThT-based fluorescence assay using the probe DNA 1 that can induce the G-quadruplex structure.
(**A**) Fluorescence emission spectra (λ_{ex} = 425 nm) of thioflavin T with decreasing concentrations of deletion mutant DNA in the probe 1 DNA system inducing the G-quadruplex/ThT-based fluorescence light-up. (**B**) Linear plot of the changes in fluorescence intensity at 490 nm against the mutant DNA concentrations. The limit of detection value was calculated to be 2.7 nM.

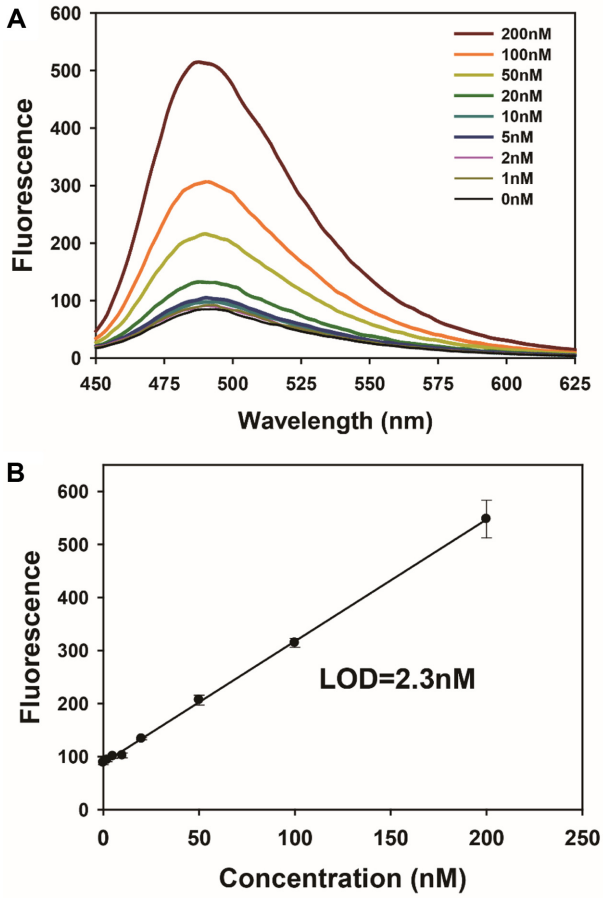


Fig. 4. Sensitivity of the G-quadruplex/ThT-based fluorescence assay using the hairpin-type probe DNA 2 that can release the G-quadruplex structure upon target DNA hybridization.
(**A**) Fluorescence emission spectra (λ_{ex} = 425 nm) of thioflavin T with decreasing concentrations of deletion mutant DNA in the probe 2 DNA system. (**B**) Linear plot of the changes in fluorescence intensity at 490 nm against the mutant DNA concentrations with a limit of detection value of 2.3 nM.

observed between fluorescence intensity and target mutant DNA concentration, with a detection limit of 2.3 nM (Fig. 4B). Thus, two probe DNAs provided a similar detection limit for the EGFR exon 19 deletion mutant DNA using the G-quadruplex formation and subsequent ThT fluorescence light-on.

In conclusion, we have developed a simple fluorometric detection method for EGFR deletion mutant genes using a G-quadruplex-inducing probe DNA designed to be complementary to target mutant DNA and G-quadruplex/ThT-based fluorescence light-up system without a label and quencher. By observing evident differences in fluorescence intensity between the target mutant DNA and wild-type DNA, we have verified transformation of the probe DNA into the G-quadruplex structure upon hybridization to the target mutant DNA. In addition, the high specificity and sensitivity of the G-quadruplex/ThT-based fluorescence assay enables efficient detection of target mutant DNA quantitatively at amounts as low as 2.3 nM. This G-quadruplex/ThT-based fluorescence detection system would be a simple method for detecting cancer-related deletion mutant DNAs for the diagnosis of cancer-causing mutations in genes.

Acknowledgments

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