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Rapid and label-free amplification and detection assay for genotyping of cancer biomarker

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ABSTRACT

As understanding of the molecular pathways that drive malignancy in human cancer improves, personalized genotype-based therapy in combination with the predictive biomarker for the efficacy of targeted therapy is becoming more popular in cancer management. Sanger sequencing, that has been the gold standard for mutation analysis in cancer since the 1970s, suffers from low sensitivity, complexity, and time-consuming and labor-intensive procedure. Although several PCR based molecular testing methods are being emerged, there is no universal assay available for genotyping of cancer biomarkers. Here we present a rapid, simple and sensitive assay for the detection of epidermal growth factor receptor (*EGFR*) mutation in non-small cell lung cancers (NSCLCs). The assay employs a novel double mis-matched primer (DMP) set to improve the detection ability of isothermal solid-phase amplification/detection (ISAD) based on silicon microring biosensor. We show that the EGFR-DMP can detect *EGFR* gene mutations within 20 min in a label-free and real-time manner. The EGFR-DMP was able to detect a mutation in a sample containing only 1% of the mutant cells in a mixture of wild-type cells. Furthermore, to validate the proposed assay for potential applications in clinical diagnostics, we examined paraffin-embedded tissue samples from 10 NSCLC patients for the presence of *EGFR* mutations by performing EGFR-DMP and direct sequencing. The EGFR-DMP assay was able to rapidly detect the mutation, with high sensitivity and specificity. The EGFR-DMP assay offers a robust and sensitive approach for the rapid identification of the *EGFR* mutation. The high sensitivity and specificity and rapidity of this approach may make it useful for predicting the clinical response to targeted *EGFR* TKIs as a companion diagnostic.

1. Introduction

Our understanding of biomarkers to aid clinical decisions in cancer treatment has increased considerably over the past two decades. Advances in the knowledge of the molecular mechanisms underlying disease have led to the development of several novel strategic drugs by using the drug-diagnostic co-development model. This requires a potential link between a certain molecular characteristic and treatment outcome (Dimou et al., 2011; Li et al., 2008; Olsen and Jorgensen, 2014; Soo et al., 2009). Thus, the use of biomarkers to predict the benefits of targeted therapy and thereby avoid delaying other effective treatment modalities is an attractive option (Eberhard et al., 2008). Consequently, the choice of treatment for cancer patients relies on molecular testing, so-called companion diagnostic (CDx), using specific biomarkers and it has become a critical tool to ensure the care and safety of patients. CDx is an emerging and promising method that can provide biological and clinical information to enable better decisions to be made regarding treatment (U.S. FDA, 2014; Khozin et al., 2014). The main aim of the CDx assay is to determine whether benefits from a particular treatment would outweigh potential side effects and risks by using an accurate and rapid test that can predict individual responses.

Non-small cell lung cancer (NSCLC) is the most common type (75–80%) of lung cancer and is a major threat to human health (Eberhard et al., 2008; Khozin et al., 2014; Rosell et al., 2009; Soo et al., 2009; Wang et al., 2013). Although surgery is the most effective treatment, more than 70% of patients who present locally advanced or metastatic disease are not eligible for surgery.

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Furthermore, the response rates to standard treatments, such as chemotherapy and drug treatment, for advanced or recurrent NSCLC are as low as 30–40% (Santos et al., 2011). Tyrosine kinase domain inhibitors (TKIs), erlotinib and gefitinib, target the epidermal growth factor receptor (*EGFR*) and are widely used as first-line treatment agents in patients with NSCLC. Treatment with these two drugs results in a diverse response that is dependent on the mutation status of the patient. The mutation profiles, including a specific point mutation (L858R) in exon 21 and a short in-frame deletion in exon 19 (Del_E747-A750), have been reported to comprise up to 90% of all EGFR-activating mutations (Ellison et al., 2013; Marchetti et al., 2006; Taniguchi et al., 2011; Wang et al., 2010; Weber et al., 2014). Therefore, accurate identification of the mutation status of *EGFR* prior to the treatment is important in NSCLC patients who might benefit from *EGFR*-TKI therapy (Ellison et al., 2013).

Recently, the U.S. Food and Drug Administration (FDA) approved companion diagnostic solutions for erlotinib treatment to allow the detection of *EGFR* mutations (exon 19 or exon 21) in NSCLCs (U.S. FDA, 2014). Testing the mutational status of *EGFR* as a companion diagnostic allows the sensitivity of tumors to erlotinib treatment to be predicted, which will provide important information to determine the optimal choice of therapy. However, several issues such as lacks of sensitivity and time consuming still need to be addressed for clinical use of it in real-life decisions on drug treatment (Nagai et al., 2005; Yu et al., 2009; Rosell et al., 2009; Kiumra et al., 2006a).

To date, many new methods to detect *EGFR* mutations have been established; however, direct sequencing, known as Sanger sequencing, is commonly used. It has low sensitivity and can only detect the mutation when $>25\%$ of mutant DNA is present. Furthermore, this method has significant limitations including high-costs associated with the large-scale equipment and reagents required, and it is time-consuming, and labor-intensive (Hoshi et al., 2007; Janne et al., 2006; Kimura et al., 2006a; Nagai et al., 2005). To overcome these limitations, a number of non-sequencing methods have been devised, such as scorpion amplified refractory mutation system (ARMS) (Kimura et al., 2006a, 2006b; Newton et al., 1989; Whitcombe et al., 1999), mutation specific antibody (Yu et al., 2009), peptide nucleic acid (PNA)-mediated PCR clamping (Nagai et al., 2005), high-resolution melting analysis (Fukui et al., 2008; Nomoto et al., 2006; Willmore-Payne et al., 2006), denaturing high-performance liquid chromatography (DHPLC) (Janne et al., 2006), and smart amplification process (SMAP) (Hoshi et al., 2007) that allow rapid and accurate mutation screening of large numbers of samples. Although some of these methods have proved effective and sensitive (the detection limit of 0.1–1% of mutated DNA) at detecting *EGFR* mutations, they are still labor intensive and may require sophisticated instruments that are not suitable for near patient testing (NPT) (Crook, 2000; Pao and Ladanyi, 2007). Currently, no NPT device is available for *EGFR* mutation testing; the future development of such devices would help ensure that treatment is not delayed while test results are pending.

Recently, we have developed the isothermal solid-phase amplification/detection (ISAD) method that can amplify and detect single nucleotide polymorphisms (SNP) with high sensitivity, specificity, and rapidity (30 min) under isothermal cycling and silicon biophotonic sensor conditions (Shin et al., 2013a). In this study, we adapted the ISAD method to include a newly designed double mis-matched primer (DMP) set, the so-called EGFR-DMP, to target the *EGFR* single substitution mutation (L858R). The aim of this study is to develop an effective assay for rapid *EGFR* mutation screening in tumor tissues and from lung cancer patients, to be applied as NPT. This method can meet the above requirements that enables the rapid (20 min), and simple detection of the mutation, and it is superior ($<1\%$ of mutant cells) to direct sequencing in its ability to detect the mutation in clinical specimens.

2. Experimental

2.1. Fabrication and functionalization of EGFR-DMP device

To design EGFR-DMP device, the modified protocol was used for the detailed structure and fabrication of silicon microring resonator (SMR) that have been previously described (Shin et al., 2013a, 2013b, 2013c). To use the EGFR-DMP device as a rapid sensor of *EGFR* mutation detection, the modified protocol was used that has been previously described (Shin et al., 2013a). All designed primers used for the *EGFR* (L858R) detection were custom-synthesized from Integrated DNA Technology (Table S1). For the immobilization of the DMP primer, the prepared sensor surface was incubated with the amine-modified primer of DMP in PBS (1 μM) containing 5 mM sodium cyanoborohydride for over-night at room temperature. After incubation, unbound DNA primers were washed away with PBS and the sensors were dried using nitrogen gas. An acrylic well [1.5 cm × 0.7 cm × 2 mm] for a microfluidic chamber was then pasted onto the chip to enclose the microring sensor area [3 mm × 4 mm × 1 mm].

2.2. Lung cancer cell lines (positive and negative mutations)

To test the EGFR-DMP method, positive and negative control DNA was extracted from lung cancer cell lines including NSCLC (A540, NCI-H1975, NCI-H1650) and Jurkat (human T lymphocyte) cells that were established as previously described (Shin et al., 2014). A549, NCI-H1650, Jurkat are wild-type for *EGFR* and NCI-H1975 contains L858R mutation in the exon 21. The cell lines were maintained in plastic culture dishes with high-glucose Dulbecco's modified Eagle's Medium (DMEM, Life Technology) supplemented with 10% fetal calf serum (FCS) in a 37 °C humid incubator with 5% ambient CO_2. The cancer cell lines were cultured, and then the genomic DNA was extracted by using AL buffer with proteinase K from a QIAmp DNA mini kit (Qiagen, Hilden, Germany), as previously reported (Shin et al., 2013a, 2014). The samples were eluted with a 100 μL volume of elution buffer. The eluted DNA was stored at −20 °C until use.

The serial dilution samples were prepared for sensitivity comparison of the EGFR-DMP and direct sequencing methods by mixing the cell lines [Jurkat (wild-type) and (NCI-H1975) mutant cell lines] at the desired percentage ranging from 0%, 1%, 5%, 10%, 20%, 50% to 100%, and then the DNA was extracted using the above mentioned protocol.

2.3. Conventional PCR (PCR and real-time PCR) and direct sequencing

End-point PCR, RT-PCR and direct sequencing were performed to compare with EGFR-ISAD assay. The forward and reverse primers were synthesized at the usual length of around 24 bp (Table S1). The positive target template (L858R mutation) for the conventional methods was DNA obtained from NCI-H1975 cells at a concentration of 5 ng μL^{-1} to 500 fg μL^{-1}. The human genomic DNA (Roche Diagnostics) was used as a non-target control (negative control). The end-point PCR process consisted of an initial denaturation step at 95 °C for 15 min; 45 cycles of 95 °C for 45 s, 60 °C for 45 s, and 72 °C for 45 s; and a final elongation step at 72 °C for 10 min. 5 μL of DNA were amplified in a total volume of 25 μL containing 1X PCR buffer (Qiagen), 2.5 mM $MgCl_2$, 0.25 mM deoxynucleotide triphosphate, 25 pmol of each primer, and 1 unit of Taq DNA polymerase (Qiagen). Gel electrophoresis was used to

separate PCR products on a 2% agarose gel containing ethidium bromide (EtBr). The gel was visualized using a Gel Doc System (Bio-Rad).

For RT-PCR, the following procedure is modified from LightCycler 2.0 Instrument protocol (Roche Diagnostics). 5 μL of DNA was amplified in a total volume of 20 μL containing 4 μL of LightCycler FastStart DNA Master mix, 25 pmol of each primer, and 2 μL of 1X PCR buffer (Qiagen), 2.5 mM $MgCl_2$, 0.25 mM deoxynucleotide triphosphate, 25 pmol of each primer, and DI water. An initial pre-incubation cycle of 95 °C for 10 min was followed by 50 cycles of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 10 s, and by cooling step of 40 °C for 30 s. The amplified products with SYBR Green signals were carried out on a LightCycler 2.0 (Roche Diagnostics).

For DNA direct sequencing, all DNA samples are amplified with the sequencing primer of *EGFR* exon 21 (Table S1), and then purified it by using the QIAquick kit (Qiagen, Germany) for the direct sequencing. The purified samples were directly sequenced using BigDye Terminal chemistry with *EGFR*-Seq-Reverse primer. The DNA sequencing reactions are electrophoresed on ABI's 3730XL DNA Analyzers, which can produce read lengths of 800–1000 bases by AIT Biotech (AITbiotech Pte Ltd.).

2.4. Tumor samples of lung cancer patients

Tissue samples were obtained from the 10 patients with NSCLC (stages III–IV) were collected and separately stocked at −80 °C until use. Institutional approval and informed consent from all patients were obtained in writing. Tumor tissues from 10 patients were obtained on protocols approved by the Institutional Review Board of NUHS. The paraffin-embedded tissues obtained from the patients were collected retrospectively. The tumor cell content was at least 50% in most samples. To extract DNA from tissues, Qiagen DNeasy Tissue Kit was used. The resulting DNA was eluted in 50 μL of elution buffer. The concentration and purify of the extracted DNA from tissues were determined by spectrophotometry. The extracted DNA was stocked at −20 °C until use.

2.5. EGFR-DMP assay for EGFR mutation detection

In order to amplify the target using the modified recombinase polymerase amplification method, we prepared the assay solution for *EGFR* mutation (L858R) detection as described previously (Shin et al., 2013a). Briefly, for optimized reaction, 29.6 μL of rehydration buffer, 15 μL of nuclease-free water, 2.5 μL of other primers (10 μM) were mixed. The forward and reverse primers generated a 180-bp product. One dried enzyme pellet provided (TwistDX, UK) was added to each solution and vortexed. Then, 2.5 μL of magnesium acetate solution was dispensed into the cap of each tube. A unidirectional shake mode mixing protocol guaranteed a homogeneous distribution of the molecules that are necessary for the reaction in the buffer. After mixing, the total volume of 50 μL reaction buffer is split into five 10 μL aliquots. To start the reactions, 2 μL of the extracted DNA from cell lines and/or clinical samples with patients were mixed with the 10 μL reaction aliquot. The EGFR-DMP chip was then placed on a thermoelectric cooler (TEC) with controller (Alpha Omega Instruments) to keep a constant temperature (37 °C). and the resonance spectrum of the device was immediately measured and used as a reference to obtain a baseline. The resonance wavelength shifts were caused by kinetic information regarding the binding of the targets to the immobilized receptors (Bogaerts et al., 2012; Kindt and Bailey, 2013).

3. Results and discussion

3.1. The principle of ISAD with DMP for EGFR mutation detection

The ISAD method is a rapid DNA analysis technology that can simultaneously amplify and detect a target template within 30 min under isothermal condition based on solid phase amplification and silicon biophotonic sensing conditions in a label-free and real-time manner (Shin et al., 2013a). Although the experimental results showed that the ISAD method is highly sensitive, considerably high background signal caused by non-target materials can be problematic for the detection of target biomarkers in real clinical samples. Therefore, in this work, we have developed a new technique, double mis-matched primer (DMP) method, to reduce the background signal caused by mis-matched sequences (non-target) and to induce amplification of the mutant-specific sequence (matched target) for the improvement of mutation detection. DMP consists of two (double) mis-matched nucleotides (the mutant nucleotide from the target (M) and additional mismatched nucleotide, Fig. 1A, left) located at the 3′ end of the forward primer (Table S1). As a pair of primers, the additional mismatched nucleotide is also placed at the 3′ end of the reverse primers. The working principle of DMP is shown in Fig. 1A, right. When the template has a non-target (in this case a wild-type DNA), the amplification is suppressed or does not occur due to the low binding affinity between the double mis-matched nucleotides (DMP) and the non-target template (Fig. 1A, right) while the template with target DNA is amplified to generate amplicons. Therefore, the detection limit for the detection of target sequences can be improved due to higher signal-to-noise ratio resulted from the reduction of the non-specific target binding using the DMP method. When DMP method is applied to ISAD, one of DMP set is covalently immobilized on the silicon microring resonator (SMR) surface to prime DNA synthesis as shown in Fig. 1B. Then, a mixture of the mutant target DNA, recombinase, and polymerase from an RPA (recombinase polymerase amplification) kit is added to the sensor surface. The recombinase enzyme recognizes both primers (immobilized primer and floating primer) and enables the formation of the recombinase–primer complex. This complex binds to double stranded target DNAs and facilitates strand exchange to begin the amplification process of ISAD. Exponential amplification from both the solid and solution phases simultaneously occur by repeating the process until all the reaction components have been used, which causes the wavelength shift of SMR and can be monitored in a real-time, label-free manner. In the case of non-target wild DNA, the recombinase–primer complex binding to double stranded target DNAs for facilitation of strand exchange would be inhibited due to double mis-matched nucleotides in DMP.

3.2. Characterization of DMP by conventional PCR and ISAD

For the proof of principle demonstration of DMP method, basic experiments were first performed with genomic DNA from lung cancer cell lines using conventional PCR. Before the test, we determined the mutation status of two cell lines by using direct sequencing (Fig. 2A). NCI-1650 cells, which carry no mutation, and NCI-H1975 cells, which carry the L858R substitution mutation, were used as the templates. When conventional mutant primers are used, both templates are amplified and the difference between mutated and wild-type target is subtle (Fig. 2B, upper-Conv.). On the other hand, when DMP are used as primer set, the amplification does not occur when the template has a wild-type non-target while a mutated target is amplified by the DMP (Fig. 2B, upper-DMP). It is clearly shown that the exponential amplification of non-specific target is inhibited by the DMP-mutant set. Although

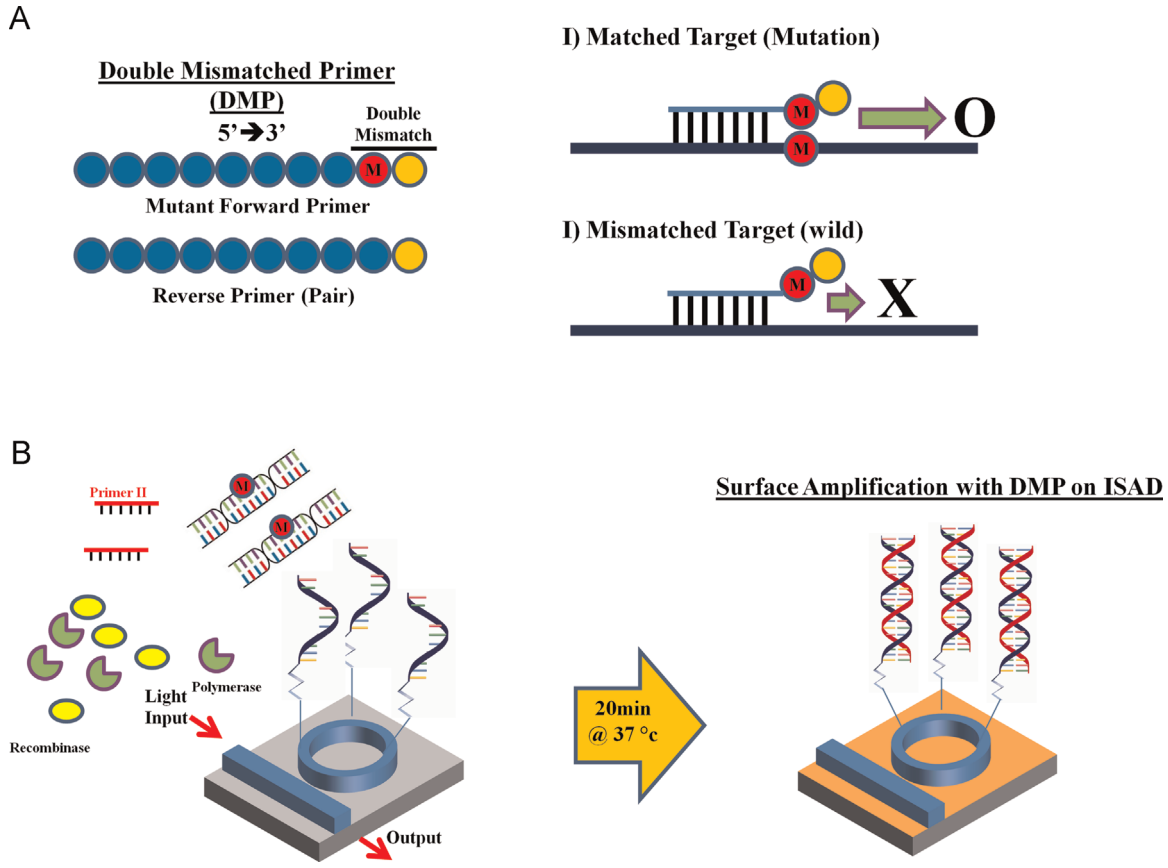

Fig. 1. Schematic representation of the principle of EGFR-DMP. (A) Double mis-matched primer (DMP) consists of two mis-matched nucleotides at the 3′-end of forward primer against the mis-matched target (wild). One mis-matched nucleotide is the mutant nucleotide of the matched target (M in red circle), and another is additional mis-matched nucleotide in order to reduce the nonspecific binding. Therefore, DMP was designed for the improvement of the detection rate of matched target without any nonspecific binding. (A-I) The DMP was only amplified the *EGFR* exon 21 (L858R) target (from NCI-H1975 cells, as a matched target), (A-II) not in the wild-type target (DNA from NCI-H1650 cells, as a mis-matched target). (B) For applying the DMP into ISAD assay, one DMP is immobilized on surface of SMR, and then added the mixture of isothermal reagent with other primer for the amplification and detection of the target simultaneously on the senor. During the amplification/detection reaction at 37 °C, the reaction was observed by the monitoring of the resonant wavelength shift for 20 min in a label-free and real-time manner. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

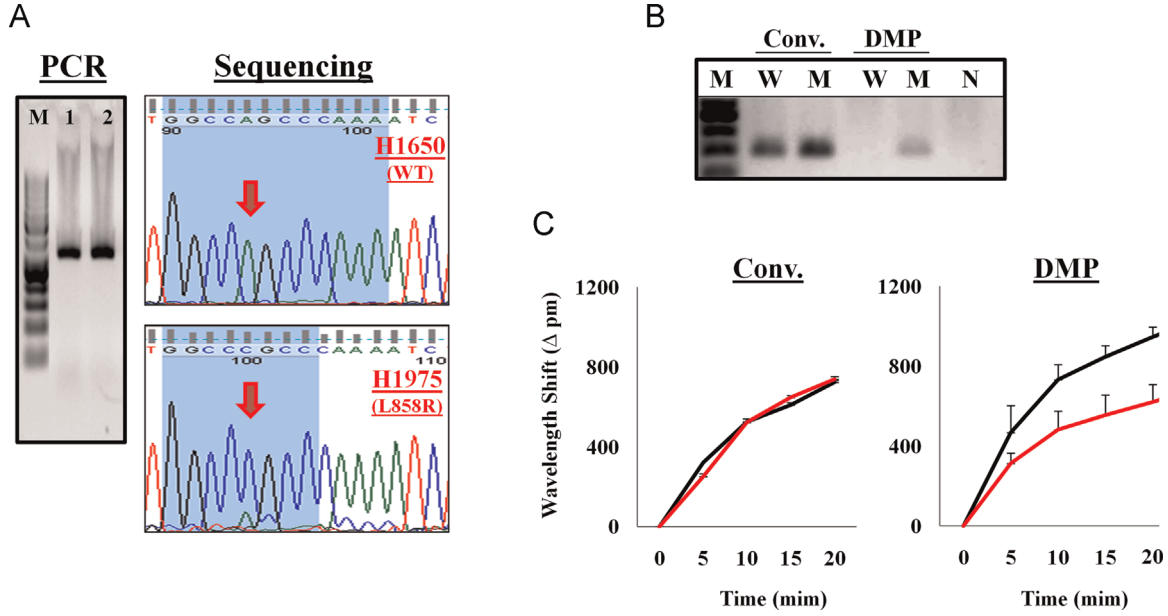

Fig. 2. Characterization of DMP using PCR and ISAD. (A) DNAs obtained from two cell lines (NCI-H1650 and NCI-H1975) were amplified by the EGFR exon 21 sequencing primer. Direct sequencing was performed to check the mutation status of the cell lines. (B) Gel electrophoresis after PCR with DMP and conventional mutant primer (Conv.) [L: 50 bp, W: H1650 DNA with Conv., M: H1975 DNA with Conv., W: H1650 DNA with DMP, and M: H1975 DNA with DMP]. (C) Resonance wavelength shift shows the results of the amplification of EGFR E21 mutation in the ISAD device together with either conventional mutant primer (Conv.) or DMP. Error bars indicated standard error of the mean based on at least 3 independent experiments. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

the overall signal intensity of the band in the agarose-gel is reduced, the data clearly shows that the background noise can be significantly reduced and consequently the sensitivity of the assay can be improved by adding one additional mis-matched sequence at the 3′ end of the DMP.

Next, we applied DMP method to ISAD for the detection of *EGFR* mutation (namely, EGFR-DMP). The SMRs are first immobilized with either conventional mutant primer or DMP mutant primer and both templates (wile-type DNA extracted from NCI-1650 and mutated DNA extracted from NCI-H1975) are introduced. When the conventional primers are used, the wavelength shifts from both templates cannot be distinguished after 20 min of amplification (Fig. 2B, lower-Conv.). For DMP immobilized SMR, the difference between mutated target (black line) and wild-type target (red line) is much more significant (Fig. 2B, lower-DMP) mainly because the amplification of wild-type target (non-target) is significantly reduced or eliminated since the hybridization between non-target template to primer is inhibited due to two (double) mis-matched nucleotides in DMP. The remaining wavelength shift (~600 pm) in non-target is mainly caused by other non-specific components such as enzymes and other chemical compounds accumulated on the sensor surface, in agreement with our previous study (Kim et al., 2013). Taken together, in the DMP combined with ISAD, the single nucleotide alteration can be detected rapidly with high sensitivity by reduction of the background signal from the non-specific binding.

3.3. Detection of EGFR exon 21 single point mutation by EGFR-DMP

Before the clinical utility of the EGFR-DMP assay was tested using specimens from NSCLC patients, we determined the detection limit of the EGFR-DMP method with serially diluted DNA (5 ng μL^{-1} to 500 fg μL^{-1}) extracted from the cell line. Fig. 3A shows that the wavelength shift increases in a concentration-dependent manner within 20 min compared to the negative control DNA (5 ng μL^{-1}). Control DNA was extracted from NCI-H1650 cells that are known to carry the wild-type allele of *EGFR* (exon 21). Moreover, Fig. 3B demonstrates that there is a linear correlation (R^2=0.9948) for different target concentrations during the reaction. Taken together, these data show that the EGFR-DMP assay can simultaneously amplify and detect target DNA compared to non-target DNA (single base difference), allowing reliable discrimination of the mutant allele from the genomic DNA. We next compared this method to conventional PCR-based methods (end-point PCR and RT-PCR) using the serially diluted DNA samples. In conventional PCR (end-point), the resultant bands (~180 bp) were obtained from the DNA samples diluted to 50 pg μL^{-1} (Fig. 3C). Similarly, the fluorescent SYBR Green signal in the RT-PCR method was observed in DNA samples diluted to 50 pg μL^{-1} (Fig. 3D). Similarly, the detection limit was observed in both conventional PCR methods (end-point PCR and RT-PCR). As expected, the detection limit of the EGFR-DMP assay was 100 times higher than that of conventional methods. Therefore, the EGFR-DMP would be useful

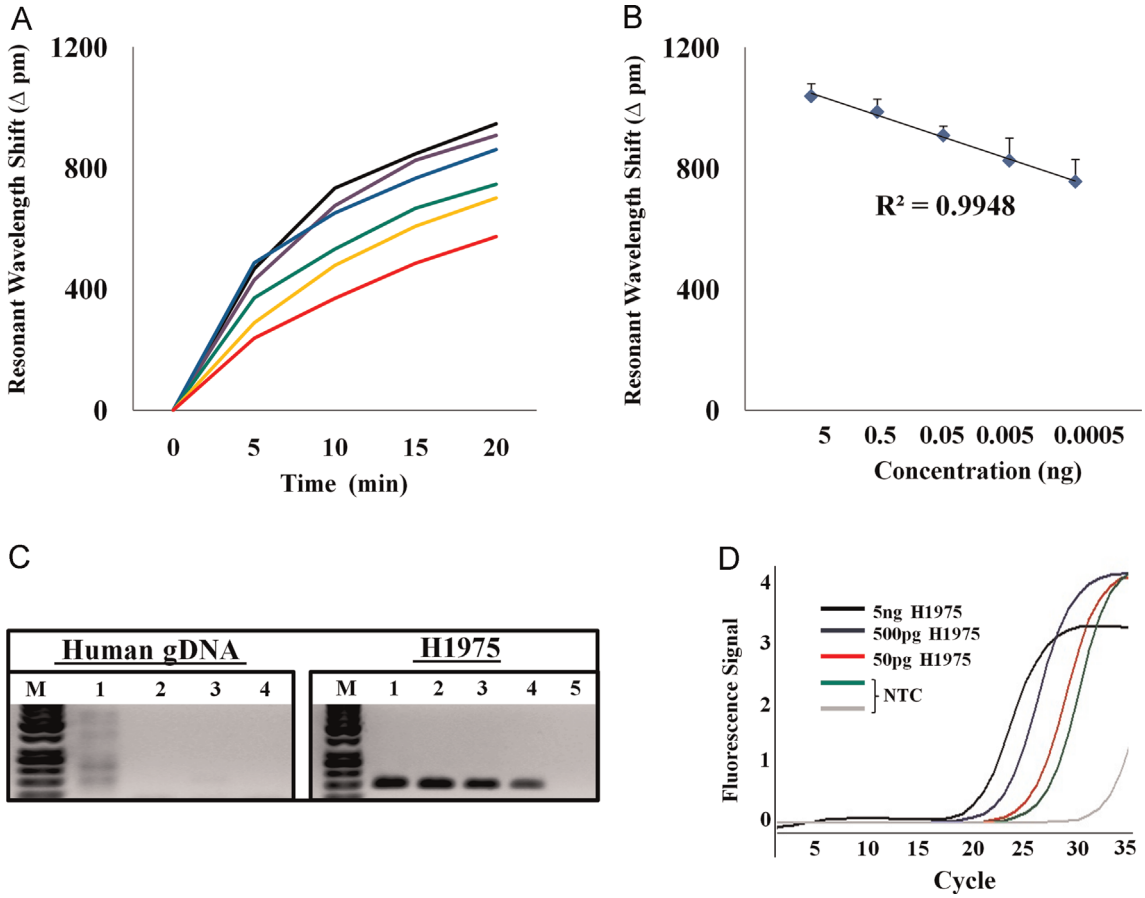

Fig. 3. Comparison of limit of detection with conventional PCR methods and EGFR-DMP. (A) Resonance wavelength shift in EGFR-DMP. The colors represent the amount of the target: black (5 ng μL^{-1}), purple (500 pg μL^{-1}), dark blue (50 pg μL^{-1}), green (5 pg μL^{-1}), orange (500 fg μL^{-1}), and red (NTC; wild-type DNA, 5 ng μL^{-1}). (B) Linear relationship between wavelength shift and the concentration of target in 20 min. Error bars indicate the standard error of the mean based on at least 3 independent experiments. (C) DNA amplification with DMP-mutant set on H1650 DNA (wild-type) and H1975 DNA (L858R mutation). Gel electrophoresis of 180 bp products by using end-point PCR. M=50 bp DNA ladder (New England), 1=50 ng μL^{-1}, 2=5 ng μL^{-1}, 3=500 pg μL^{-1}, 4=50 pg μL^{-1}, 5=no DNA (negative control). (D) Fluorescence signal by real-time PCR. The colors represent the amount of the target: black (5 ng μL^{-1}), dark blue (500 pg μL^{-1}), red (50 pg μL^{-1}) and green and gray (NTC; green (H1650 DNA, 5 ng μL^{-1}), and gray (no DNA)). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

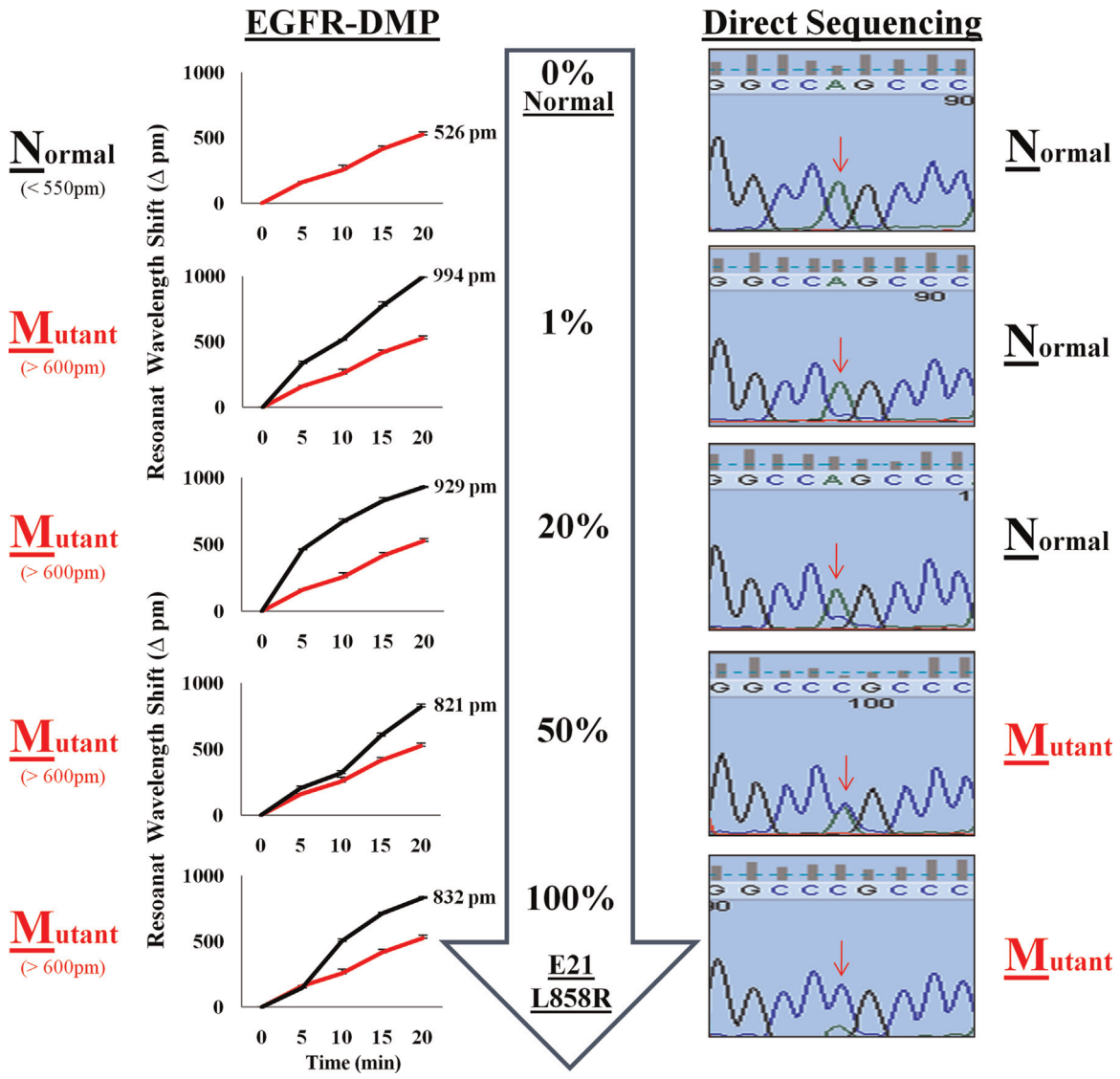

Fig. 4. Analysis of *EGFR* mutation with serial diluted cell lines. Serial dilution of H1975 cells containing a *EGFR* exon 21 single point mutation (L858R). H1975 cells were diluted with Jurkat cells (*EGFR* wild-type). The percentages of mutant cells were 0%, 1%, 20%, 50%, and 100%. DNA obtained from the mixture cell lines was extracted and amplified for analysis of *EGFR* exon 21 using EGFR-DMP and direct sequencing. In the result of the EGFR-DMP, the resonance wavelength shift range for wild allele is below 550 pm (picometer), and the range for mutant allele is above 600 pm. Error bars indicated standard error of the mean based on at least 3 independent experiments.

as a rapid (within 20 min) and highly sensitive (up to 500 fg μL^{-1}) method for the analysis of genetic variation of *EGFR* in human cancers (Fig. 3).

3.4. Sensitivity of EGFR-DMP in mixed cell population

Clinical specimens from lung cancer patients frequently consist of numerous subpopulations of cancer cells (Nagai et al., 2005; Kiumra et al., 2006b). A diagnostic device used to detect mutations in heterogeneous genomic DNA samples is desirable. Therefore, we performed serial dilutions of samples to determine the sensitivity of EGFR-DMP for detection of the mutant allele in a mixed cell population with wild-type DNA. Using serial dilutions, genomic DNA extracted from NCI-H1975 and Jurkat cell lines were used as target templates for the heterozygous *EGFR* L858 mutation and the wild-type *EGFR*, respectively. To determine the minimal detection limits for the mutant allele against a wild-type DNA background, genomic DNA was isolated from cell mixtures containing 100%, 50%, 20%, 1%, and 0% mutant cells. Fig. 4 shows that the mutant allele is easily detected by EGFR-DMP by measuring the differences in the wavelength shift between the target series (1%, 20%, 50%, and 100% mutant cells) and non-target sample (0% mutant cells). Using the EGFR-DMP assay, the mutated alleles were rapidly detected (20 min) in dilutions containing as little as 1% mutant cells. However, the mutated alleles by using direct sequencing were only able to detect a mutation in $>25\%$ of the mutant cells (Fig. 4). Therefore, the EGFR-DMP assay could be used as a companion diagnostic to identify mutant status in clinical NSCLC samples.

3.5. Mutation analysis of EGFR-DMP in clinical samples

Finally, we validated the clinical utility of the EGFR-DMP assay using 10 tissue samples obtained from lung cancer patients (stages III–IV) (Fig. 5). The genetic status of the *EGFR* gene was evaluated in all specimens using both the EGFR-DMP assay (result time: 20 min) and direct sequencing (result time: 2 days) methods (Table S2). In the tissues samples, two were found to have the L858R mutation by both methods (Fig. 5A and Table S2). Among the eight samples where no mutation was detected by sequencing, seven samples were verified as carrying wild-type alleles using the EGFR-DMP assay. Even in one sample, the mutation in case No.

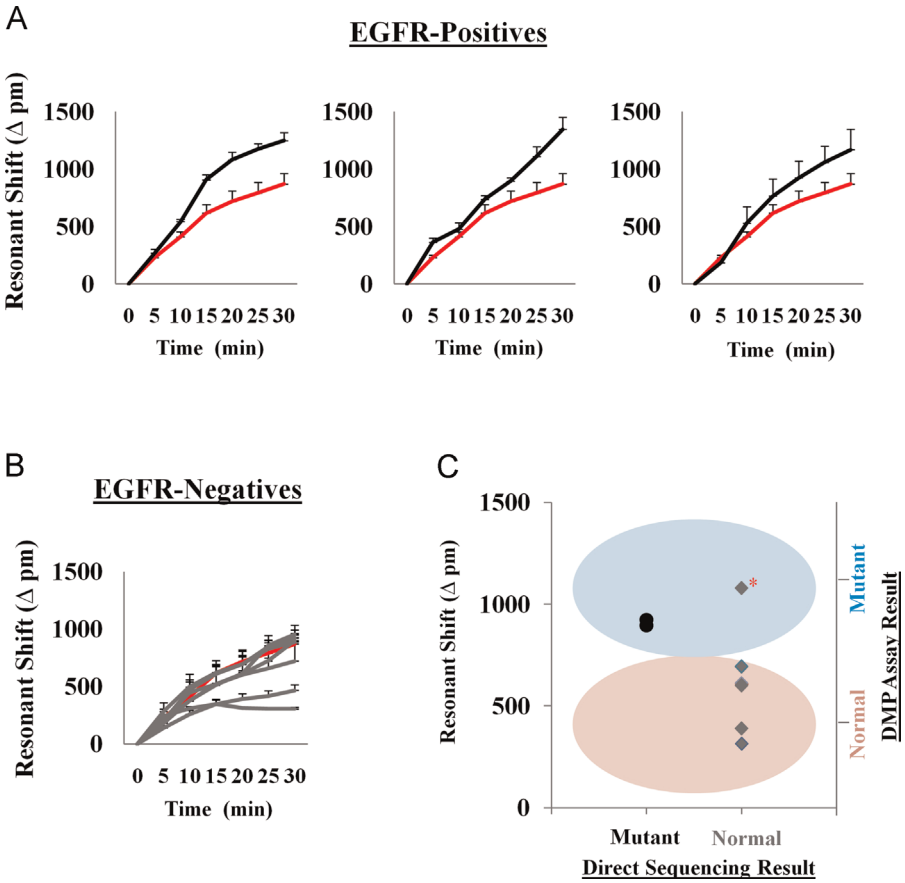

Fig. 5. Validation of the EGFR-DMP in clinical samples. Clinical utility of the EGFR-DMP with clinical samples in a blind test. Tissues from 10 lung cancer patients were analyzed with the EGFR-DMP assay. (A) The EGFR-DMP was found in 3 patients with the mutation. (B) The EGFR-DMP was found in 7 patients with no mutation. The colors represent wild-type control (red), mutant allele (black), and wild-type allele (gray). Error bars indicate the standard error of the mean based on at least 3 independent experiments. (C) 3 mutants and 7 wild-type samples were screened by EGFR-DMP. Based on the result of the EGFR-DMP, light blue oval (mutant area) and pink oval (wild-type area). On the other hand, mutant allele (black) and wild allele (gray) based on the result of direct sequencing. (*) indicated mis-matched result of sample between EGFR-DMP and direct sequencing. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

7 was detected by the EGFR-DMP only (Fig. 5B and C) due to the higher sensitivity of the assay than the sequencing method, albeit further validation with large number of clinical sample would be needed. Additionally, we have also tested small volume of plasma samples (200 μL) from sample patients in order to test the detection ability of the EGFR-DMP for mutation detection (data not shown). Unfortunately we were not able to detect the mutation mainly because of very low mutant-containing cells in the small volume of plasma (less than 1%). According to the result of previous reports, the concentration of the typical CTCs is only 1–2 CTCs in 1 mL of whole blood (Coumans et al., 2012, 2013). Thus, the CTC was not enough to detect in 200 μL of plasma sample. Therefore, further study using a larger volume of lung cancer samples (tissues and plasma) containing the mutation is required to optimize the assay. Nevertheless, the ability of the EGFR-DMP assay to rapidly detect the *EGFR* L858 mutation in clinical specimens will ensure that the patients receive appropriate treatment in a timely manner, and will simplify the equipment needed by using an isothermal cycling and biophotonic sensor technique.

4. Conclusions

In this study, we have shown that the EGFR-DMP assay can rapidly detect the *EGFR* L858R mutation in human specimens, and can be used as a tool to diagnose NSCLC tumors that may benefit from specific treatments. The EGFR-DMP assay is fast (< 20 min), highly sensitive (1% of mutant allele), accurate, and cost-effective because it is a label-free method and does not involve thermal cycling. It therefore offers the opportunity for the use in NPT settings. The EGFR-DMP assay described in this study is capable of *EGFR* mutation detection and has been shown to overcome the limitations associated with other screening methods in NPT settings. Our results show that EGFR-DMP is a suitable method for detecting mutated DNA from patient tissues samples with rapid, high sensitivity and specificity compared to direct sequencing. The EGFR-DMP assay needs further validation to allow more robust and diverse mutations detection including exon 19 (in-frame deletion) in a single reaction with a large cohort of NSCLC patient samples including both the early and the late tumor stages of cancer. Notably, we are integrating a recently developed DMA (Dimethyl adipimidate) with a microfluidic-based DNA extraction technique (Shin et al., 2014) that combines with the current EGFR-DMP assay onto a single chip to generate a fully integrated *EGFR* detection system as a companion diagnostic tool as a NPT. We envision that the development of a system with enhanced accuracy, sensitivity, specificity, time efficiency, and cost effectiveness will facilitate NSCLC diagnosis and ensure that the correct treatments are administered to the right patients to optimize efficacy.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2014.12.039.

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