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

Yong Shin [a], Ross A Soo [b], Jaeyun Yoon [a,c], Agampodi Promoda Perera [a], Yong-Jin Yoon [c], Mi Kyoung Park [a,*]

[a] Institute of Microelectronics, A*STAR (Agency for Science, Technology and Research), 11 Science Park Road, Singapore Science Park II, Singapore 117685, Singapore
[b] Department of Hematology-Oncology, National University Cancer Institute of Singapore, National University Health System, Singapore
[c] School of Mechanical & Aerospace Engineering, Nanyang Technological University, 50 Nanyang Avenue, Singapore 639789, Singapore

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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.

* Corresponding author.
E-mail address: parkmk@ime.a-star.edu.sg (M.K. Park).

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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