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Label-free and high-sensitive detection of Kirsten rat sarcoma viral oncogene homolog and epidermal growth factor receptor mutation using Kelvin probe force microscopy

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ABSTRACT

Assessment of Kirsten rat sarcoma viral oncogene homolog (*KRAS*) and epidermal growth factor receptor (*EGFR*) mutations are essential for targeted therapies of patients with non–small–cell lung cancer. In this report, we propose a label-free and high-sensitive detection method of *KRAS* and *EGFR* mutations using KPFM and a gold nanoparticle (AuNP)–based platform that densely adsorbs probe DNA and minimizes the sensing area. The detection is based on the evaluation of the surface potential of each AuNP. When AuNPs are modified with probe DNA (AuNP–pDNA), the surface potential is shifted towards the negative potential due to the negatively charged DNA backbone. When AuNP–pDNA further captures target mutant DNA through DNA hybridization, an additional surface potential shift occurs. The platform is able to detect *KRAS* mutant DNA (13 mer) and *EGFR* mutant DNA (84 mer) with a limit of detection (LOD) of 3.3 pM. Furthermore, the platform is able to detect selectively the *KRAS* mutant DNA from its wild-type DNA. Our proposed label-free and high-sensitive KPFM method has shown potential glimpses of a personalized medical diagnosis for cancer patients.

1. Introduction

Lung cancer is one of the most common malignances that lead to human death (Sharma et al., 2007). Lung cancer can be divided into two groups: small–cell lung cancer (SCLC) and non–SCLC (NSCLC); about 80% of lung cancers fall under the category of NSCLC. In several studies, the correlation between epidermal growth factor receptor (*EGFR*) and NSCLCs has been investigated (Paez et al., 2004; Yatabe et al., 2006). From the results of these studies, *EGFR* inhibitor drugs such as gefitinib and erlotinib have been used as lung cancer treatments (Kuang et al., 2009; Paez et al., 2004). However, the treatment results showed a low response (~10–20%) in clinical trials due to *EGFR* mutations that cause drug resistance (Yatabe et al., 2006). Patients with a resistance to *EGFR* inhibitor drugs are usually positive for the Kirsten rat sarcoma viral oncogene homolog (*KRAS*) mutation (Pao et al., 2005). Therefore, assessment of *EGFR* and *KRAS* mutations are essential for targeted therapies of patients with NSCLC.

In order to overcome the limitations of conventional therapies, overall *EGFR* and *KRAS* mutations have to be monitored (Do et al., 2008). Currently, obtaining a solid biopsy is a widely used approach to monitor *EGFR* and *KRAS* mutations; however, this form of biopsy has its limitations such as an invasive procedure that cannot be performed in real-time, and the biopsy does not represent the overall mutations (Crowley et al., 2013).

Owing to the limitations of solid biopsies, liquid biopsies have been investigated recently (Crowley et al., 2013). A liquid biopsy is a non-invasive approach that can detect *EGFR* and *KRAS* mutations in metastatic cancer patients in real-time. Liquid biopsies enable detection based on the quantification of biomarkers such as circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), and tumor-derived *exo*somes from patient blood samples (Bettegowda et al., 2014; Speicher and Pantel, 2014). ctDNA is a cell-free DNA that is known to be released from dead tumor cells and the DNA is selectively isolated from the plasma (Spellman and Gray, 2014). The correlation of ctDNA with the presence of a tumor has been investigated in several studies (Newman et al., 2014; Punnoose et al., 2012; Taly et al., 2013). These studies were able to monitor the genomic alterations in real time and have shown that ctDNA can be used as a biomarker for targeted therapy. CTCs and exosomes are also widely investigated for genomic alterations; however, the insufficient DNA in the cell and a reduced sensitivity when compared with ctDNA restricts CTCs as biomarkers (Speicher and Pantel, 2014). Exosomes as biomarkers are confined in use due to the difficult isolation process requirements, such as

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ultracentrifugation and size-based isolation (Speicher and Pantel, 2014).

Various techniques have been presented for the detection of gene mutation (Kosaki et al., 2013; Zhao et al., 2016): The standard detection method of gene mutation in ctDNA relies on real-time polymerase chain reaction (PCR) (Taly et al., 2013; Taniguchi et al., 2011; Tuononen et al., 2013). This method provides a high-sensitive detection performance; however, as the amplification step increases, cost and complexity increase as well. Other types of technology such as approach using beads, emulsion, amplification, and magnetics components (BEAMing) (Richardson and Iglehart, 2012), electrochemical (Xu et al., 2016), silicon nanowire field-effect transistor (NWFET) (Wu et al., 2009), fluorescent (Eftekhari-Sis et al.) and optical sensors (Liu et al., 2015) are also presented (Table S1). However, these technologies carry low sensitivities, high costs, and complexities in their data analyses. In detail, BEAMing and optical sensor perform the detection based on PCR amplification which lead to increase of cost and complexity. NWFET sensor possess high sensitivity of 0.88 fM, however, the detection platform requires very complex fabrication procedures including nanowire patterning, source/drain implantation and annealing, contact pad deposition and passivation layer deposition processes that lead to the increase of cost and complexity. Electrochemical and fluorescent sensors perform the detection by simple procedure, but the sensitivity is limited to nanomolar level.

In recent years, Kelvin probe force microscopy (KPFM) and atomic force microscopy (AFM) have been employed to detect specific biomolecules and their interactions (Nakahara et al., 2015; Park et al., 2011). KPFM is a variant of atomic force microscopy (AFM). It measures the difference between the work function of the AFM tip and the sample on the molecular scale. The great advantages of KPFM are high sensitivity and label-free detection that originate from the molecular-scale detection of native properties of the target sample (Sinensky and Belcher, 2007). Accordingly, KPFM has been employed for the detection of protein, DNA, small molecules, and metal ions (Park et al., 2015, 2014, 2011; Sinensky and Belcher, 2007). In this report, we propose a label-free and high-sensitive detection method of *KRAS* and *EGFR* mutant DNA using KPFM and a gold nanoparticle (AuNP)-based platform that densely adsorbs probe DNA and minimizes the sensing area, providing the beneficial features. In the KPFM used here, the detection is based on the evaluation of the surface potential of each AuNP, using a single droplet of solution containing AuNPs. When AuNPs are modified with probe DNA (AuNP–pDNA), the surface potential is shifted towards the negative potential due to the negatively charged DNA backbone. When AuNP–pDNA further captures target mutant DNA through DNA hybridization, an additional surface potential shift occurs. The platform was able to detect *KRAS* mutant DNA (13 mer) and *EGFR* mutant DNA (84 mer) with a limit of detection (LOD) of 3.3 pM. Furthermore, the platform was able to detect selectively the *KRAS* mutant DNA from its wild type DNA.

2. Materials and methods

2.1. Materials

All oligonucleotides were purchased from Integrated DNA technologies (Coralville, IA, USA). Tris-EDTA buffer solution (TE buffer), Tris (2-carboxyethyl) phosphine hydrochloride solution (TCEP), ethyl alcohol (99.9%), 20 nm-diameter gold nanoparticles (AuNPs) in citrate buffer, and NaCl were purchased from Sigma-Aldrich (St. Louis, MO, USA). The Innova microscope, Nanodrive controller, platinum–iridium coated silicon cantilever (SCM-PIT), and Nanoscope software V1.20 were purchased from Bruker (Santa Barbara, CA, USA).

2.2. AuNP DNA modification (AuNP-pDNA and AuNP-EDNA)

In this experiment, probe DNA with the sequences of 5′-thiol-ACGCCAACAGCTC-3′ (*KRAS* mutant) and 5′-TCGGAGATGTCTTGA-TAGCG-thiol-3′ (*EGFR* mutant) were added to TE buffer solution and the final concentration of probe DNA and TE buffer were 1 mM and 30 mM. The preparation of probe DNA functionalization on AuNP was based on the protocol described by Jeuwen Lin & Yi Lu, with a few modifications (Liu and Lu, 2006). In detail, the TCEP solution (1.5 μl, 10 mM) was added to the probe DNA solution (10 μl, 1 mM) to activate the thiol-modified DNA, which was then incubated at room temperature for 1 h. Then the solution was mixed with the solution containing 20 nm-diameter AuNPs in citrate buffer (988.5 μl). The volume of the as-produced solution was 1 ml. The concentration of probe DNA was controlled during this process. The produced solution was stirred with a magnetic bar, at room temperature, in a dark room for 16 h. The resulting, functionalized AuNPs were centrifuged at 16,000 × *g* and room temperature for 15 min to discard free probe DNA in the supernatant. The discarded amount was replaced with 30 mM of TE buffer. Then the solution (AuNP-pDNA) was stored in refrigerator for further use. For the confirmation of the probe DNA functionalization on AuNPs, 5 mM of NaCl was added to a solution of bare AuNP and AuNP-pDNA, and the subsequent color change of each solution was observed after 24 h. The absorbance of bare AuNP and AuNP-pDNA solution was measured using an UV/Vis spectrophotometer (Hach, DR-4000). For further confirmation, each DNAs with sequences of 5′-thiol-GAGCTGTTGGCGT-3′ (cDNA) and 5′-thiol-TGTTGTGGTTTTG-3′ (ncDNA) was introduced to AuNP solution in order to modify AuNP with the DNA. After the modification, each of solution was mixed with the AuNP-pDNA solution in volume ratio of 1:1 with 0.15 M of NaCl and the color change of each solution was observed.

2.3. KRAS and EGFR mutant DNA detection

Target DNA with sequences of 5′-GAGCTGTTGGCGT-3′ (*KRAS* mutant, G12V) and 5′-GGACTCTGGATCCCAGAAGGTGA-GAAAGTTAAAATTCCCGTCGCTATCAAGGCATCTCCGAAAGCCAA-CAAGGAAATCCTCGAT-3′ (*EGFR* mutant, 746–750 (del ELREA) in EGFR exon 19) were dissolved in 30 mM of TE buffer and the final concentration of target DNAs were 1 mM. Then the solution was added to the AuNP-pDNA solution and the target DNA concentration was adjusted during this process. After that, the solution was stored in dessicator for 1 h. Then a single droplet (2 μl) of the solution was dropped on a silicon wafer that was priorly washed with ethyl alcohol (99.9%) and triple-distilled water (DI-water). The wafer was dried for 5 h before usage in the desiccator in order to reduce the error signal that would otherwise arise from the humidity of the sample. After complete dehydration process of the dropped solution, the product was lightly rinsed via DI-water to eliminate aggregated AuNPs, followed by another dehydration process.

2.4. Tapping mode AFM and KPFM analysis

Measurements and analyses of tapping mode AFM and KPFM were performed using an Innova microscope with a Nanodrive controller in air at ambient pressure (1 atm) and room temperature. For all imaging, SCM-PIT cantilever with a resonance of ~75 kHz and 20 nm radius was used, and the wafer was grounded with carbon tape and silver paste (Dotite, Japan). A close-loop scanner was used to obtain exact images of the sample at each state. All the images in this experiment were obtained with 1 μm × 1 μm size areas at 0.65 Hz scanning. The topography and surface potential were measured simultaneously via feedback