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Detection of epidermal growth factor receptor (EGFR) mutations in non-small cell lung cancer (NSCLC) using a fully automated system with a nano-scale engineered biomagnetite

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Abstract

A fully automated system using nano-scale engineered biomagnetite was developed to detect mutations in the epidermal growth factor receptor (EGFR) gene in non-small cell lung cancer (NSCLC). Bacterial magnetic particles (BacMPs) were isolated from the magnetic bacterium *Magnetospirillum magneticum* AMB-1 and conjugated to streptavidin. Biotin-labeled target PCR products were then captured with the BacMPs, hybridized with the detection probe and detected by fluorescence signaling. The process was performed using a newly designed automated processor equipped with an XYZ mobile arm containing a 96-way automated pipetter, reagent dispenser and fluorescence detector. Two types of somatic mutations (in-frame deletions and point substitutions) in the EGFR gene were successfully identified within 3.5 h using this system, suggesting that this system could be used in clinical tests of EGFR gene mutations in lung cancer, and potentially other cancer, patients. Additionally, a very low mutation rate could be detected in these samples.

Keywords: Nano-scale engineered biomagnetite; Epidermal growth factor receptor (EGFR) mutation; Non-small cell lung cancer (NSCLC); Bacterial magnetic particles (BacMPs); Allele-specific oligonucleotide hybridization; Automated system

1. Introduction

Automated systems for nucleic acid purification, amplification, labeling and signal detection have become necessary for post-genomic analyses of large numbers of samples (Meldrum, 2000). These automated systems could potentially eliminate problems associated with long processing time, human error, inconsistencies due to multiple operators and limitations in multiple sample processing (Marziali et al., 1999; Itoh et al., 1999). However, the design of fully automated systems has not been completely successful due to the difficulty in automating nucleic acid purification processes. In recent years the utilization of magnetic beads has facilitated the development of semi-automated instruments that purify DNA (Obata et al., 2001, 2002). For example, bacterial magnetic particles (BacMPs), which are synthesized by magnetotactic bacteria, are nano-scale (50–100 nm) magnetites, and are enveloped by a lipid bilayer, have been used in various applications including DNA extraction (Nakagawa et al., 2006) and DNA marker detection (Takeyama et al., 2000; Matsunaga et al., 2001; Yoshino et al., 2003; Ota et al., 2003). Previously, a novel automated instrument using BacMPs successfully separated and washed magnetic samples with highly uniform thermal control (Tanaka et al., 2003). The system was then successfully used for DNA extraction (Yoza et al., 2003) and SNP detection (Maruyama et al., 2004). However, this system was only semi-automated, and obviously a fully automated system using BacMPs would be beneficial to such experimental endeavors.

Lung cancer is the most fatal cancer in many developed countries. Gefitinib (Iressa®, Astra Zeneca, London, UK), an inhibitor of the epidermal growth factor receptor (EGFR) tyrosine kinase, has been approved in Japan and the United States

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for the treatment of non-small cell lung cancer (NSCLC). This drug significantly shrinks tumors in 27.5% of Japanese NSCLC patients, and in 10.4% of Caucasian (Giaccone and Rodriguez, 2005). Several reports have identified somatic mutations in the tyrosine kinase domain of the EGFR gene in NSCLC, especially adenocarcinoma, and that clinical responsiveness to gefitinib was significantly associated with these mutations (Lynch et al., 2004; Paez et al., 2004; Pao et al., 2004). Therefore, EGFR mutations, specifically at exons 19 and 21, may be useful molecular markers for treatment of NSCLC. The most common method for detecting EGFR mutations requires the initial separation of tumor cells from normal cells (Endo et al., 2005). This process is time-consuming and thus its potential as a method for choosing patients for specific therapeutic treatments is limited (Janne et al., 2006). Furthermore, in the detection of DNA mutation within lung cancer patients, it is rather common for the extraction of lung cancer tissue to be conducted. Therefore, in order to avoid such hassle, methods involving the extraction of cancer cells from peripheral blood and from phlegm or the extraction of DNA from cancer cells are becoming popular. Currently, peripheral blood has been one of the spotlights in attaining lung cancer DNA for higher level DNA analysis and detection (Asano et al., 2006).

In this study, a fully automated system using BacMPs was developed to detect DNA mutations. We used this system to detect somatic mutations in the EGFR gene with the goal of developing a specific therapeutic treatment of NSCLC. Furthermore, we evaluated the minimum detectable content of a tumor cell in a specimen in an effort to avoid the tedious exclusion process of normal cells.

2. Materials and methods

2.1. Cell lines

NSCLC cell lines H1975, H385, H1650, and H1666 were purchased from American Type Culture Collection (Manassas, VA). H1650 contained an exon 19 L747-E749 deletion while H1975 contained an exon 21 L858R substitution in the EGFR gene.

2.2. Nano-scale engineered biomagnetite

BacMPs were extracted and purified from *Magnetospirillum magneticum* AMB-1. Aminosilane-modified BacMPs for DNA capture were coated by 3-[2-(2-aminoethylamino)-ethyl amino]-propyltrimethoxysilane (AEEA), a type of silane coupling agent, as previously described (Nakagawa et al., 2006). The streptavidin-immobilized BacMPs used in the capture of PCR products were prepared according to a previously described method (Maruyama et al., 2004).

2.3. A fully automated processor

Fully automated detection of mutations in the EGFR was performed using a newly designed automated processor (Fig. 1A). This processor is equipped with an XYZ mobile arm (Fig. 1B), containing a 96-way automated pipetter, reagent dispenser and a fluorescence detector, in addition to two reaction units (Fig. 1C) for 96-well microplates (65 mm × 46.5 mm) that fall within a magnetic field formed by neodymium–iron–boron (Nd–Fe–B)-sintered magnets positioned on its underside. The reaction units are fitted with a heat block capable of regulating temperatures within the range of 4–99 °C. The presence of the magnets and the heat block within the reaction units results in simultaneous heating and magnetic separation in a single well. DNA extraction and PCR amplification were performed in Reaction Unit I, positioned on the left-hand side of the processor, while hybridization of samples with a detection probe and fluorescence detection were performed in Reaction Unit II, positioned on the right-hand side of the processor (Fig. 1D).

2.4. Capture of cultured cell-derived DNA on aminosilane-modified BacMPs and DNA amplification of the EGFR gene

Cultured cell-derived DNA was captured on aminosilane-modified BacMPs according to a method described previously (Nakagawa et al., 2006). Cultured cells (2×10^5 cells) were lysed in 10 μL of lysis buffer (20 mM MES, 5 M urea, 4% Tween 20, 0.1 mg/mL proteinase K, pH 5.0) containing 10 μg of aminosilane-modified BacMPs. The mixture was incubated at 56 °C for 20 min. DNA captured by the aminosilane-modified BacMPs or DNA-BacMP complexes was then collected magnetically. Cell lysates were subsequently removed and the complexes were washed five times with PBS containing 2% BSA (pH 7.5).

The DNA amplification was performed using DNA-BacMP complexes. As preliminary experiments, amount of DNA-BacMP complex and pH of reaction solution in PCR amplification were optimized. PCR amplification was performed using primers designed to detect a 69 bp region found in the TGF-β1 gene (Yamada et al., 1998) as a model target. The forward primer, 5′-CGCCTCCCCCATTCCGCCCT-3′ and the reverse primer, 5′-ACCAGTAGCCACAGCAGCGGTAGCA-3′ were used. PCR was performed with a PTC-100 Peltier Thermal Cycler (MJ Research Inc., Watertown, MA) using the following protocol; preheating (95 °C, 5 min), 35 cycles at 95 °C for 30 s, 56 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 5 min. The PCR reaction buffer contained various amount of DNA-BacMP complexes (1–10 μg), 1× buffer with various pH (pH 8.3–11), 1.5 mM $MgCl_2$, 0.2 μmol of each primer, 0.25 mM dNTPs, and 0.6 U of AmpliTaq (Perkin/Elmer) in a final volume of 25 μL. PCR products were visualized using a 3% gel.

Exons 19 and 21 of the EGFR gene were PCR-amplified from DNA-BacMP complexes. The 69-bp fragment of exon 19 of the EGFR gene was amplified using sense (5′-TTAAAATTCCCGTCGCTATCAA-3′) and biotin-labeled antisense (5′-CGAGGATTTCCTTGTTGGCTT-3′) primers while the 67-bp fragment of exon 21 of the EGFR gene, containing the SNP site located 22 bases from the 5′-end, was amplified using biotin-labeled sense (5′-TCAAGATCACAGATTTTGGGC-3′) and antisense (5′-CCTTCTGCATGGTATTCTTTCTC-3′)