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Highly sensitive and selective colorimetric genotyping of single-nucleotide polymorphisms based on enzyme-amplified ligation on magnetic beads

Xiaojun Chen [a], Andrew Ying [a], Zhiqiang Gao [b,*]

[a] Institute of Bioengineering and Nanotechnology, 31 Biopolis Way, Singapore 138669, Singapore
[b] Department of Chemistry, National University of Singapore, 3 Science Drive 3, Singapore 117543, Singapore

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ABSTRACT

Herein we report a new strategy for highly sensitive and selective colorimatric assay for genotyping of single-nucleotide polymorphisms (SNPs). It is based on the use of a specific gap ligation reaction, horseradish peroxidase (HRP) for signal amplification, and magnetic beads for the easy separation of the ligated product. Briefly, oligonucleotide capture probe functionalized magnetic beads are first hybridized to a target DNA. Biotinylated oligonucleotide detection probes are then allowed to hybridize to the already captured target DNA. A subsequent ligation at the mutation point joins the two probes together. The introduction of streptavidin-conjugated HRP and a simple magnetic separation allow colorimetric genotyping of SNPs. The assay is able to discriminate one copy of mutant in 1000 copies of wild-type KRAS oncogene at 30 picomolar. The detection limit of the assay is further improved to 1 femtomolar by incorporating a ligation chain reaction amplification step, offering an excellent opportunity for the development of a simple and highly sensitive diagnostic tool.

1. Introduction

Single-nucleotide polymorphisms (SNPs) are single nucleotide variations in a DNA sequence. SNPs are the most abundant mutations in human genome (Sachidanandam et al., 2001). They have been shown to be associated with risks to various diseases (Jorde, 2000; Risch and Merikangas, 1996). It was reported that SNPs are the main cause for many types of cancer or the important biomarkers to indicate the high risk of cancer (Imyanitov et al., 2004; Oh et al., 2007). For example, over 90% mutations in KRAS gene at condons 12, 13 and 61 eventually lead to the development of pancreatic cancer (Forrester et al., 1987). Owing to their importance in medicine, many methods for screening unknown SNPs and quantifying known SNPs have been reported, primarily based on allele-specific hybridizations (Cho et al., 2007; Ding et al., 2010) and enzymatic allele discriminations (Chen et al., 2009; Forrester et al., 1987; Li et al., 2005b; Mattes and Seitz, 2001; Wan et al., 2009; Zhang et al., 2009). The allele-specific hybridization makes use of little difference in the thermal stability arising from SNPs (Yu et al., 2001). Such mechanism requires highly stringent hybridization conditions. The enzymatic allele discriminations, such as allele-specific enzymatic ligation (Baron et al., 1996; Chen et al., 1998; Hashimoto et al., 2005; Li et al., 2005a; Liu and Lin, 2007; Lowe et al., 2010; Mattes and Seitz, 2001; Yeh et al., 2006), allele-specific enzymatic cleavage (Gunderson et al., 2005; Nollau and Wagener, 1997), and allele-specific single-base primer extension (Hirschhorn et al., 2000; Kwok, 2001; Myakishev et al., 2001), are more selective approaches for SNP genotyping.

Among the enzymatic allele discrimination methods, the allele-specific enzymatic ligation exhibits high specificity for known SNPs, which has been coupled to various signal transduction and amplification schemes for the development of sensitive assays for SNP genotyping. So far, assays based on colorimetry (Lee et al., 2010; Li et al., 2005b; Storhoff et al., 1998; Xue et al., 2009; Zhu et al., 2010), fluorometry (Nakayama et al., 2003; Yeh et al., 2006), mass spectrometry (Mattes and Seitz, 2001), surface-enhanced Raman spectroscopy (Lowe et al., 2010), and electrochemical methods (Long et al., 2004; Yu et al., 2001; Zhang et al., 2008b) have been proposed. A variety of reporters, such as nanoparticles (Qin and Yung, 2007; Storhoff et al., 1998), redox compounds (Huang et al., 2009; Wu et al., 2007), fluorescent dyes (Nakayama et al., 2003; Yeh et al., 2006), and enzyme (Liu et al., 2008; Zhong et al., 2003) have been coupled to the ligation reaction to transduce the signal of the ligated product. For instance, Xue et al. (2009) covalently linked gold nanoparticles onto oligonucleotide-modified glass slides via the ligation reaction. The perfectly matched target can subsequently be visualized after a silver enhancement, whereas SNPs show no or little change due to their low ligation efficiency. Wu and co-workers (Wu et al.,

* Corresponding author. Tel.: +65 65163887; fax: +65 67791691.
E-mail address: chmgaoz@nus.edu.sg (Z. Gao).

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2007) developed an electrochemical approach by specifically ligating ferrocene (Fc)-tagged oligonucleotide probes onto a gold electrode. Compared to other reporters, enzyme shows some attractive advantages, such as high sensitivity and ample choices of substrates for colorimetric, fluorescent, and chemiluminescent detections (Ruzgas et al., 1996).

In most of the above mentioned assays, the ligation reaction is carried out heterogeneously on a solid support to capture the ligated product in order to separate it from unligated probes and avoid stringent washing (Xue et al., 2009; Yu et al., 2001; Zhang et al., 2008b). The heterogeneous nature of the ligation reaction implies that those assays intrinsically suffer from low hybridization efficiency and steric hindrance, resulting in poor reproducibility, poor sensitivity and a narrow dynamic range. On the other hand, the steric hindrance can be significantly minimized by performing the ligation reaction in a homogeneous environment. In the heterogeneous approaches where unligated probes can be conveniently eliminated by a simple rinsing. In the homogeneous environment, however, the unligated probes co-exist with the ligated product, posing a great threat to the detection of the ligated product. To circumvent this problem, a fluorescence resonance energy transfer (FRET)-based approach was utilized to detect the ligated product in the homogeneous environment (Nakayama et al., 2003). Nonetheless, some fundamental issues, such as photobleaching, low sensitivity, and spectral overlap among the dyes, largely limit the application of the FRET approach in the ligation-based SNP detections.

It is therefore more desirable to develop an assay that combines the high efficiency of the homogeneous ligation reaction and the simplicity of the heterogeneous approach to eliminate the unligated probes. Towards this end, functionalized magnetic beads may be good candidates as carriers to facilitate the homogeneous ligation reaction and to provide a simple and fast separation upon applying a magnetic field. Here we present the proof-of-concept of an assay for sensitive and selective detections of SNPs that combines the high efficiency of the homogeneous ligation reaction, the signal transduction/amplification of an enzymatic reporter, horseradish peroxidase (HRP), and the ease of use of the functionalized magnetic beads in the separation of the ligated product. HRP was coupled to the biotinylated detection probes ligated on the magnetic beads by the ligation reaction. After a non-stringent wash and a magnetic separation step, colorimetric quantification of the ligated product could be realized upon the addition of a HRP substrate, 3,3,5,5-tetramethylbenzidine (TMB). This assay offered a simple, selective and sensitive way for SNP detections.

2. Experimental

2.1. Materials and reagents

Long-arm carboxyl-terminated magnetic beads (~1 μm, 20 mg/ml) were purchased from Bioclone Inc (San Diego, USA). 1-ethyl-3-3-dimethylaminopropyl carbodiimide hydrochloride (EDC) and the SuperBlock blocking buffer were acquired from Pierce (Rockford, USA). All other chemicals were from Sigma-Aldrich (St Louis, MO). All synthetic oligonucleotides used in this work were from Proligo (Singapore). T4 DNA ligase and a 10x buffer for the ligation reaction were purchased from New England Biolabs Inc (Ipswich, MA). Ampligase® thermal stable ligase kit was from Epicenter Biotechnologies (Madison, USA). Concentrated streptavidin-conjugated HRP (SA-HRP) solution and its corresponding one-step TMB substrate and stop solutions were from BD Biosciences (San Jose, CA). 10 mM Tris buffer (pH=8.0) containing 0.5 M NaCl and 0.1% Tween 20 was used as washing buffer. DNase-free ultrapure water was used throughout. Sequences of the amine-terminated capture probes, the biotinylated detection probes and KRAS oncogene targets used in this study are listed in Table S1.

2.2. Functionalization of the magnetic beads

The magnetic beads were functionalized with the amine-terminated capture probes following the protocol provided by the company with a slight modification (Scheme 1A). Briefly, the carboxyl-terminated magnetic beads (2 μL) were washed with water and suspended in 1 mL of pH 5.5 10 mM potassium phosphate containing 0.5 M NaCl. The capture probes were added into the above solution with a final concentration of 2 micromolar (μM) together with 100 μL of 0.057% freshly-prepared EDC. The reaction mixture was kept for 24 h at room temperature with gentle shaking. Afterwards, the supernatant was separated and the capture probe content in it was analyzed. It was found that an average of $\sim 2 \times 10^5$ capture probe strands are attached to each magnetic bead. The unreacted carboxyl groups were blocked by incubating the functionalized magnetic beads in 1 M glycine for 2 h. After three thorough washings with water, the functionalized magnetic beads were kept at 4 °C and ready for use.

2.3. Ligation reaction

The ligation reaction was performed in a 20 μL reaction solution containing 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 1 mM adenosine triphosphate (ATP), 500 U T4 DNA ligase, 2 μM the biotinylated detection probes, and the DNA target of specified concentration. The ligation reaction was carried out at room temperature for 20 min. After the ligation reaction, the magnetic beads were washed twice with washing buffer and three times with water to denature the hybridized DNA target.

2.4. Ligation chain reaction

In order to further improve the sensitivity, ligation chain reaction (LCR) was applied to amplify the target DNA. Ampligase® Thermal stable ligase was used during the thermal cycling to facilitate the exponential amplification. In a typical experiment, 100 μL of reaction solution contains 1x Ampligase reaction buffer, 50 U Ampligase, 1 μM the capture probe, 1 μM the biotinylated detection probe, 0.5 μM WT target half A, 0.5 μM WT target half B, and the KRAS wild-type target. The mixture was vortexed and divided into five equal aliquots. The aliquots were undergone 5, 10, 15, 20 and 25 thermal cycles, respectively. Each thermal cycle was composed of a 2 min ligation at 45 °C and a 1 min denaturation at 90 °C. After the thermal cycling, the magnetic beads were washed twice with the washing buffer and three times with water to denature the hybridized DNA target.

2.5. HRP attachment and colorimetric detection

After the ligation reaction or LCR, the magnetic beads were suspended in 250 μL of the blocking buffer and incubated for 10 min. One μL of the SA-HRP solution was added and incubated for 30 min at room temperature to attach SA-HRP to the ligated detection probes on the magnetic beads. After the HRP attachment, the magnetic beads were washed with the washing buffer for five times. 100 μL of the TMB solution was then added to the magnetic beads and incubated for 30 min at room temperature in the dark. Finally, 50 μL of the stop solution was added and the absorbance of the solution was measured at 450 nm with a background correction at 570 nm.