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Electrochemiluminescent assay for detection of extremely rare mutations based on ligase reaction and bead enrichment

Changming Cheng [a,b,*,1], Jie Wang [a,1], Chao Yang [a], Yin Zhou [a], Juan Chen [a], Jie Zhang [a], Nengqin Jia [c,*], Hongmei Cao [c], Guoping Zhao [a,b]

[a] National Engineering Center for Biochip at Shanghai, Shanghai 201203, People's Republic of China
[b] Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, People's Republic of China
[c] Key Laboratory of Resource Chemistry of Ministry of Education, Department of Chemistry, College of Life and Environmental Sciences, Shanghai Normal University, Shanghai 200234, People's Republic of China

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ABSTRACT

The detection of rare mutations is particularly essential in many areas of biomedical research. Here, we report an ultrasensitive method to detect extremely rare point mutations based on electrochemiluminescent assay. The point mutation among large excess wild-type alleles is exclusively amplified through ligase detection reaction. The products corresponding to the amplification of mutant alleles are selectively captured by magnetic beads and then labeled with electrochemiluminescent substrates. Thus, point mutations with a percentage as small as 0.01% in the DNA population can be detected by electrochemiluminescent assay. Moreover, because the electrochemiluminescent signal of the mutation is proportional to the percentage of mutant alleles in the DNA population, the percentage of mutant alleles can be roughly accessed.

It is essential to detect the mutations in many areas of biomedical research such as molecular diagnosis, personalized therapy, and drug development [1–3]. Recently, the detection of mutations in the circulating DNA of tumor patients' blood has attracted increasing interest of researchers [4–7]. The mutations in circulating DNA usually bear the uncommon variations consistent with those of DNA isolated from tumor cells [5]. Thus, the detection of these mutations may afford abundant information about the stage of tumor, prognosis, and drug efficiency of therapy. However, it is difficult to detect such mutations in circulating DNA through the routine techniques of genetic testing owing to the interference of wild-type alleles [8,9]. Actually, there are predominant wild-type alleles derived from the apoptosis of normal cells in the circulating DNA. The mutant alleles represent only a tiny fraction of total circulating DNA in the blood, in some cases even less than 0.01%. Moreover, there is only one nucleotide variation between point mutations and wild-type alleles in their DNA sequences. Both situations make the detection of rare mutations very complicated. Although a variety of methods for detecting genetic variants have been reported so far, it is still a challenge to detect such point mutations among a large excess of wild-type alleles. Direct DNA sequencing is usually considered as the "gold standard" in genetic testing, which can identify the mutations only among the DNA population containing at least 25% mutant alleles. Many methods based on polymerase chain reaction (PCR)[2] have been proposed to improve the sensitivity of mutation detection, including restriction fragment length polymorphism (RFLP), single-strand conformation polymorphism (SSCP), and TaqMan real-time PCR [10–12]. However, these methods are still incompetent at detecting point mutations with a percentage less than 0.1% due to the intrinsic limitations of PCR in sensitivity and specificity.

Ligase detection reaction (LDR) is a sensitive method that was developed for detection of single base pair variation by Barany and coworkers in 1999 [13]. LDR uses DNA ligase enzyme to exclusively integrate the neighboring oligonucleotides hybridized to target DNA sequences in which there is perfect complementation at the nick junction [14–17]. By repeating thermal cycles, the one strand of target DNA sequence can be amplified linearly. Because

* Corresponding authors. Addresses: National Engineering Center for Biochip at Shanghai, Shanghai 201203, People's Republic of China. Fax: +86 21 51320266 (C. Cheng), Key Laboratory of Resource Chemistry of Ministry of Education, Department of Chemistry, College of Life and Environmental Sciences, Shanghai Normal University, Shanghai 200234, People's Republic of China (N. Jia).
E-mail address: cmcheng2005@gmail.com (C. Cheng).

[1] These authors contributed equally to this article.

[2] *Abbreviations used:* PCR, polymerase chain reaction; LDR, ligase detection reaction; CL, chemiluminescence; ECL, electrochemiluminescence; Ru(II)$(bpy)_3$, tris(2,2-bipyridine) ruthenium(II); dNTP, deoxynucleoside triphosphate; TPA, tripropylamine; Ru-NHS, Ru$(bpy)_2$(mcbpy-*O*-Su-ester)$(PF_6)_2$; CV, cyclic voltammetry; GCE, glassy carbon electrode; EDTA, ethylenediaminetetraacetic acid; DMF, dimethylformamide.

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the detection of LDR products usually depends on gel electrophoresis assay, the sensitivity of LDR for mutation detection is limited owing to the low sensitivity of gel electrophoresis. Many methods have been developed to improve the sensitivity of LDR by adopting other assays with high sensitivity, but these modified strategies are usually complicated and time-consuming.

Chemiluminescence (CL) has been a sophisticated technique for the clinical immunoassay owing to its high sensitivity [18]. Electrochemiluminescence (ECL) is a form of CL in which the light emission is motivated by an electrochemical reaction [19,20]. Not only are the analytical advantages of CL retained, but also ECL possesses a distinct advantage with ease of control [21]. It allows controlling the time and position of light emission by alteration of applying potential. The light emission can be precisely confined in the region approaching the detector in order to obtain the maximum ratio of signal to noise. Thus, ECL becomes an ideal tool for detection of trace biomolecules [20,22,23]. The commercial apparatus and reagents based on the ECL method have been developed and applied in the immunoassay of clinical practice, but the application of ECL in genetic testing has still been limited so far. It may be contributed to the complication of genetic testing. For the detection of rare mutations, the interference of predominant wild-type alleles usually leads to failed detection. Thus, the sensitivity is the key parameter for the detection of rare mutations. Here, the sensitivity is defined as the minimal percentage of mutant alleles among the whole DNA population that can be detected. Although there have been a few recent reports about the detection of genes based on the ECL method, most of these reports focused solely on the detectable minimal concentration of genes in the absence of wild-type alleles [24–26]. The sensitivity of mutation detection has not been investigated. Other studies have investigated the sensitivity of mutation detection based on ECL, but the resulting sensitivity often depends on the strategies for the identification and amplification of mutant alleles [23,27].

Here, we report an ultrasensitive method for the detection of extremely rare point mutations through ECL assay. A point mutation of the *TP53* gene (674A > G) was used as the model for evaluation of the sensitivity of mutation detection in this study. The discrimination and amplification of mutant alleles were obtained through the LDR process. The LDR products corresponding to the amplification of mutant alleles are selectively enriched to the surface of magnetic beads and then labeled with tris(2,2-bipyridine) ruthenium(II) [Ru(II)$(bpy)_3$], the ECL substrate. Thus, point mutations with a percentage as small as 0.01% in the DNA population can be detected through ECL assay. For comparison, the sensitivity of the LDR based on gel electrophoresis was evaluated. Moreover, the relationship between the ECL signal of the LDR product and the percentage of mutant alleles was investigated to evaluate the feasibility of quantitative detection of rare mutations.

Materials and methods

Reagents and apparatus

Wild-type and mutant genomic DNA molecules were abstracted from human liver hepatocellular carcinoma cell lines—*HepG2* and *HUH-7*, respectively—by a Blood and Cell Culture DNA Mini Kit (Qiagen). The oligonucleotides used in this work were synthesized by Shanghai Sangon Biological Engineering Technology and Services (Shanghai, China). The sequences of the primers and probes in detail are listed in Table 1. Hot Start DNA polymerase, PCR buffer, deoxynucleoside triphosphate (dNTP) mix, and DNA marker (DL1000) were purchased from Takara Biotechnology (Dalian, China). Taq DNA ligase and its buffer were purchased from New England Biolabs (Beijing, China). Streptavidin-activated magnetic beads (Dynabeads M-270 streptavidin) were purchased from Invitrogen Dynal (Oslo, Norway).

Tripropylamine (TPA) was purchased from Sigma (St. Louis, MO, USA), and Ru$(bpy)_2$(mcbpy-*O*-Su-ester)$(PF_6)_2$ (Ru-NHS) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Other chemical reagents were purchased from Sinopharm Chemical Reagent Shanghai (Shanghai, China). All reagents were used without further purification.

ECL was recorded with an MPI-E electrogenerated chemiluminescence analyzer (Xi'an Remax Electronic Science Technology, China). A CHI 660A electrochemical analyzer (Shanghai Chenhua Instruments, China) was used to carry out cyclic voltammetry (CV) measurements in an analytical cell of 10 ml. The electrochemical reaction cell was composed of a work electrode (glassy carbon electrode, GCE), a counter electrode (platinum), and a reference electrode (Ag/AgCl).

Preamplification

Wild-type and mutant genomes were extracted from the cultured cell lines as per the manufacturer's instructions. The *TP53* gene was preamplified to generate amplicons with a length of 175 bp for the subsequent LDR procedure. To evaluate the sensitivity for mutation detection of this method, the mutant genome was diluted by wild-type genome to form the DNA solutions containing various percentages of mutant alleles. Then, the diluted genome solution was used as the DNA template to amplify the *TP53* gene that contained the point mutation (674A > G) in PCR. The PCR mixture consisted of 50 ng of genomic DNA, 1× PCR buffer, 0.1 mM dNTP mix, 0.2 μM primers, and 2.5 U of Hot Start DNA polymerase and PCR-grade water in a total volume of 50 μl. PCR was performed under the following cycling conditions: 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. The product of preamplification without purification was directly used as the DNA template for the subsequent LDR procedure.

LDR of rare mutation

Two kinds of oligonucleotides were designed as the probes for LDR in this study. To avoid confusion in the context, one was named as the detecting probe and the other was named as the reporting probe. Two probes were designed to perfectly complement the mutant allele in which they were adjacent at the site of mutant nucleotide. The detecting probe has at its 3′ end the nucleotide corresponding to the mutant nucleotide and has at its 5′ end the nucleotide modified by biotin. The reporting probe has at its 5′ end the nucleotide phosphorylated and has at its 3′ end the nucleotide modified by the amino group.

LDR was performed using the following procedure. The LDR mixture (30 μl) consisted of 2 pmol of the probes, 1× ligase buffer, and 5 μl of DNA template. The mixture was incubated at 94 °C for 2 min and then mixed with 20 U of Taq DNA ligase, followed by 29 cycles of 30 s at 94 °C and 4 min at 65 °C. The length of the integrated probe was 94 bp.

Bead enrichment and labeling of $Ru(bpy)_3^{2+}$ *of LDR products*

Enrichment of LDR products to the magnetic beads (streptavidin activated) was performed as per the manufacturer's instructions for the beads. In brief, 50 μl of hybridization buffer (20 mM Tris–HCl, 2 mM ethylenediaminetetraacetic acid (EDTA), and 600 mM NaCl, pH 7.4), 2.5 μl of streptavidin-activated magnetic beads (5 mg/ml), and 17.5 μl of H_2O were mixed with 30 μl of LDR products. The mixture was incubated at 37 °C for 30 min. After that, the beads were pelleted by a magnetic separator for 60 s and washed three times with the binding buffer (10 mM Tris–HCl,