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PCR-free mutation detection of *BRCA1* on a zip-code microarray using ligase chain reaction

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Abstract

We describe here ligation-based strategy to detect mutations in *BRCA1* utilizing zip-code microarray technology. In our first approach, PCR was performed to amplify the genomic regions containing the mutation sites. The PCR products were then used as templates in a subsequent ligation reaction using two ligation primers that flanked the mutation site. The primary allele-specific primer is designed to contain a base of mutation site at its 3′ end with 5′ complementarity to the respective zip-code sequence while the secondary common primer is modified by biotin at its 3′ end. Depending on the genotype of samples at the mutation site, the nick between the two ligation primers can be sealed in the presence of DNA ligase. The ligation products were then hybridized on the zip-code microarray followed by staining with streptavidine-cy3 to generate a fluorescent signal. Using this strategy we successfully genotyped selected Korean-specific mutation sites in exon 11 of *BRCA1* with a wild type and two heterozygote mutant samples. Furthermore, we also demonstrated that ligase chain reaction using unamplified genomic DNA as direct templates is enough to generate sufficient signals for correct genotypings in a multiplexed manner, verifying first that PCR is not essential for this microarray-based strategy.

Keywords: Mutation detection; Ligase chain reaction; Zip-code microarray; BRCA mutation

1. Introduction

Genetic mutations are responsible for attacks of various diseases and even cause cancer. Most typical examples are mutations in *BRCA1* and *BRCA2*, which are associated with a greater risk of developing breast, ovarian, prostate and other cancers [1]. The incidence of breast cancer and its associated death rate in Korea are increasing at a more rapid rate than the world average [2–4]. Therefore, we are in great need to develop an efficient tool to detect *BRCA* mutations for the medical benefit of breast cancer patients.

Wide arrays of methods and technologies have been proposed for screening of *BRCA* mutations and some of them are currently used in clinical and research laboratories worldwide. They include single-strand conformation polymorphism assay, direct DNA sequencing, clamped denaturing gel electrophoresis, heteroduplex analysis, and protein truncation assay [5–10]. Recently, DNA microarray-based strategy [11–14] has emerged as one of the most powerful technologies for routine clinical detection of such human genetic mutations due to its high power of multiplexity, high processing speed, and low cost. Of several microarray-based methods, zip-code microarray technology offers many promising opportunities due to its unique advantages over the conventional technology [15]. The zip-code microarray contains unique zip-code sequences with similar T_m values which are used to monitor products generated from allele-specific reaction, such as single base extension and ligation reactions. Most attractive feature of this approach is that once developed, the zip-code microarray can be used for different sets of human genetic mutations. Therefore, over the past few years, several methods for mutation genotyping have been reported based on the zip-code microarray technology [16,17].

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Along with the single base extension reaction, ligation reaction by DNA ligase can also be efficiently combined to achieve a diagnostic method for human genetic mutations, which was mainly developed by Barany group [18]. This ligation-based technology utilizes the ability of DNA ligase to seal adjacent oligonucleotides hybridized to target DNA only when the perfect complementation is present at the nick junction. Barany group very successfully demonstrated that several genetic variations can be detected by employing this technology [19–21]. In their studies, so called PCR/LDR (polymerase chain reaction/ligase detection reaction) strategy is usually employed, in which PCR precedes LDR to generate sufficient templates for the main ligation reaction.

Since the LDR method can provide an elegant tool for the detection of point mutations, we herein, describe ligation-based genotyping strategy on the zip-code microarray with a wild type and heterozygote mutant samples from breast cancer patients. Two Korean-specific mutation sites were selected in *BRCA1* exon11 (1942 and 3459) for this study [4,22]. First, we successfully genotyped two mutation sites using PCR products as templates for the main ligation reaction, which is a similar approach with other reports [20,21]. However, we found that the PCR amplification is not essential with this strategy and unamplified genomic DNA can be directly used to generate sufficient ligated products by ligase chain reaction. Finally, using this advanced PCR-free strategy, we correctly genotyped the Korean-specific *BRCA* mutations in a multiplexed manner.

2. Materials and methods

2.1. Oligonucleotides

Oligonucleotides for PCR primers, zip-code sequences, and ligation primers (Tables 1 and 3) were synthesized and purified by Bioneer (Seoul, Korea). Concentrations of all nucleotides were measured by CARY 100 Conc UV–Vis spectrophotometer (Varian, Australia) at λ_{260} nm based on the calculated molar extinction coefficient and their identities were confirmed by matrix-assisted laser desorption/ionization — time-of-flight (MALDI-TOF).

2.2. Microarray fabrication

The zip-code sequences were diluted in 200 mM carbonate buffer (pH 9) with 10% (v/v) dimethyl sulfoxide (Sigma-Aldrich) and spotted on aldehyde-activated glass slides (CEL-Associates, Inc. Pearland, Texas) by using a VersArray Chip Writer Compact Systems (BioRad Laboratories (Canada) Ltd.) according to the manufacturer's instructions. They were then allowed to covalently bind to the glass surface for 12 h in a humid chamber. The spots were about 200 μm in diameter and the centre to centre distance between adjacent spots was 300 μm. To verify the reproducibility six spots were printed for each oligonucleotide on the same glass slide.

Table 1
DNA zip-code sequences used in this study

Zip-code	Sequence (5′→3′)
Z1	NH_2 — TGCGGGTAATCG
Z2	NH_2 — ATCGTGCGACCT
Z3	NH_2 — ATCGGGTATGCG
Z4	NH_2 — ACCTGACCATCG

2.3. DNA isolation, sequencing, and PCR amplification

We prepared genomic DNA (gDNA) from whole blood of an apparently healthy subject and two breast cancer patients by using genomic DNA extraction kit (Qiagen, Hilden, Germany) and our laboratory standard protocol [14]. After PCR amplification, direct sequencing was performed using an ABI Dye Terminator Cycling Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions, followed by analysis on an ABI3700 DNA sequencer (Applied Biosystems).

Each PCR amplification was carried out in a thermocycler (Applied Biosystems) using a 50 μl total reaction volume containing 100 ng gDNA, 0.5 μM each primer [for 1942 mutation site: 5′-AGATTTGGCAGTTCAAAAGA-3′ (forward), 5′-TGCAATTCAGTACAATTAGG-3′ (reverse), for 3459 mutation site: 5′-TGGAAGTAATTGTAAGCATCCT-GAAATAAAAA-3′ (forward), 5′-GGGAAGCTCTTCATCCT-CACTAGATAA-3′ (reverse)], 5 μl 10× Pyrobest (Takara Bio Inc, Japan) reaction buffer (20 mM tris–HCl, pH 8.3 at 37 °C, 10 mM KCl, 2 mM $MgCl_2$), 0.2 mM dNTPs (Pyrobest) and 0.5 U *Taq* DNA polymerase (Pyrobest). The reaction tubes were heated at 94 °C for 5 min, followed by 35 cycles of 30 s at 94 °C, 30 s at 57 °C and 1 min at 72 °C. The reaction ended with 5 min extension at 72 °C and then cooling at 4 °C for preservation. After the amplification, the PCR products were purified by Qiaquic purification kit (Qiagen) and amplification was confirmed by agarose-gel electrophoresis.

2.4. Ligation reaction

Ligation reactions were performed by using PCR products or direct gDNA as template DNA (tDNA) in a 20 μl volume containing tDNA (40 ng), two primary primers corresponding to both of wild and mutant sequences (5 pmol), one common secondary primer (5 pmol), reaction buffer (30.5 mM Tris–HCl, 27.5 mM KCl, 10 mM $MgCl_2$, pH 8.3), 1 mM DTT, 2.5 mM NAD^+ and 5 units Tfi (*thermus filiformis)* DNA ligase (Bioneer, Korea). The reaction tubes were heated at 95 °C for 5 min followed by 25 cycles of 30 s at 95 °C and 2 min at 65 °C and then a final 5 min extension at 95 °C. Finally the reaction mixture was cooled at 4 °C for preservation.

2.5. Hybridization and staining

10 μl ligated products were mixed with 20–30 μl 1× SSPET buffer (0.01 M phosphate buffer, 0.15 M NaCl, 0.001 M EDTA, pH 7.4, Triton X-100 0.01%) and applied to the zip-code microarray followed by an incubation at 38–45 °C for 6 h. Then, the hybridized slides were washed by 6× SSPET buffer (0.9 M NaCl, pH 7.4, Triton X-100 0.005%) and distilled water for 8 min and 5 min respectively, and finally air dried at room