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Improvement of Position of Label (POL) Influence by Fluorescently-labeled Helper Probe

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Abstract

DNA microarray-based technologies provide for high-throughput methods and the simultaneous analysis of a large number of genetic information at one assay. However, several important aspects of microarray technology such as the selection of optimal oligonucleotide probes and improvement of hybridization kinetics still have to be resolved. Recently, Lei Zhang *et al.* proposed that one of the important things in applications of a short oligonucleotide microarray is the position of label (POL) effect. In this study, we fabricated a microarray for the identification of polymorphisms in human mitochondrial DNA hypervariable region II (HVII), as well as a fluorescently-labeled helper probe applied to a fabricated microarray. In conclusion, we report on the results obtained with additional fluorescently-labeled oligonucleotide probes, called helper probes, in an attempt to improve the relative POL influences. Microarray results were improved and reduced the POL effect. These results may contribute to better hybridization patterns, data interpretation, and a new aspect for the selection of an efficient probe in microarray applications.

Keywords: Helper probe, POL, DNA chip, Microarray, mtDNA

Introduction

DNA microarray-based technologies provide for high-throughput methods and the simultaneous analysis of a large number of genetic information at one assay. Therefore, DNA microarray-based technologies have become powerful tools in biological studies such as gene expression, species identification, single nucleotide polymorphisms (SNP), and forensic DNA typing[1-5]. A DNA microarray can discriminate genetic variations using a specific probe hybridized with fluorescent-labeled target DNA containing genetic information and significant miniaturization[6], as thousands of specific probes can be printed onto a modified glass slide[1]. In an experiment of a DNA microarray, the important thing is correctly hybridized with the target DNA. Various factors such as hybridization temperature, time, and concentration of probe and target materials can affect the hybridization efficiency and specificity[7]. Also, other parameters such as the secondary structure of target products, steric hindrance, and the relative position of label (POL) influence the efficiency of hybridization[8-10]. For correct results, microarray technology has to obtain a high specificity and efficiency of probes, particularly for the application of diagnostics using single-nucleotide polymorphism (SNP)[11]. Recently, one of the key issues in applications of short oligonucleotide based microarray is the POL relative to the probe-target duplex in oligonucleotide microarrays[10]. A comparison of microarray signal patterns of a probe-target identified POL as a critical factor affecting signal intensity.

Here, we report on the results obtained with additional fluorescently-labeled oligonucleotide probes, called helper probes, in an attempt to improve the relative position of label (POL) influences. In this study, we fabricated a microarray for the identification of a human mitochondrial DNA variation and then tested the POL effect of duplex formation in human mitochondrial DNA, as well as a fluorescently-labeled helper probe applied to the same micorarray. The microarray results were improved and reduced the POL effect. This result may contribute to better hybridization patterns and data interpretation in microarray applications.

Results and Discussion

Sequence-specific Probes

The sequences of HV II were determined for 12 unrelated persons. Compared to the Revised Cambridge Reference Sequence (rCRS) of human mitochondrial DNA, 7 sites of variation on HV II region were identified. In particular, changes of nucleotide position

263 were found in over 90% of the subjects, and most variations of the HV II region were positioned at 93-263. This result is in agreement with a previous study[9]. In this study, we selected two variation points (nucleotide positions 93 and 150) to verify the effect of the fluorescently-labeled helper probe.

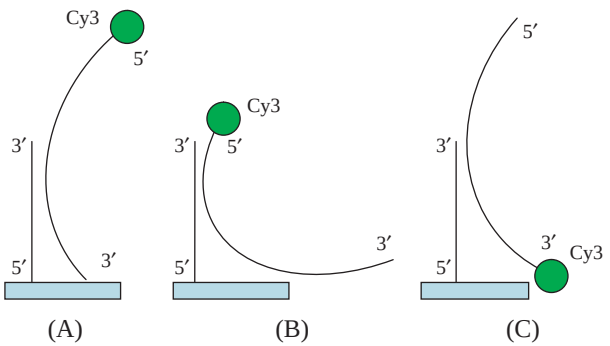

Figure 1. Schematic representation of the signal intensity relative to distance between probe and fluorescently-labeled position: (A) Long distance between oligonucleotide probe and fluorescently-labeled position of target DNA: low level signal intensity, and (B), (C) short distance between oligonucleotide probe and fluorescently-labeled position of target DNA: high level signal intensity.

To detect a sequence variation in an HV II segment, specific probes were designed based on the sequencing results of the samples. Each probe included variation sites at the central region[5], and to relieve steric hindrance, an oligonucleotide spacer was added at the 5′ end of the probe. The specific probe for nucleotide position 93 was located in 83-103 of the HV II region. In order to judge the POL effect, reverse complementary probes with the same variation were chosen. The helper probe sequence was based on the position in the amplified products of the HV II region. Each helper probe was located in approximation to variation points. Detailed target variation sites as well as the specific probes and helper sequence are shown in Tables 1 and 2.

Hybridization Patterns of Oligonucleotide Microarray

To determine the performance of the fluorescently-labeled helper probe, specific probes including the target variation sequence were printed on a slide, and after fabrication of the microarray, hybridized with

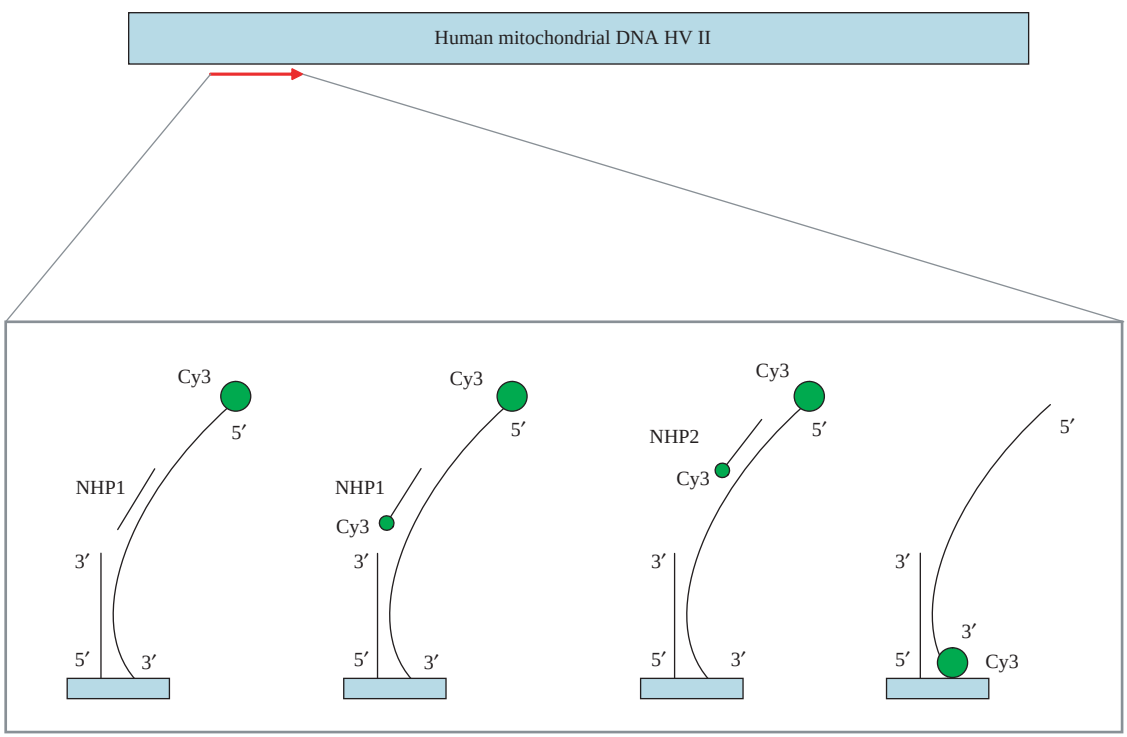

Figure 2. Schematic representation of improvement by fluorescently-labeled helper probe. Human mitochondrial DNA HV II products include polymorphisms (93C > G, 150A > T). The reverse primer was labeled with cyanine 3 fluorescent dyes during the primer synthesis. Fluorescently-labeled dyes are incorporated at the site in the 5′-end of reverse strand amplified products. To improve the POL effect, cyanine 3 fluorescently-labeled and unlabeled helper probes are added to the reaction of hybridization.

Table1. List of helper and specific probes.

Name	Nucleotide sequence	Nucleotide position*
NHP1	5′-GGA GCA CCC TAT GTC GCA GTA TCT-3′	106-129
HP1	5′-cyanine 3-GGA GCA CCC TAT GTC GCA GTA TCT-3′	106-129
HP2	5′-cyanine 3-AGC CGC TTT CCA CAC AGA CAT C-3′	259-278
93G	5′-aminolink-TTT TTT TTT TAG CAT TGC GGG ACG CTG GAG-3′	83-103
RC93G	5′-aminolink- TTT TTT TTT CTC CAG CGT CCC GCA ATG CTA-3′	103-83
150T	5′-aminolink- TTT TTT TTT ATT CCT GCC TCA TTC TAT TAT TT-3′	137-159
RC150T	5′-aminolink- TTT TTT TTT AAA TAA TAG AAT GAG GCA GGA AT-3′	159-137

*The nucleotide positions are described in previous studies[15,16].

Table 2. Nucleotide sequence variation in HV II region of 12 unrelated persons. A dot indicates that the sequence was the same as that of the Revised Cambridge Reference Sequence (rCRS) of human mitochondrial DNA.

No	1	2	3	4	5	6	7
Nucleotide position	93	150	152	199	210	235	263
Reference	A	C	T	T	A	A	A
Nucleotide variation	G	T	C	C	G	G	G
mt1	G	·	·	·	G	·	G
mt2	·	T	·	·	·	·	G
mt3	·	T	·	·	·	·	G
mt4	·	T	·	·	·	·	G
mt5	·	T	·	C	·	·	G
mt6	·	·	C	·	·	·	G
mt7	·	·	·	·	·	G	G
mt8	·	·	·	·	·	·	G
mt9	·	·	·	·	·	·	G
mt10	·	·	C	·	·	·	G
mt11	·	·	·	·	·	·	G
mt12	·	·	C	·	·	·	G
n=12	1	4	3	1	1	1	12

the target samples. When compared with an unlabeled helper probe, in the case of variation position 93, the hybridization signal intensity of the 93G probe was varied by up to about 3-4-fold by fluorescently-helper probes HP1. In the case of the reverse complementary probe, it was about 3-fold if not applied with a fluorescently-labeled helper probe (Figure 3). This phenomenon is in agreement with a previous study by Lei Zhang[10]. In the case of variation position 150, the signal intensity was varied up to about 3-8-fold by HP2. On the other hand, HP1 did not work. The reverse complementary probe of variation site 150 increased in signal intensity but was given an equivocal basecalling of mitochondrial variation (Figure 3).

Recently, one of the key issues is the position of label relative to the probe-target duplex in an oligonucleotide microarray experiment. Lei Zhang *et al*. proposed that the position of the label is a critical factor affecting signal intensity[10]. In most previous studies, probe specificity and efficiency were attributed to the effect of a secondary structure, steric hindrance, melting temperature of probe contents, hybridization time, and concentration of the probe or target. However, the signal intensity differences of a probe in the same target DNA proposed the importance of the POL effect. Also, Régis Peytavi *et al*. demonstrated that the difference of signal intensity is dependent on the reassociation of the free overhanging region of the target DNA strand with its complementary strand, and they proposed evidence that the presence of the complementary strand in a hybridization reaction was associated with the low level of signal intensity of 5′ immobilized probes targeting the 3′ end of a product[9]. To apply DNA microarray-based technologies, this phenomenon must be resolved. Particularly, polymorphisms such as the human leukocyte antigen (HLA) gene or the control region of human mitiochondrial DNA are show an extensive variation over the target DNA[3,7]. These targets are difficult to apply in a DNA microarray because they are weaker than other target DNA by the POL effect[7].

In this study, the same phenomenon was presented in the mitochondrial DNA HV II region. When compared with 93G and 150T probe signal intensity, the unlabeled helper probe was poorer than the fluorescently-labeled helper probe. This result shows that signal intensity depends on the distance between the specific probe and helper probe. To increase the signal intensity, a long-distance probe from the labeled position needs an auxiliary probe. Fluorescently-labeled probes offer two benefits to the low-level signal intensity of a long distance from the labeled position of the target DNA. First, a helper probe decrease can open an inaccessible secondary structure by a long target DNA[13]. Second, an additional fluorescent dye can increase the signal intensity and decrease the effect of the POL. Therefore, it is possible to improve the probe performance.

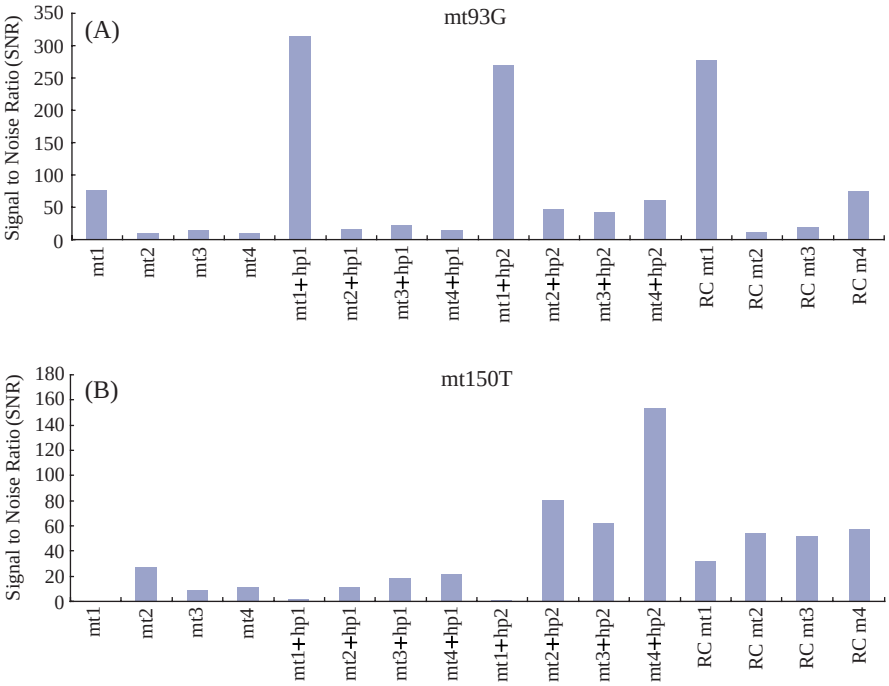

Figure 3. Signal intensities of unlabeled helper probe (106-129), cyanine 3 fluorescent dye-labeled helper probes (106-129, 259 -278) and reverse complementary probe: (A) hybridized results of mt93G marker (includes 93A > G, positive sample is mt1), and (B) hybridization result of mt150T marker (includes 150C > T, positive samples are mt2, mt3 and mt4).

Conclusion

In this study, we reconfirmed the importance of selecting the appropriate probe position to ensure an efficient hybridization and the performance of short capture probes, and proposed that fluorescently-labeled helper probes can improve the POL effect. These results should contribute to the establishment of a new aspect for the selection of an efficient probe, which should help to improve the sensitivity and specificity of microarray experiments.

Materials and Methods

Samples

DNA samples from 12 unrelated persons were extracted using salting out methods. DNA samples were quantified using a spectrophotometer and gel electrophoresis.

DNA Amplification and Sequencing

To amplify mitochondrial hypervariable segment II (HV II), we used one primer as follows: F15, 5′-CAC CCT ATT AAC CAC TCA CG-3′ and R448, 5′-TGA GAT TAG TAG TAT GGG AG-3′[14]. The amplification reaction mixture consisted of 0.5 U of Taq DNA polymerase (Enzynomics, KOREA), a 1X Taq buffer with 1.5 mM magnesium chloride, 0.5 μM of each primer, a 200 mM dNTP mixture, and 10 ng of genomic DNA. PCR was carried out in a DNA-ENGENE (MJ Research, USA), with a denaturation at 94˚C for 30 s, then 32 cycles with a denaturation at 94˚C for 20 s, annealing at 56˚C for 10 s, an extension at 72˚C for 30 s, and followed by a 5˚C soak. To confirm the amplification process, the PCR products were analyzed by electrophoresis with 1.5% agarose gels containing 0.5 μg/mL ethidium bromide. Successfully amplified HV II segment products were purified with an Exspin PCR purification kit (GeneAll, KOREA) and used for PCR direct sequencing.

Sequencing reactions were carried out using the fluorescent dye-termination methods (Big Dye, Applied Biosystems, Foster City, CA) and a genetic analyzer (ABI PRISM 3100 Avant, Applied Biosystems) according to the manufacturer's instructions. Chromatograms were visually checked and sequences aligned manually. Analysis of the sequences was carried out using ABI Prism sequence software (PE/Applied Biosystems, Foster City, CA), and chromatograms were used to visually check the sequences

manually.

Microarray for Improvement of POL Effect and Hybridization

To obtain reverse strand HV II fragments from human genomic DNA, enzymatic digestion of the forward strand of amplified PCR products was carried out using a Lambda exonuclease. The forward primer was modified by phosphate functional group and amplified products treated with 1 unit of the Lambda exonuclease (Fermentas, USA) in a 1X Taq buffer at 37˚C followed by inactivation of the enzyme at 80˚C for 15 min. The reverse primer was labeled with cyanine 3 fluorescent dye during primer synthesis to detect the target DNA. The treated PCR products were directly hybridized to the microarray without an additional purification process.

A total of 4 amino-modified oligonucleotide probes were located on the slide. Aminolinker-modified oligonucleotide probes were resuspended at 50 μM in a Spot buffer (3X SSC, 1.5 M betaine) and then stored in 384-well microtiter plates. Each probe was printed onto silylated slides (Cell Associate, USA) using a robotic microarrayer (Cartesian Technology, USA), and printed oligonucleotide microarrays were incubated in a humidity chamber with a controlled environment of 25˚C and 70% humidity. After incubation, the printed oligonucleotide microarrays were washed out by a treatment of 0.1% SDS solution and immobilized by a sodium borohydride solution (NaBH4 1 g : PBS 300 mL: Ethanol 100 mL). The remaining microarray solution was washed using nuclease-free water three times repeatedly and then dried with centrifugation for 5 min at 800 rpm. The hybridization solution was composed of 100 μL of 3 × SSC, 0.3% sarcosyl, and 10 UL of treated PCR products, and hybridization was performed for one hour at 55˚C. To prevent the evaporation of the hybridization mixture, a hybridization chamber was filled with distilled water to maintain humidity. After hybridization, elimination of non-reacting probes and hybridization components was accomplished by washing with 1X SSC plus 0.1% sarcosyl for 3 min, 1X SSC for 3 min, and 0.1X SSC for one min, and then drying with centrifugation. The dried micorarrays were stored at room temperature before scanning.

Scanning of Microarray

Hybridization signals on the microarrays were detected using a Genepix 4000B microarray scanner (Axon instrument, USA) at 545 nm. Scanning conditions were a laser power of 99% and PMT gain of 500, as well as quantification using Genepix 4.1 software.

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