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A mass spectrometry-based multiplex SNP genotyping by utilizing allele-specific ligation and strand displacement amplification

Jung Hun Park[a], Hyowon Jang[a], Yun Kyung Jung[a,b], Ye Lim Jung[a,c], Inkyung Shin[d], Dae-Yeon Cho[d], Hyun Gyu Park[a,*]

[a] Department of Chemical and Biomolecular Engineering (BK21+ Program), Korea Advanced Institute of Science and Technology (KAIST), 291 Daehak-ro, Yuseong-gu, Daejeon 305-701, Republic of Korea
[b] School of Biomedical Engineering, Inje University, Inje-ro 197, Gimhae, Gyeongnam 50834, Republic of Korea
[c] Department of Business Opportunity Analysis, Technology Innovation Analysis Center, Korea Institute of Science and Technology Information (KISTI), Seoul 130-741, Republic of Korea
[d] Labgenomics Co., Ltd. Korea Bio Park, 694-1 Sampyeong-dong, Bundang-gu, Seongnam-si, Gyeongi-do 463-400, Republic of Korea

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ABSTRACT

We herein describe a new mass spectrometry-based method for multiplex SNP genotyping by utilizing allele-specific ligation and strand displacement amplification (SDA) reaction. In this method, allele-specific ligation is first performed to discriminate base sequence variations at the SNP site within the PCR-amplified target DNA. The primary ligation probe is extended by a universal primer annealing site while the secondary ligation probe has base sequences as an overhang with a nicking enzyme recognition site and complementary mass marker sequence. The ligation probe pairs are ligated by DNA ligase only at specific allele in the target DNA and the resulting ligated product serves as a template to promote the SDA reaction using a universal primer. This process isothermally amplifies short DNA fragments, called mass markers, to be analyzed by mass spectrometry. By varying the sizes of the mass markers, we successfully demonstrated the multiplex SNP genotyping capability of this method by reliably identifying several BRCA mutations in a multiplex manner with mass spectrometry.

1. Introduction

Genetic variations in human genome can affect how humans develop diseases and respond to pathogens, chemicals, medication, vaccines, and other agents. Single nucleotide polymorphisms or SNPs are the most abundant form of genetic variations in the human genome and have been extensively studied as key genetic markers for efficient diagnosis and prognosis of various human diseases because they may be responsible for individual differences in the effectiveness and tolerability of drugs (Cardon and Bell, 2001; Feuk et al., 2006; Manolio et al., 2009; Tabor et al., 2002). Therefore, the development of efficient strategies for SNP identification has become one of the most important areas of medical research (Kim and Misra, 2007; Kwok, 2001; Syvanen, 2005; Wang et al., 1998). Among various methods to identify SNP genotypes, fluorescence-based methods have been intensively developed over the past decades (Duan et al., 2007; Hardenbol et al., 2003; Jung et al., 2008; Liew et al., 2004; Lowe et al., 2010; Mun et al., 2009; Park et al., 2015; Schouten et al., 2002; Shen et al., 2005; Shin et al., 2014; Zhang et al., 2006). Although fluorescent methods possess high sensitivity and specificity, they usually require a complicated and expensive labeling step to prepare fluorophore-modified probes. To overcome this limitation, alternative label-free genotyping methods have been developed based on electrophoresis (Gross et al., 1999), colorimetric assay (Guo et al., 2011; Jung et al., 2011), electrochemistry (Won et al., 2009), and surface-enhanced Raman spectroscopy (Papadopoulou and Bell, 2011). These methods successfully eliminate the requirement for fluorophore-labeled probes; however, they are not suitable to achieve multiplex genotyping because multiple signals from multiple analysis sites are generally hard to distinguish from each other. Therefore, there is great incentive for the development of a more accurate and convenient strategy enabling multiplex SNP genotyping in a label-free manner.

Along this line, mass spectrometry-based analysis has been intensively used to achieve label-free multiplex genotyping because it enables rapid and accurate multiple measurements of a sample in a wide detection range (Hong et al., 2004; Jurinke et al., 1996; Mauger et al., 2006). Representatively, mass spectrometry has been used with single base extension (SBE) (Di Giusto and King, 2003; Kim et al., 2002; Ross et al., 1998) and restriction fragment mass polymorphism (RFMP) (Hwang et al., 2007; Kim et al., 2005) to achieve multiplex

* Corresponding author.
E-mail address: hgpark@kaist.ac.kr (H.G. Park).

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genotyping. In the SBE-based method, SBE primers terminated just one base before the polymorphic sites are single base extended by different dideoxynucleotide triphosphates (ddNTPs) according to the base sequence at the polymorphic sites. By analyzing the molecular weight of the extended primers, the base sequences at the polymorphic sites are readily identified.

In the RFMP assay, polymerase chain reaction (PCR) is first conducted by employing the PCR primers which are designed to hybridize to the template near the polymorphic site and additionally contain restriction endonuclease recognition sequences at the middle part. The nuclease recognition sequences form a loop shape when the primers are annealed to the template. The resulting PCR amplicons contain the nuclease recognition sequences at their ends where the cleavage reaction is promoted by restriction enzyme generating short oligonucleotide fragments. Because the sequences of the restriction fragments are different according to the sequence variations at the polymorphic sites, the genotypes at the polymorphic sites can be identified by analyzing the fragments on mass spectrometry. These two methods enable multiplex genotyping by varying the size of SBE primers and restriction fragments; however, multiple peaks generated from a single polymorphic site might be hard to interpret and limit the number of multiple genotypes to be simultaneously determined.

Thus, herein, we developed an advanced multiplex genotyping method based on matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) using allele-specific ligation to discriminate between alleles at polymorphic sites and the strand displacement amplification (SDA) reaction to produce the corresponding mass markers. Because a single mass marker is designed to be produced only in the presence of a specific allele, the number of mass peaks on the final spectrum will be the same with that of polymorphic sites to be simultaneously analyzed. Therefore, the interpretation of the mass spectrum is easier for the analysis of multiple polymorphic sites in target DNA when compared with the previously developed strategies. To verify the multiplex genotyping capability of this method, several mutations in the BRCA1 gene, which is the breast and ovarian cancer susceptibility gene (Cho et al., 2014; Han et al., 2011; Seo et al., 2004), were successfully genotyped in a multiplex manner.

2. Materials and methods

2.1. Oligonucleotides and reagents

All DNA oligonucleotides used in this study were synthesized by Integrated DNA Technologies (IDT; IA, USA). The 5′ phosphate-modified primary ligation probes and the molecular beacon probe were purified by high-performance liquid chromatography (HPLC) while the other oligonucleotides including the primers and the secondary ligation probes were purified according to the standard desalting procedure. The OligoAnalyzer 3.1 program from IDT was used to design the primer and probe sequences by checking their dimer formation. Thermopol buffer, NEBuffer 3.1, dNTPs, 9°N™ DNA ligase, Vent (exo-) polymerase, and Nt. BstNBI nicking enzyme were purchased from New England Biolabs (MA, USA). i-proof polymerase for multiplex PCR was purchased from Bio-Rad (CA, USA). The G-DEXTM IIb Genomic DNA Extraction Kit was purchased from iNtRON Biotechnology (Korea), and the Nucleospin Gel & PCR clean-up kit was purchased from Macherey-Nagel (Germany). All chemical reagents were purchased from Sigma-Aldrich (MO, USA), and desalting resin was purchased from Sequenom, Inc. (CA, USA). Doubly distilled water with a specific resistance over 18 MΩ/cm was purchased from Bioneer (Korea) and used in all the experiments.

2.2. DNA extraction and target gene amplification

To demonstrate the genotyping capability of this strategy, we studied four mutation sites of the exon 11 region of the BRCA1 gene (Table 1). Clinical whole blood samples were obtained from Labgenomics Clinical Laboratories (Korea). The genotypes of both wild-type samples from normal persons and mutant samples from breast cancer patients were previously determined by direct sequencing. Approval was obtained from the Institutional Review Board of Labgenomic, and written informed consents were obtained from all participants.

Total genomic DNA was extracted with the G-DEXTM IIb Genomic DNA Extraction Kit according to the manufacturer's instructions. To cover the whole region of exon 11 in the BRCA1 gene, we performed multiplex PCR with five primer pairs (Table S1). Multiplex PCR was carried out on a C1000 thermo-cycler (Bio-Rad) in a 50 μL solution containing 5 ng of isolated genomic DNAs, 500 nM of each primer, 1× i-proof HF buffer, 200 μM dNTPs, and 1 U of DNA polymerase. PCR was performed using initial denaturation at 98 °C for 30 s, followed by 40 cycles of 98 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s, and then a final extension at 72 °C for 60 s. After the amplification, the PCR products were purified with the Nucleospin Gel & PCR clean-up kit according to the manufacturer's instructions. To verify the amplified products, gel electrophoresis was done with 2% agarose gel (Fig. S1).

2.3. Allele-specific ligation

Allele-specific ligation was carried out on the C1000 thermo-cycler in a 20 μL solution containing 2 μL of the purified PCR products, 1 μM of each ligation probe, 1×9°N™ DNA ligase reaction buffer (10 mM Tris-HCl, 600 μM ATP, 2.5 mM $MgCl_2$, 2.5 mM Dithiothreitol, and 0.1% Triton X-100; pH 7.5 at 25 °C), and 20 U of 9°N™ DNA ligase. The reaction tube was heated at 95 °C for 5 min, followed by 20 cycles of 1 min at 95 °C and 5 min at 55 °C. The reaction ended with a 5 min incubation at 55 °C and then cooling at 4 °C for preservation.

2.4. Strand displacement amplification

The SDA reaction was carried out on the C1000 thermo-cycler in a 50 μL solution containing 5 μL of the ligated product, 1× Thermopol buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, and 0.1% Triton® X-100; pH 8.8 at 25 °C), 0.5× NEBuffer 3.1 (50 mM NaCl, 25 mM Tris-HCl, 5 mM $MgCl_2$, and 50 μg/ml BSA; pH 7.9 at 25 °C), 1 μM universal primer, 100 μM dNTPs, 2 U of Vent (exo-) polymerase, and 2.5 U of Nt. BstNBI nicking enzyme. The sequence of the universal primer is 5′-AGCATCGGCTTGACGATG-3′. The reaction tube was incubated at 55 °C for 2 h, followed by a heat inactivating step at 80 °C for 20 min. In the experiments to optimize the SDA reaction condition, a chemically synthesized ligated template (5′-CTGGGCAGTGTTGAGACTCTCCTTGCTAAGCCAGGCTTTTGCTTTAT-TACAGAATTCAGCCTTTTCCATCGTCAAGCCGATGCT-3′) was used.

2.5. Fluorescent analysis

For the fluorescent analysis of the SDA products, 1 μM of a molecular beacon probe (5′-FAM-CACTGCTTTCTGGGCAGTG-Dabcyl-3′) in 1× Thermopol buffer was first heated at 95 °C for 1 min, cooled slowly to 20 °C (0.1 °C/s), and incubated at 20 °C for 20 min in the C1000 thermo-cycler. 30 μL of the SDA products were then mixed with 5 μL of the molecular beacon probe and incubated at 25 °C for 30 min. The fluorescence signal was measured with the Infinite® 200 PRO microplate reader (Tecan Group Ltd., Switzerland) with an excitation and emission wavelength of 470 and 520 nm, respectively.

2.6. MALDI-TOF MS analysis

To remove salts from the SDA products, 20 μL of the SDA products were thoroughly mixed with 5 mg of desalting resin. The mixture was

Table 1
Mutation sites in the exon 11 region of the BRCA1 gene and their corresponding mass markers studied in this work.

Mutation ID	Nucleotide change	Effect on protein	Mass marker sequence (5′ → 3′)	Molecular weight
1–43	c.3627dupA	p. E1210RfsX9	CACTGCCCAGG	3380 Da
1–81	c.3813dupT	p. N1272X	CACTGCCCAGAT	3668 Da
1–93	c.928C > T	p. Q310X	CACTGCCCAGGG	3709 Da
1–143	c.4092_4093delCT	p. L1365RfsX2	CACTGCCCAGCCCC	4208 Da

fully rotated for one minute at room temperature and centrifuged at 8000 rpm for 30 s. After centrifugation, the supernatant was analyzed by mass spectrometry. A matrix solution was prepared by dissolving 50 mg of 3-hydroxypicolinic acid and 10 mg of diammonium hydrogen citrate in 1 ml of 50% acetonitrile. On a MTP 384 ground steel MALDI-TOF MS plate (Hudson Surface Technology, NJ, USA), 1 μL of the matrix was first loaded and fully dried at room temperature. Then, 1 μL of the desalted sample was loaded onto the dried matrix and fully dried again. The sample was finally analyzed with the Autoflex III (Bruker Daltonics, Germany) MALDI-TOF MS instrument. Detection procedure described above is summarized in Fig. S2 for a better understanding.

3. Results and discussion

3.1. Overall procedure

In this study, we verified the SNP genotyping capability of our mass spectrometry-based method by applying it to the identification of BRCA mutations in a multiplex manner. We first performed multiplex PCR with genomic DNA as a template to amplify the genomic region containing the mutation sites under analysis. For the amplified PCR products, allele-specific ligation is conducted with ligation probe pairs to discriminate between wild and mutant alleles at the mutation sites schematically shown in Fig. 1. The primary ligation probe anneals up to one base before the mutation site at the 5′ side of the template DNA and is extended by an additional universal primer annealing sequence at the 3′ end to promote the SDA reaction. The 5′ end of the primary ligation probe is chemically phosphorylated for ligation with the hydroxyl group at the 3′ end of the secondary ligation probe. The secondary ligation probe annealing to the 3′ side of the template DNA is terminated by a complementary mutant base at the 3′ end and perfectly matched to the mutant allele at the mutation site. The secondary ligation probe additionally contains a 5′ end overhang with a nicking enzyme recognition site and complementary mass marker sequence.

Through the ligation process, the ligation probe pairs are linked by DNA ligase only at the mutant alleles while they remain intact at the wild-type alleles. The universal primer is then annealed at the 3′ end of the ligated products and extended by DNA polymerase. Next, the SDA reaction takes place by continuously repeating the cleavage at the nicking site and by strand displacement extension. This process produces mass markers corresponding to the mutation sites, which are finally subjected to analysis by mass spectrometry. By designing mass markers with different sizes to be produced from different mutation sites, multiple mutation sites can be simultaneously genotyped based on the peak positions on the mass spectrum. The strategy to produce mass markers only from mutant alleles simplifies the final mass spectrum when considering that more than 1000 BRCA mutations have been identified, and the mutations are very rarely observed among people (Seo et al., 2004). Furthermore, this strategy enables immediate visual interpretation of the tested samples for the presence of mutations.

3.2. Fluorescent molecular beacon probe-based optimization and confirmation of the SDA reaction

In this work, the SDA reaction uses two enzymes, a polymerase and nicking enzyme, which determines the sensitivity of our strategy by producing the mass markers to be analyzed by mass spectrometry. We first optimized the SDA reaction conditions with a chemically synthesized ligated probe as a template for the SDA reaction. For this purpose, the mass marker produced from the SDA reaction was fluorescently analyzed with a molecular beacon probe whose hairpin sequence is complementary to the mass marker. The fluorescence signal is generated only when the molecular beacon probe is opened, revealing the presence of the mass marker while the molecular beacon probe maintains a quenched form in the absence of the mass marker. Based on this experimental design principle, we analyzed the fluorescent signals resulting from the SDA reactions using various buffer compositions (Fig. S3) and various amounts of enzymes (Fig. S4), and finally determined the optimized conditions, 1× Thermopol buffer and 0.5× NEBuffer3.1, and 2 U of polymerase and 2.5 U of nicking enzyme, which were used in further experiments in this study.

To confirm the production of the mass markers from the SDA reaction following the allele-specific ligation of the ligation probes in Table S2, we conducted singleplex genotyping for the selected four mutations and fluorescently analyzed the produced mass markers in the same manner. As the results show in Fig. 2, highly enhanced fluorescence signals were observed only from the mutant samples while the wild-type samples showed quenched fluorescence signals for all the tested mutation sites. We then repeated the same genotyping experiments for the four mutation sites but in a multiplex manner by using all the ligation probe pairs in a single reaction tube (Fig. 3). Again, all four mutant samples showed highly enhanced fluorescence signals while the fluorescence signals from the two wild-type samples were quite low. All these observations clearly indicate that the mass markers were successfully produced by the SDA reaction after the ligation probe pairs were ligated only in the presence of mutant samples.

3.3. Mass spectrometry-based multiplex genotyping for BRCA mutations

To achieve multiplex genotyping for BRCA mutations, we used MALDI-TOF MS to analyze the mass markers produced by the SDA reaction. Fig. 4 shows the mass spectra obtained from the singleplex analysis at the four mutation sites for the wild-type and mutant samples. As envisioned, only the mutant samples showed the mass marker peaks labeled with ∇ at the specific position corresponding to the mutation sites described in Table 1 while there was no mass marker peak observed for the wild-type samples. Therefore, the genotypes of each SNP site in the target genomic DNA can be simply determined by the existence of the mass marker peaks. A peak for the unreacted universal primer was observed for both the wild-type and mutant samples, which could serve as an internal control peak in the mass spectra to provide operational assurance for the prior procedures including the desalting process. We finally applied our mass spectrometry-based method to the multiplex genotyping of BRCA mutations (Fig. 5). As a result, this method successfully identified all the mutations present in the four mutant samples by correctly producing

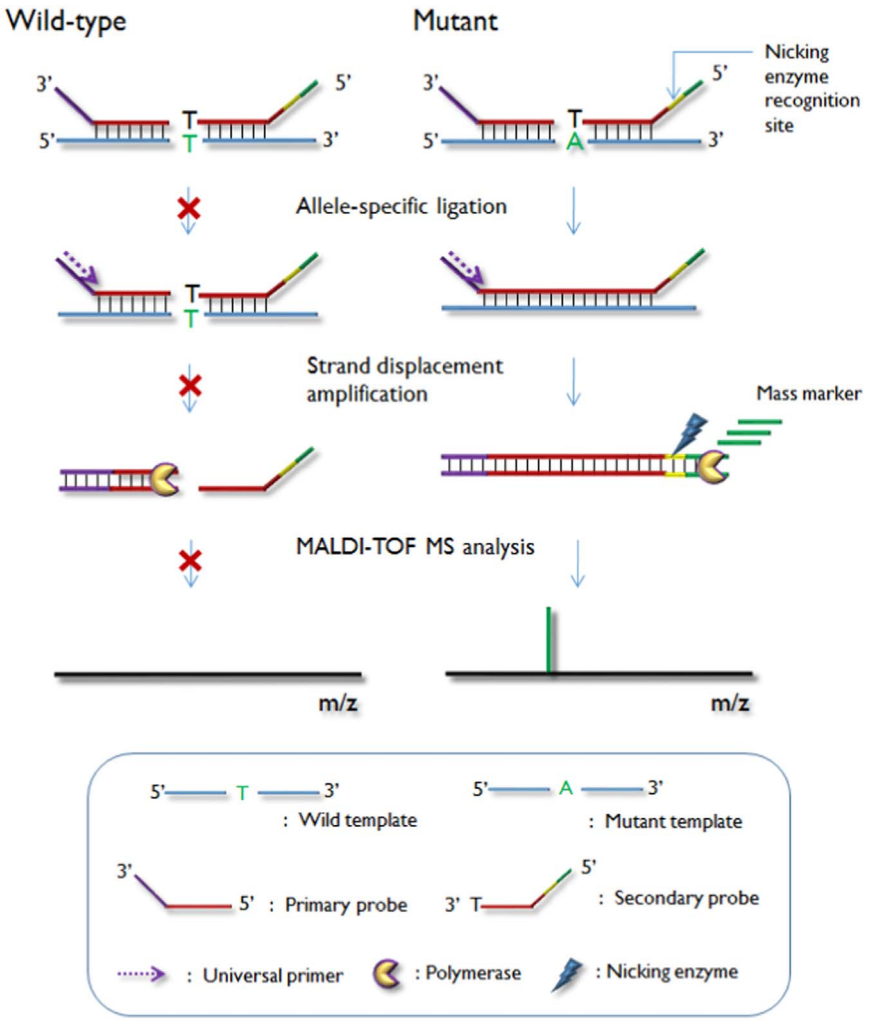

Fig. 1. Schematic representation of the mass spectrometry-based method for multiplex mutation genotyping.

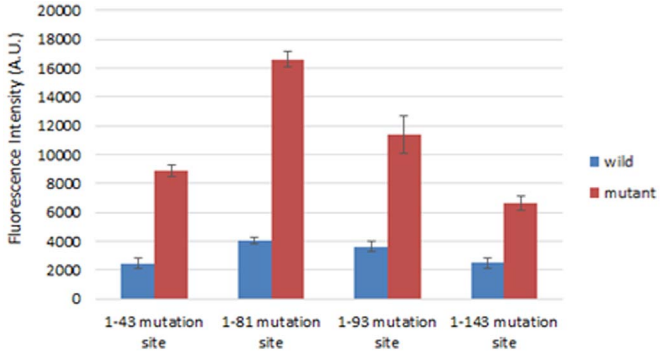

Fig. 2. Fluorescence signals obtained by singleplex genotyping of the four selected mutation sites.

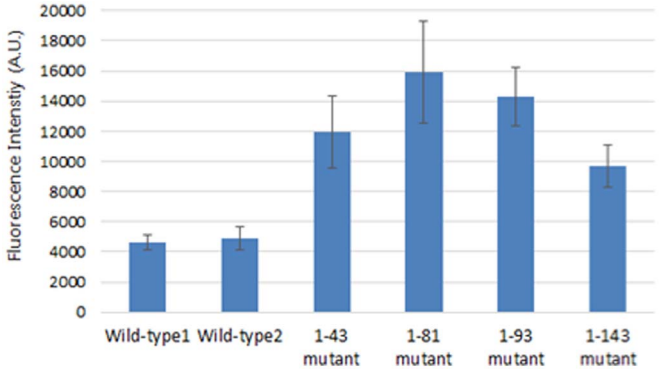

Fig. 3. Fluorescence signals obtained by multiplex genotyping of the four selected mutation sites.

the mass peaks corresponding to the specific mutant alleles (Fig. 5(C-F)). On the other hand, there were no mass marker peaks observed for the two wild-type samples, correctly indicating that the two wild-type samples do not have any mutant alleles at the four mutation sites (Fig. 5(A) and (B)). It should be noted that previous fluorescent analyses could only provide information about the presence of one or more mutant alleles for the analyzed mutation sites but not identify the specific mutation site; however, mass spectrometry-based analysis is able to identify specific mutation sites in a multiplex manner based on the position of the peaks.

The mass spectrometry has been considered as one of the most promising and versatile tools for the detection of biomolecules including protein (Yates et al., 2009) microorganism (Carbonnelle et al., 2011), and nucleic acids (Sauer et al., 2003) due to its easy automation, high-throughput, and high accuracy. Accordingly, the mass spectrometry has continuously received more and more attention from many biological researchers, and nowadays the mass spectrometry has been employed not only for physicochemical analysis (Medriganac and Espinat, 2007, , Wakankar et al., 2011, Sandquist et al., 2013) but also for biosensor studies as a detector like fluorescence scanner (Gopal et al., 2011, 2013; Lee et al., 2012; Ahmad et al., 2012). In accord with

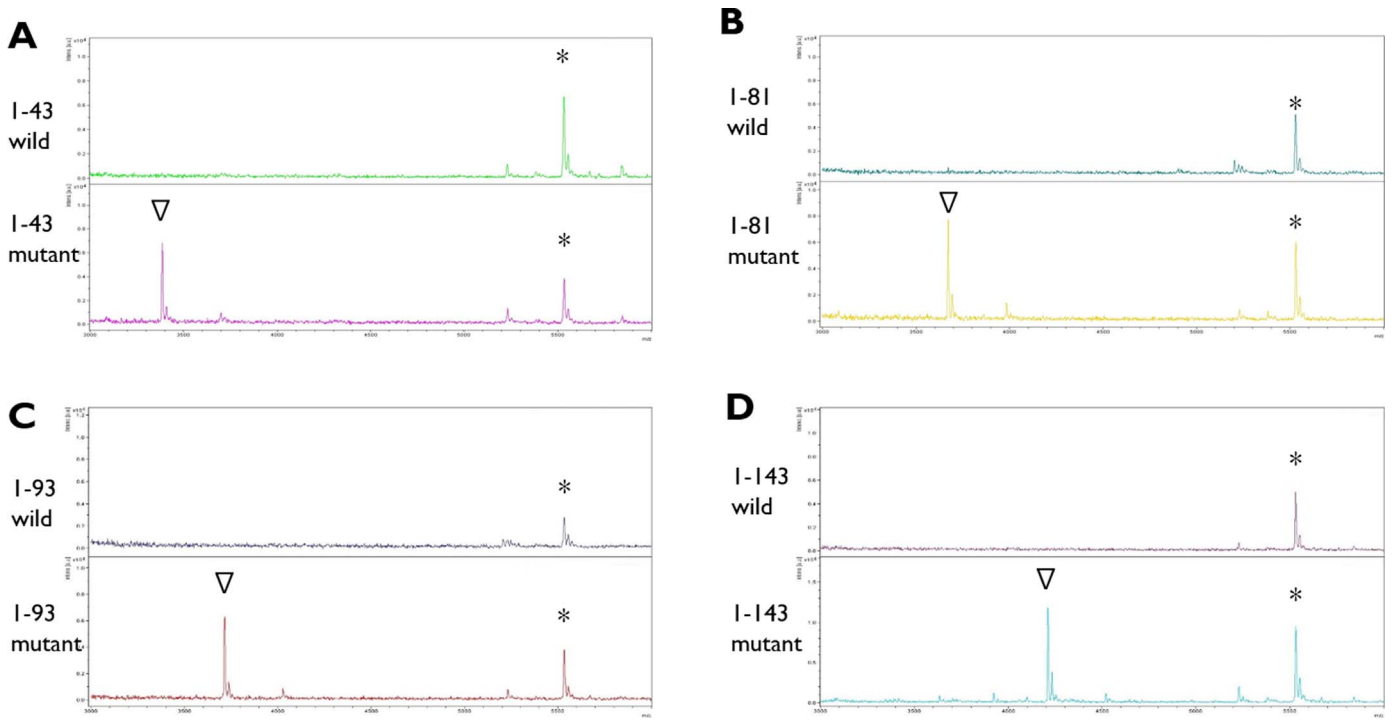

Fig. 4. MALDI-TOF mass spectra from singleplex genotyping of the four selected mutation sites. (A-D) are mass spectra from wild-type and mutant samples at 1–43, 1–81, 1–93, and 1–143 mutation sites, respectively. ∇ indicates a mass marker peak and * indicates a universal primer peak.

this recent research trend, we herein utilized the mass spectrometry as a biosensing instrument to achieve label-free multiplex SNP genotyping by taking advantage of its great multiplexing capability. When compared with many other SNP genotyping methods (Chen et al., 2009, Li et al., 2014, Ho et al., 2014, Knez et al., 2014, He and Ma, 2014, Cheng et al., 2014, Zhi et al., 2014, Chang et al., 2014, Hu et al., 2015), the multiplexing capability of this strategy could be further enhanced by simply modulating the length of the mass markers within the ligation probe. Remarkably, the strategy to produce a short oligonucleotide through the allele-specific ligation and isothermal amplification is quite novel itself, which could be utilized for other biosensor studies.

4. Conclusion

In this study, we describe a new method for mass spectrometry-based multiplex SNP genotyping using allele-specific ligation and the SDA reaction. The SDA reaction following the mutant allele-specific ligation produces mass markers only in the presence of mutant alleles in the target DNA, which are then subject to mass spectrometry analysis. By varying the size of arbitrarily designed short DNA fragments, denoted as mass markers, we successfully identified the mutant genotypes at the four selected BRCA mutation sites in a multiplex manner using clinical patient samples. Because the mass markers are designed to be produced only from the mutant alleles, the interpretation of the final mass spectrum is quite simplified. This method should serve as a powerful strategy to achieve high-throughput label-free genotyping for a more number of BRCA mutations and also other genetic variations.

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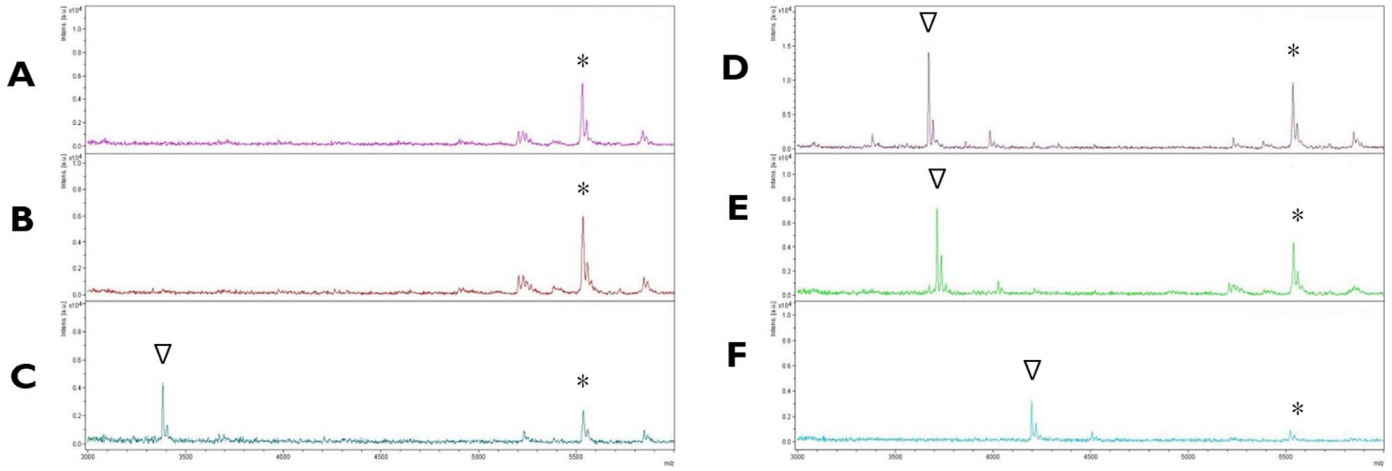

Fig. 5. MALDI-TOF mass spectra from multiplex genotyping of the four selected mutation sites. (A) and (B) are mass spectra from the wild-type samples, and (C-F) are mass spectra from the mutant samples at 1–43, 1–81, 1–93, and 1–143 mutation sites, respectively. ∇ indicates a mass marker peak and * indicates a universal primer peak.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2016.10.065.

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