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A single nucleotide polymorphism genotyping method using phosphate-affinity polyacrylamide gel electrophoresis

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Abstract

To date, various methods have been developed to facilitate the genotyping of a single nucleotide polymorphism (SNP) for aiding in the diagnosis and treatment of inherited diseases. The most commonly used method for SNP genotyping is an allele-specific hybridization procedure using an expensive fluorochrome-labeled oligonucleotide probe and a specialized fluorescence analyzer. Here, we introduce a simple and reliable genotyping method using a 1:1 mixture of 5′-phosphate-labeled and nonlabeled allele-specific polymerase chain reaction (PCR) primers. The method is based on the difference in mobility of the phosphorylated and nonphosphorylated PCR products (in the same number of basepairs) on phosphate-affinity polyacrylamide gel electrophoresis. The phosphate-affinity site is a polyacrylamide-bound dinuclear zinc(II) complex, which preferentially captures the 5′-phosphate-labeled allele-specific product compared with the corresponding nonlabeled product. The obtained DNA migration bands can be visualized by ethidium bromide staining. We demonstrate the genotyping of a SNP reported in a human cardiac sodium channel gene, *SCN5A*, using this novel procedure.

Keywords: SNP genotyping; Allele-specific PCR; Phosphate-affinity PAGE; Zinc

Single nucleotide polymorphisms (SNPs)[1] are DNA sequence variations that occur when a single nucleotide in the genome sequence is altered. The use of SNPs is expected to lead to a greater elucidation of the genetic basis for diseases and to realize the potential for clinical diagnostics and pharmacogenetics [1]. Now, a number of methods are available for SNP genotyping, and the choice of the method depends on the assay scale. In a large-scale assay, an allele-specific hybridization method using a TaqMan probe [2], a fluorescence resonance energy transfer probe [3], or a molecular beacon [4] is most commonly used for SNP genotyping. The method allows accurate allele discrimination in a one-step procedure without separation of the analytes or removal of the surplus fluorescent contaminants. However, expensive fluorochrome-labeled oligonucleotides are required, in addition to a special apparatus for the fluorescence measurement. On the other hand, the genotyping methods for a small-scale assay require complicated processes and a skillful analyst; many of these methods are gel electrophoresis-based techniques, such as single-strand conformation polymorphism [5], denaturing gradient gel electrophoresis [6], and conformation-sensitive gel electrophoresis [7].

In 2002, we reported a gel electrophoresis-based SNP detection method, Zn^{2+}–cyclen PAGE, for the small-scale screening of various disease-causing mutations [8]. The method is based on the principle that the binding of Zn^{2+}–cyclen (i.e., a mononuclear zinc(II) complex) to the thymine base changes the local DNA conformation, resulting in differences in the electrophoretic mobility of a mutant DNA. The Zn^{2+}–cyclen PAGE was applied to the comprehensive screening of heterozygous mutations scattered throughout a human cardiac sodium channel gene, *SCN5A*, which is related to inherited arrhythmia syndromes [9]. In this report, we introduce a newly developed

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[1] *Abbreviation used:* SNPs, single nucleotide polymorphisms.

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gel electrophoresis-based method for SNP genotyping. The principle of the method is based on our recent findings on a phosphate-binding tag molecule, Phos-tag (i.e., a dinuclear metal (Mn^{2+} or Zn^{2+}) complex of 1,3-bis[bis(pyridin-2-ylmethyl)amino]propan-2-olate) [10–16]. The Phos-tag molecule has a vacancy on two metal ions that is suitable for the access of a phosphomonoester dianion (R-OPO_3^{2-}) as a bridging ligand. This fact has resulted in the development of phosphate-affinity gel electrophoresis (Mn^{2+}–Phos-tag SDS–PAGE) for the mobility shift detection of phosphoproteins from their nonphosphorylated counterparts [13–15]. We have adapted the electrophoretic separation method for the SNP genotyping in the present study; the separation of phosphate-labeled and nonlabeled PCR products (i.e., allele-specific DNA in the same number of basepairs) was conducted by a similar phosphate-affinity PAGE but with a Phos-tag complex with two zinc(II) ions (Zn^{2+}–Phos-tag PAGE). As the first practical example, we demonstrated the SNP genotyping of a silent mutation reported in the *SCN5A* gene [9] using Zn^{2+}–Phos-tag PAGE.

Materials and methods

Materials

The acrylamide-pendant Phos-tag ligand (*N*-(5-(2-acryloylaminoethylcarbamoyl)pyridin-2-ylmetyl)-*N*,*N*′,*N*′-tris(pyridin-2-yl-methyl)-1,3-diaminopropan-2-ol) was obtained from the Phos-tag consortium (http://www.phos-tag.com, Japan). The 100 bp DNA ladder as a DNA size marker was purchased from Takara Bio (Otsu, Japan). T4 polynucleotide kinase was obtained from New England Biolabs (Beverly, MA). KOD-plus-DNA polymerase was purchased from Toyobo (Osaka, Japan). All oligonucleotide PCR primers were obtained from Invitrogen Japan (Tokyo, Japan). Ethidium bromide was purchased from Nacalai Tesque (Kyoto, Japan). All reagents and solvents used were of the highest commercial quality and were used without further purification.

Preparation of genomic DNA

Peripheral blood (10 mL) was obtained from seven healthy blood donors, and genomic DNA was extracted from the leukocytes according to the standard protocol using the QIAamp DNA Blood Maxi Kit (Qiagen, Hilden, Germany). Written informed consent for participation was obtained from all donors. The DNA concentration was determined by using an ethidium bromide fluorescent quantitative method [17].

Design of the allele-specific primers and procedure of PCR for genotyping

Allele-specific PCR primers containing a single nucleotide variation of G and A in the exon 2 of the *SCN5A* gene (a G-allele-specific primer, 5′-GCCGCGGGCTTGCTTCTCCG-3′, and an A-allele-specific primer, 5′-GCCGCGGGCTTGCTTCTCTG-3′, underlined at the variation site) and a reverse PCR primer, 5′-GGTCTGCCCACCCTGCTCTC-3′, were designed by referring to the sequence of GenBank Accession No. M77235 (cDNA of *SCN5A*). The allele-specific PCR primers for the SNP genotyping are illustrated in Fig. 1. The G-allele-specific primer (50 μM) was phosphorylated with T4 polynucleotide kinase (5 U) in a reaction mixture (final volume, 20 μL) consisting of 70 mM Tris–HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM dithiothreitol, and 1 mM ATP at 37 °C for 30 min. The reaction mixture was incubated at 75 °C for 5 min to stop the kinase reaction. The A-allele-specific primer and the reverse primer were used without the kinase treatment. PCR for amplification of the target region (220 bp) was performed with the following reaction

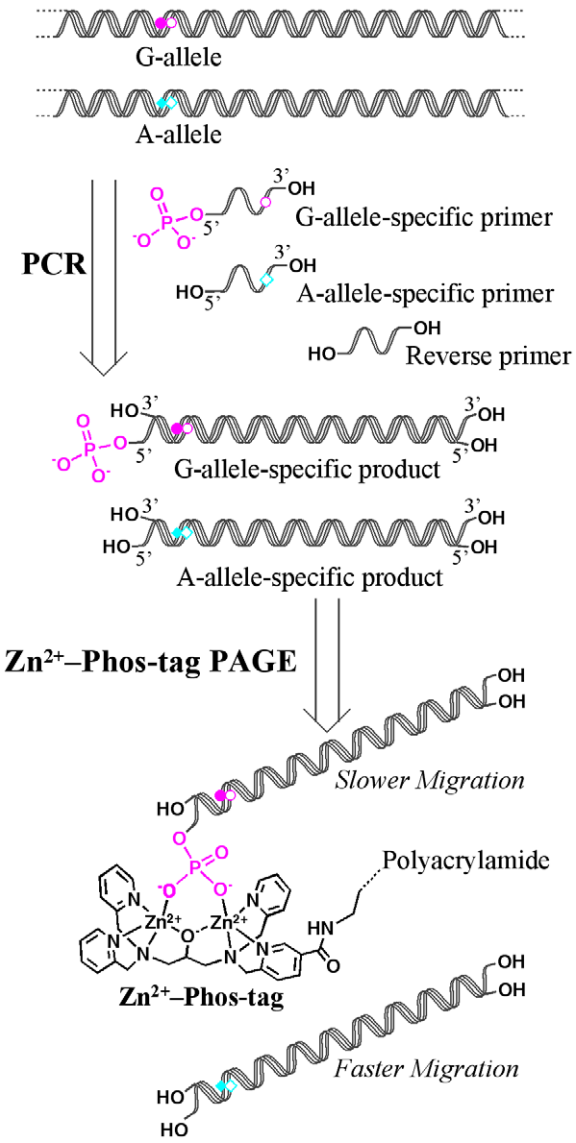

Fig. 1. Novel SNP genotyping using a two-step procedure of allele-specific PCR and Zn^{2+}–Phos-tag PAGE.