Genotyping and mapping assay of single-nucleotide polymorphisms in *CYP3A5* using DNA-binding zinc(II) complexes

Category: Analytical Investigation

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Running title: Genotyping and mapping assay of single-nucleotide polymorphisms

Abbreviations: CYP, cytochrome P450; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SNP, single-nucleotide polymorphism.

Abstract

Objective: It is clinically important to detect the single-nucleotide polymorphism (SNP) of *CYP3A5*3* (6986A>G) associated with enzymatic activity for drug metabolism. The aim of this study was to establish an accurate strategy for SNP screening.

Design and Methods: Polyacrylamide gel electrophoresis (PAGE) using zinc(II) complexes were applied for SNP detection. Genomic analyses of 19 healthy subjects were conducted by using both Zn^{2+}–Phos-tag-PAGE and Zn^{2+}–cyclen-PAGE methodologies.

Results: Zn^{2+}–Phos-tag PAGE permitted identification of the following allele genotypes: the A/A homozygote (*CYP3A5*1/*1*) in 3 individuals, the G/G homozygote (*CYP3A5*3/*3*) in 14 individuals, and the A/G heterozygote (*CYP3A5*1/*3*) in 2 individuals. Zn^{2+}–cyclen PAGE demonstrated not only reproducibility of the genotyping but also existence of a novel heterozygous SNP (6929G>A) in the subject with *CYP3A5*1/*1*.

Conclusion: We demonstrated reliable SNP genotyping and mapping in *CYP3A5* using the combination method of Zn^{2+}–Phos-tag PAGE and Zn^{2+}–cyclen PAGE.

Keywords: CYP3A5; SNP; Mutation; Zinc(II) complex; Phos-tag; Cyclen; PAGE; Electrophoresis

Introduction

The human cytochrome P450 (CYP) enzymes metabolize endogenous and xenobiotic compounds to more water-soluble products that are easily eliminated from the body [1]. CYP3A is the most abundant subfamily in the human liver and intestine, and plays a major role in metabolism of many therapeutic drugs and toxins [2]. One family isoform, CYP3A5, is highly polymorphic [3,4] and this polymorphism often leads to marked differences in protein expression and catalytic activity [5–9]. The most frequent single-nucleotide polymorphism (SNP) 6986A>G (in intron 3, responsible for *CYP3A5*3* allele) is known to be associated with defective alternative splicing, resulting in the formation of a truncated protein and extremely low enzymatic activity [8]. Thus, it is very important to detect the SNP and identify the allele genotype to obtain theoretical and practical information on CYP3A5 metabolic activity.

We have developed two kinds of polyacrylamide gel electrophoresis (PAGE)-based SNP detection methods, Zn^{2+}–cyclen PAGE [10–14] and Zn^{2+}–Phos-tag PAGE [15–17]. Regarding the Zn^{2+}–cyclen-PAGE methodology, the principle is based on the thymine-recognizing property of Zn^{2+}–cyclen (cyclen = 1,4,7,10-tetraazacyclododecane) in double-stranded DNA [18–20]. The procedure combined with a polymerase chain reaction (PCR)-based heteroduplexing method permits the visualization of heteroduplex bands on the PAGE gel and enables SNP screening. As for the Zn^{2+}–Phos-tag-PAGE methodology, the principle is based on the difference in mobility of the phosphorylated and nonphosphorylated DNA fragments (in the same number of base pairs) on the phosphate-affinity-PAGE gel containing immobilized phosphate-binding tag molecules, polyacrylamide-bound dizinc(II) complexes, Zn^{2+}–Phos-tag (Phos-tag = 1,3-bis[bis(pyridin-2-ylmethyl)amino]propan-2-olate) [21–27]. The procedure combined with allele-specific PCR method using a 1:1 mixture of 5'-phosphate-labeled and non-labeled allele-specific primers permits the separation of the 5'-phosphate-labeled PCR product as a slower migration band from the non-labeled counterpart and enables the determination of genotype as heterozygote or homozygote.

In this study, we introduce a combination method of Zn^{2+}–Phos-tag PAGE and Zn^{2+}–cyclen PAGE, and discuss its resolving power for SNP genotyping and mapping. As the first practical example of using the combination method and human genomic DNA samples, we demonstrate reliable screening of polymorphisms containing the SNP of 6986A>G, responsible for the major phenotype of CYP3A5 activity.

Methods

Preparation of genomic DNA

Genomic DNA was extracted from saliva (1 mL) obtained from 19 unrelated healthy Japanese donors (7 males and 12 females) according to the standard spin procedure for isolation of genomic DNA from saliva and mouthwash using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany). Written informed consent for participation was obtained from all the donors. This study was conducted in accordance with the principles of the Declaration of Helsinki.

Design of the allele-specific primers and procedure of PCR for Zn^{2+}–Phos-tag PAGE

Allele-specific PCR primers containing a single-nucleotide variation of A (**1*) and G (**3*) in the intron 3 of the *CYP3A5* gene (a *CYP3A5*1*-allele-specific primer, 5'-TGGTCCAAACAGGGAAGAGATATT-3', and a *CYP3A5*3*-allele-specific primer, 5'-TGGTCCAAACAGGGAAGAGATACT -3', underlined at the variation site) and a reverse PCR primer, 5'-ATGGAGAGTGGCATAGGAGATACC-3', were designed by referring to the DNA sequence of GenBank Accession No. NG_000004.3 (Fig. 1). The *CYP3A5*3*-allele-specific primer (25 μM) was phosphorylated with 5 U of T4 polynucleotide kinase (New England Biolabs, Beverly, MA, USA) in a reaction mixture (final volume, 40 μL) consisting of the kinase special buffer 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 *CYP3A5*1*-allele-specific primer and the reverse primer were used without the kinase treatment. PCR for amplification of the target region (128 bp) was performed with the following reaction mixture: a 1:1 mixture of allele-specific primers (each at 0.10 μM), a 0.20 μM reverse primer, the genomic DNA template (1 μL), dNTPs (each at 0.20 mM), 0.80 mM $MgSO_4$, 0.10 U KOD-plus- DNA polymerase (Toyobo, Osaka, Japan), and its special buffer

in a volume of 5 μL. After initial denaturation at 95 ˚C for 3 min, the PCR amplification was carried out for 30 cycles of 15-sec denaturation at 95 ˚C, 30-sec annealing at 64 ˚C, and extension at 68 ˚C to obtain 5'-phosphate-labeled and/or non-labeled DNA fragments.

Zn^{2+}–Phos-tag PAGE

Electrophoresis was performed using a 1-mm-thick, 9-cm-wide, and 9-cm-long gel prepared with 18% (w/v) polyacrylamide (99:1 ratio of acrylamide and *N,N'*-methylenebisacrylamide), an acrylamide-pendant Phos-tag ligand (20 μM) obtained from the Phos-tag Consortium (http://www.phos-tag.com/english/index.html, order product name: Phos-tag acrylamide AAL-107), 2 equivalents of $Zn(NO_3)_2$, and 375 mM Tris-HCl (pH 8.8) on a standard mini-slab PAGE apparatus (model AE-6500; ATTO, Tokyo, Japan) at 15 mA/gel for 100 min at room temperature. The electrophoresis running buffer (pH 8.4) was 25 mM Tris and 192 mM glycine. Each PCR reaction mixture prepared as described above was dissolved in 5.0 μL of a loading buffer consisting of 50 mM EDTA (pH 8.0), 30% (v/v) glycerol, and 0.05% (w/v) bromophenol blue. Then, 1.0 μL of each sample solution was applied to the polyacrylamide gel containing gel-bound Zn^{2+}–Phos-tag. The DNA bands were visualized with 10,000-fold-diluted SYBR green I (Cambrex Bio Science Rockland, Rockland, ME, USA) staining (15 mL/gel) after electrophoresis.

Design of the primers and procedure of PCR for Zn^{2+}–cyclen PAGE

A primer 1, 5'-CCATACCCCTAGTTGTACGACACA-3', and the same reverse primer were designed as described above (see Fig. 1). PCR for amplification of the target region (244 bp) was performed with the following reaction mixture: a 0.20 μM primer 1, a 0.20 μM reverse primer, the genomic DNA template (1 μL), dNTPs (each at 0.20 mM), 1.0 mM $MgSO_4$, 0.10 U KOD-plus- DNA polymerase, and its special buffer in a volume of 20 μL. After initial denaturation at 95 ˚C for 3 min, the PCR amplification was carried out for 30

cycles of 15-sec denaturation at 95 ˚C, 30-sec annealing at 60 ˚C, and extension at 68 ˚C.

Zn^{2+}–cyclen PAGE

Electrophoresis was performed in the mini-slab PAGE apparatus (model AE-6500; ATTO) at 20 mA/gel for 100 min at room temperature. The gel consisted of 8.5 mL of a polyacrylamide gel (5.0 mM Zn^{2+}–cyclen, 15% [w/v] polyacrylamide, and 375 mM Tris-HCl at pH 8.8). The acrylamide stock solution was the same mixture of a 99:1 ratio of acrylamide and *N,N'*-methylenebisacrylamide described above. The Zn^{2+}–cyclen complex was used as a dinitrate salt as reported previously [10]. The cathode buffer was 25 mM Tris and 192 mM glycine, and the anode buffer was 25 mM Tris and 192 mM glycine containing 5.0 mM $Zn(NO_3)_2$. Each PCR reaction mixture (0.5 μL) prepared as described above was dissolved in 0.5 μL of the loading buffer and then applied to the polyacrylamide gel containing 5.0 mM Zn^{2+}–cyclen. The DNA bands were visualized with SYBR green I staining as described above.

DNA sequence analysis

For checking the DNA sequence of all subjects, each remaining PCR reaction mixture (19.5 μL) prepared for the Zn^{2+}–cyclen-PAGE analysis was used as a template after ethanol precipitation and directly sequenced on both DNA strands using the BigDye Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and the primer 1 or the reverse primer (see Fig. 1) as a probe of each strand. Sequencing products after ethanol precipitation were dissolved with 13 μL of Hi-Di Formamide (Applied Biosystems) and then applied onto an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Results

*Genotyping of CYP3A5*1 or CYP3A5*3 allele using Zn^{2+}–Phos-tag PAGE*

To apply the Zn^{2+}–Phos-tag-PAGE methodology for the genotyping of the SNP 6986A>G responsible for *CYP3A5*3* allele, we first performed allele-specific amplifications of the target region of *CYP3A5* (see Fig. 1, a 128-bp sequence of 247062–247189 entered as GenBank Accession No. NG_000004.3) using a 1:1 mixture of 5'-phosphate-labeled *CYP3A5*3*-allele-specific and non-labeled *CYP3A5*1*-allele-specific primers, the reverse non-labeled primer, and each genomic template from 19 unrelated healthy Japanese subjects. Subsequently, the resultant PCR products were analyzed using 20 μM Zn^{2+}–Phos-tag PAGE. Figure 2A shows that two kinds of allele-specific PCR products were clearly observed as a slower migration band (the 5'-phosphate-labeled *CYP3A5*3*-allele product) and a faster one (a non-labeled *CYP3A5*1*-allele product). In the genotyping of the **1/*3* heterozygote, both allele amplifications were observed almost equally in the lane of the electrophoresis gel. We demonstrated that Zn^{2+}–Phos-tag PAGE permitted identification of the following allele genotypes: the **1/*1* homozygote in 3 individuals (subject numbers 8, 15, and 17), the **3/*3* homozygote in 14 individuals (subject numbers 1, 3–7, 9–12, 14, 16, 18, and 19), and the **1/*3* heterozygote in 2 individuals (subject numbers 2 and 13).

Genetic analysis using Zn^{2+}–cyclen PAGE

Next, the 244-bp DNA sequence of 247062–247305 in *CYP3A5* gene shown in Fig. 1 was amplified using each genomic template from the 19 subjects for the Zn^{2+}–cyclen-PAGE analysis. We examined the zygosity (homo or hetero) using the resultant PCR products and compared with the results of the genotyping by the Zn^{2+}–Phos-tag-PAGE methodology described above. The results of 5.0 mM Zn^{2+}–cyclen PAGE are shown in Fig. 3 (see the electrophoresis data). The Zn^{2+}–cyclen-PAGE methodology supported all the results of

genotyping by Zn^{2+}–Phos-tag PAGE on the SNP site of 6986A>G. We could observe the difference in the electrophoresis migration degree between two kinds of PCR products from the **1/*1* homozygote (2 individuals, subject numbers 15 and 17) and the **3/*3* homozygote (14 individuals, subject numbers 1, 3–7, 9–12, 14, 16, 18, and 19). This demonstrates high resolution of Zn^{2+}–cyclen PAGE, permitting the separation of two homoduplexes from each *CYP3A5*1* or *CYP3A5*3* allele. In addition, three additional differentially migrating bands representing the presence of heteroduplexes were confirmed clearly in 2 individuals (subject numbers 2 and 13) with the **1/*3* heterozygote and 1 individual (subject number 8) with the **1/*1* homozygote. Moreover, there was a difference in the electrophoresis migration pattern between two kinds of PCR products from the **1/*1* homozygote and the **1/*3* heterozygote. The appearance of multiple bands and a differential pattern in the individual with the **1/*1* homozygote (subject number 8) suggests the existence of another SNP.

DNA sequence analysis

To check the DNA sequence of all subjects, direct sequencing analyses were conducted using each of the PCR products prepared for the Zn^{2+}–cyclen PAGE analysis. As for the SNP site of 6986A>G, typical sequencing data are shown in Fig. 2B. All concerns of the homozygosity and heterozygosity were consistent with the genotyping results by Zn^{2+}–Phos-tag PAGE. The direct sequencing demonstrated that our genotyping assays for identification of *CYP3A5*1* or *CYP3A5*3* allele using the Zn^{2+}–Phos-tag-PAGE methodology were complete. As for another SNP site in one individual (subject number 8), a heterozygous nucleotide substitution of 6929G>A was newly discovered (see the sequencing data in Fig. 3 and the asterisked site in Fig. 1). The direct sequencing disclosed no other variation in the sequence of all subjects, indicating that all heterozygous substitutions in *CYP3A5* region tested were detected by our screening assays using the Zn^{2+}–cyclen-PAGE methodology. All subjects containing the PCR product showing additional/other bands due to the novel SNP were confirmed by direct sequencing analyses for the sense and anti-sense strands.

Discussion

In this article, we described a combination method of Zn^{2+}–Phos-tag PAGE and Zn^{2+}–cyclen PAGE for SNP genotyping and mapping. As the first practical example of using the combination method, we demonstrated reliable screening of polymorphisms containing the key SNP of 6986A>G responsible for *CYP3A5*3*. The Zn^{2+}–Phos-tag-PAGE method enabled a simple genotyping assay on the SNP site of 6986A>G without using expensive fluorescent primers or a special apparatus for real-time PCR, just by the mobility shifting of allele-specific PCR products. Successive Zn^{2+}–cyclen-PAGE method permitted not only detection of the *CYP3A5*3* genotypes as shown by the Zn^{2+}–Phos-tag-PAGE analysis, but also discovery of a novel heterozygous SNP (6929G>A) in the individual carrying the *CYP3A5*1* homozygote, followed by the standard and more robust/reliable DNA sequence analysis. The Zn^{2+}–cyclen-PAGE methodology also demonstrated high-resolution power distinguishable separately between the *CYP3A5*1* and *CYP3A5*3* homozygotes. A single DNA band on the Zn^{2+}–cyclen-PAGE gel indicates no mutation: the detection specificity of the screening would not be less than that (generally 70 to 90%) of other gel-based approaches such as a single-strand conformation polymorphism and a denaturing gradient gel electrophoresis [11]. Since both electrophoretic methods using the DNA-binding zinc(II) complexes require a general PCR apparatus and a mini-slab PAGE system, it may be a very convenient tool for clinical researchers and physicians to obtain the theoretical and practical information on CYP3A5 activity from a small number of patients. One hundred mini-slab gels for Zn^{2+}–Phos-tag-PAGE (20 μM Zn^{2+}–Phos-tag, 1-mm-thick, 9-cm-wide, 9-cm-long gels) can be prepared from 10 mg of the commercially available Phos-tag acrylamide AAL-107 (see Methods section). The cost of Phos-tag acrylamide used per one gel is about 6 US dollars. Regarding the Zn^{2+}–cyclen molecule, the cost for one electrophoresis run (in this case of 19 DNA fragments) is less than 1 US dollar [11]. In conclusion, we provided information regarding a new assay for allelic discrimination between the three different genotypes of the *CYP3A5* (6986A>G). The gel-based assay using DNA-binding zinc(II)

complexes was able to easily discriminate between all three genotypes in the population studied and even contributed to discover a novel SNP. Use of this strategy is thus worthy of consideration for an accurate analysis of SNP in a small-scale screening.

Acknowledgements

This work was supported by Grants-in-Aid for Scientific Research (B) (19390011) and (C) (19590040) from the Japan Society of the Promotion of Science, a Grant-in-Aid for Young Scientists (B) (20790036) from the Ministry of Education, Culture, Sports, Science, and Technology, and a research grant from the Ube Foundation (The Watanabe Memorial Award). We thank the Research Center for Molecular Medicine, Graduate School of Biomedical Sciences, Hiroshima University, Japan, for the use of its facilities.

References

[1] Shimada T, Yamazaki H, Mimura M, Inui Y, Guengerich FP. Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. J Pharmacol Exp Ther 1994;270:414–23.

[2] de Wildt SN, Kearns GL, Leeder JS, van der Anker JN. Cytochrome P450 3A: ontogeny and drug disposition. Clin Pharmacokinet 1999;37:485–505.

[3] Saeki M, Saito Y, Nakamura T, et al. Single nucleotide polymorphisms and haplotype frequencies of CYP3A5 in a Japanese population. Hum Mutat 2003;21:653.

[4] Park SY, Kang YS, Jeong MS, Yoon HK, Han KO. Frequencies of CYP3A5 genotypes and haplotypes in a Korean population. J Clin Pharm Ther 2008;33:61–5.

[5] Aoyama T, Yamano S, Waxman DJ, et al. Cytochrome P450 hPCN3, a novel cytochrome P-450IIIA gene product that is differentially expressed in adult human liver. J Biol Chem 1989;264:10388–95.

[6] Wrighton SA, Brian WR, Sari MA, et al. Studies on the expression and metabolic capabilities of human liver cytochrome P450IIIA5 (HLp3). Mol Pharmacol 1990;38:207–13.

[7] Tateishi T, Watanabe M, Moriya H, Yamaguchi S, Sato T, Kobayashi S. No ethnic difference between Caucasian and Japanese hepatic samples in the expression frequency of CYP3A5 and CYP3A7 proteins. Biochem Pharmacol 1999;57:935–9.

[8] Kuehl P, Zhang J, Lin Y, et al. Sequence diversity in CYP3A4 promoters and characterization of the genetic basis of polymorphic CYP3A5 expression. Nature Genet 2001;27:383–91.

[9] Lin YS, Dowling ALS, Quigley SD, et al. Co-regulation of CYP3A4 and CYP3A5 and contribution to hepatic and intestinal midazolam metabolism. Mol Pharmacol 2002;62:162–72.

[10] Kinoshita-Kikuta E, Kinoshita E, Koike T. A novel procedure for simple and efficient

genotyping of single nucleotide polymorphisms by using the Zn^{2+}–cyclen complex. Nucleic Acids Res 2002;30:e126.

[11] Kinoshita E, Kinoshita-Kikuta, E, Kojima H, Nakano Y, Chayama K, Koike T. Reliable and cost-effective screening of inherited heterozygosity by Zn^{2+}–cyclen polyacrylamide gel electrophoresis. Clin Chem 2005;51:2195–7.

[12] Nakano Y, Tashiro S, Kinoshita E, et al. Non-SCN5A related Brugada syndromes: verification of normal splicing and trafficking of SCN5A without exonic mutations. Ann Hum Genet 2007;71:8–17.

[13] Kinoshita E, Kinoshita-Kikuta E, Yoshimoto M, Koike T. Detection of the Gua/Cyt-to-Cyt/Gua mutation in a Gua/Cyt-lined sequence using Zn^{2+}–cyclen polyacrylamide gel electrophoresis. Anal Biochem 2008;380:122–7.

[14] Kinoshita-Kikuta, E, Kinoshita E, Koike T. Zn(II)–cyclen polyacrylamide gel electrophoresis for SNP detection. In: Komar AA, Ed. Single nucleotide polymorphisms: methods and protocols. 2nd ed. Totowa, NJ: Humana Press, 2009 (in press).

[15] Kinoshita E, Kinoshita-Kikuta, E, Koike T. A single nucleotide polymorphism genotyping method using phosphate-affinity polyacrylamide gel electrophoresis. Anal Biochem 2007;361:294–8.

[16] Kinoshita-Kikuta E, Kinoshita E, Koike T. A mobility shift detection method for DNA methylation analysis using phosphate-affinity polyacrylamide gel electrophoresis. Anal Biochem 2008;378:102–4.

[17] Kinoshita E, Kinoshita-Kikuta E, Koike T. Phosphate-affinity polyacrylamide gel electrophoresis for SNP genotyping. In: Komar AA, Ed. Single nucleotide polymorphisms: methods and protocols. 2nd ed. Totowa, NJ: Humana Press, 2009 (in press).

[18] Shionoya M, Kimura E, Shiro M. A new ternary zinc(II) complex with [12]aneN_4 (=1,4,7,10-tetraazacyclododecane) and AZT (= 3'-azido-3'-deoxythymidine). Highly selective recognition of thymine and its related nucleotides by a zinc(II) macrocyclic tetraamine complex with novel complementary associations. J Am Chem Soc

1993;115:6730–7.

[19] Kikuta E, Murata M, Katsube N, Koike T, Kimura E. Novel recognition of thymine base in double-stranded DNA by zinc(II)–macrocyclic tetraamine complexes appended with aromatic groups. J Am Chem Soc 1999;121:5426–36.

[20] Kinoshita E, Kinoshita-Kikuta E, Koike T. A heteroduplex-preferential T_m depressor for the specificity-enhanced DNA polymerase chain reactions. Anal Biochem 2005;337:154–60.

[21] Kinoshita E, Takahashi M, Takeda H, Shiro M, Koike T. Recognition of phosphate monoester dianion by an alkoxide-bridged dinuclear zinc(II) complex. Dalton Trans 2004;1189–93.

[22] Kinoshita E, Kinoshita-Kikuta E, Takiyama K, Koike T. Phosphate-binding tag, a new tool to visualize phosphorylated proteins. Mol Cell Proteomics 2006;5:749–57.

[23] Kinoshita-Kikuta E, Aoki Y, Kinoshita E, Koike T. Label-free kinase profiling using phosphate affinity polyacrylamide gel electrophoresis. Mol Cell Proteomics 2007;6:356–66.

[24] Kinoshita E, Kinoshita-Kikuta E, Matsubara M, et al. Separation of phosphoprotein isotypes having the same number of phosphate groups using phosphate-affinity SDS-PAGE. Proteomics 2008;8:20094–3003.

[25] Kinoshita E, Kinoshita-Kikuta E, Matsubara M, et al. Two-dimensional phosphate-affinity gel electrophoresis for the analysis of phosphoprotein isotypes. Electrophoresis 2009;30:550–9.

[26] Kinoshita E, Kinoshita-Kikuta E, Ujihara H, Koike T. Mobility shift detection of phosphorylation on large proteins using a Phos-tag SDS-PAGE gel strengthened with agarose. Proteomics 2009 (in press).

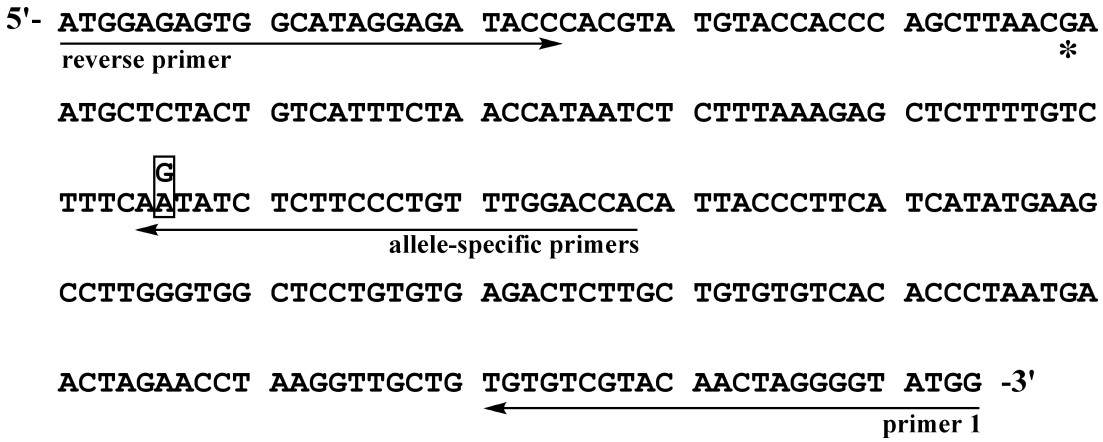
[27] Kinoshita E, Kinoshita-Kikuta E, Koike T. Separation and detection of large phosphoproteins using Phos-tag SDS-PAGE. Nat Protoc 2009 (in press).

Figure legends

Fig. 1. Partial DNA sequence of *CYP3A5* gene (a 244-bp sequence of 247062–247305 entered as GenBank Accession No. NG_000004.3) and the location of the PCR primers (arrows) used in this study (see Methods section for details). The squared nucleotides in the sequence of the allele-specific primers indicate the SNP site of 6986A>G responsible for the major phenotype of CYP3A5 activity [8]. The asterisked nucleotide shows a novel SNP site discovered in this study.

Fig. 2. Genetic analysis using Zn^{2+}–Phos-tag PAGE (A) and Zn^{2+}–cyclen PAGE (B) followed by DNA sequence analysis. The allele-specific PCR products (128 bp) amplified from 19 healthy individuals were applied to a Zn^{2+}–Phos-tag-PAGE gel (20 μM polyacrylamide-bound Zn^{2+}–Phos-tag and 18% (w/v) polyacrylamide) (A; electrophoresis data). The allele genotype (**1/*3* heterozygote, **1/*1* homozygote, and **3/*3* homozygote) and subject number of each individual are shown above each lane. The *CYP3A5*1* and *CYP3A5*3* alleles are arrowed on the left side of the PAGE gel. Typical direct sequencing data around the SNP site of 6986A>G from the anti-sense strand using primer 1 (see Fig. 1) for 5 individuals (subject numbers of 2, 8, and 13–15) are shown below the electrophoresis data. The PCR products (244 bp) amplified from 19 healthy individuals were applied to a 15% (w/v) polyacrylamide gel containing 5.0 mM Zn^{2+}–cyclen (B; electrophoresis data). The subject number of each individual is shown above each lane. Typical direct sequencing data around a novel SNP site of 6929G>A using the primer 1 for subject number of 8 is shown on the right-hand side of the electrophoresis data.

Fig. 1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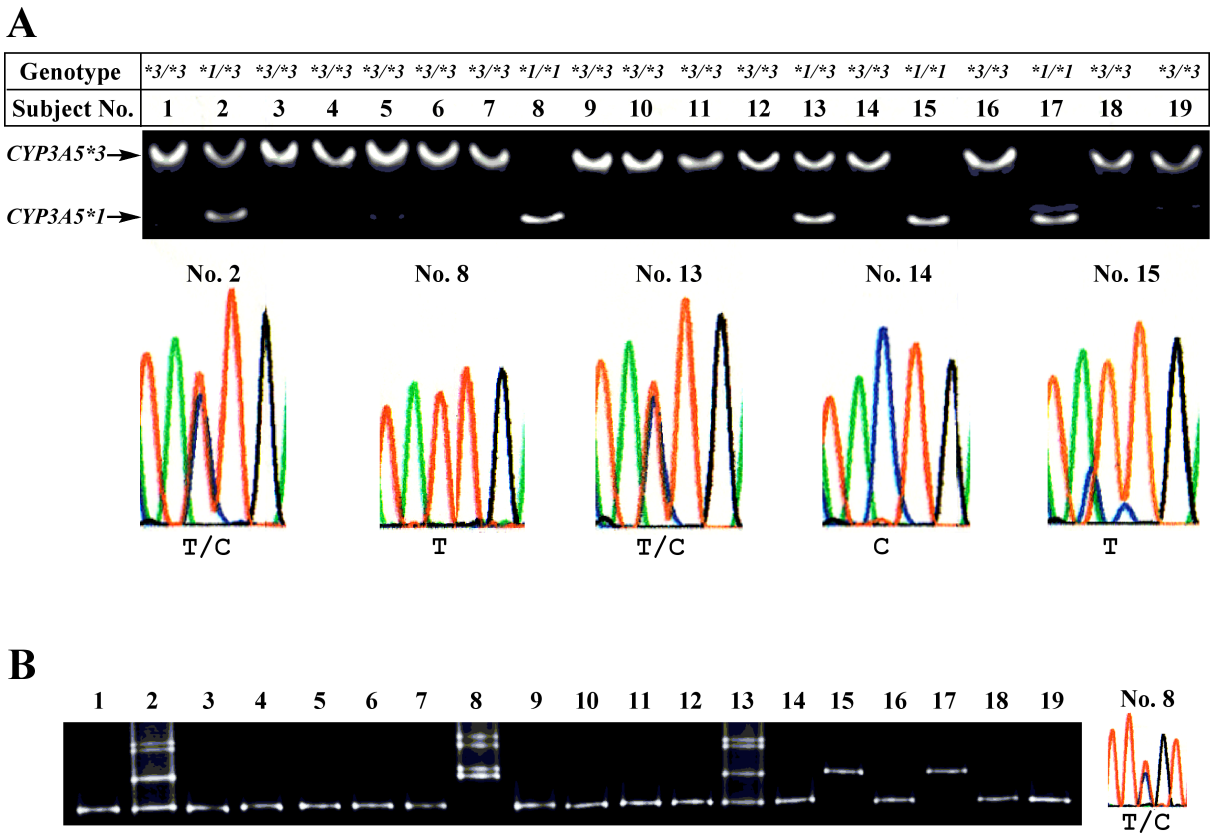
reverse primer
*
ATGCTCTACT GTCATTTCTA ACCATAATCT CTTTAAAGAG CTCTTTTGTC
G
TTTCAATATC TCTTCCCTGT TTGGACCACA TTACCCTTCA TCATATGAAG
allele-specific primers
CCTTGGGTGG CTCCTGTGTG AGACTCTTGC TGTGTGTCAC ACCCTAATGA
ACTAGAACCT AAGGTTGCTG TGTGTCGTAC AACTAGGGGT ATGG -3'
primer 1

Fig. 2


A
Genotype
*3/*3 *1/*3 *3/*3 *3/*3 *3/*3 *3/*3 *3/*3 *1/*1 *3/*3 *3/*3 *3/*3 *3/*3 *1/*3 *3/*3 *1/*1 *3/*3 *1/*1 *3/*3 *3/*3
Subject No.
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
CYP3A5*3
CYP3A5*1
No. 2
No. 8
No. 13
No. 14
No. 15
T/C
T
T/C
C
T
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
No. 8
T/C