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Time-saving multiplex detection of single nucleotide polymorphisms by ultrasensitive DNA microarray

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Rapid and multiplex detection system using an ultrasensitive DNA microarray was developed and utilized for the analysis of six pharmacokinetically relevant single nucleotide polymorphisms (SNPs) (*MDR1*-C1236T, *MDR1*-G2677TA, *MDR1*-C3435T, *CYP3A5*-A6986G, *CYP2C19*-G681A, *CYP2C19*-G636A) from blood samples derived from liver transplant patients. The SNP detection system is comprised of three processes: multiplex PCR, single base extension with fluorescently labelled di-deoxy-nucleotides and detection by DNA microarray. The entire workflow of this system completes within 5 h. The final genotype call was obtained statistically by Mahalanobis distance which was calculated from the bi-coloured fluorescent signals detected by the microarray. In order to detect the six SNPs, this system required only 50 copies of genomic DNA, and the obtained detection calls completely matched with the results by the sequencing-based genotyping method. With the high sensitivity and rapid processing, our SNP detection system utilizing ultrasensitive microarray is a promising device applicable for diagnostic utility.

Keywords: diagnostic tool/microarray/multiplex/SNP/transplantation.

Abbreviations: CYP, cytochrome P450; ddNTP, di-deoxy NTP; MDR, multidrug resistance; PMT, Photo Multiplier Tube; POCT, point of care testing; PPI, proton pomp inhibitor; SBE, single base extention; SNP, single nucleotide polymorphism.

Our knowledge of the human genome has increased rapidly in recent decades due to the completion of the Human Genome Project and other large-scale endeavours, and these discoveries are now applied for personalized medicine and genome-based drug discovery.

Calcineurin inhibitor tacrolimus (FK-506) has been used as a primary immuno- suppressive agent in liver transplantation (*1*). Proton pump inhibitors (PPIs) are often co-administrated with tacrolimus in patients receiving allograft transplantation to avoid surgical stress-related gastric bleeding or gastrointestinal ulceration. However, this co-administration sometimes adversely affects the concentration/dose (C/D) ratio of tacrolimus, which leads to side effects such as nephrotoxicity, neurotoxicity and life-threatening infection (*2*, *3*). It is known that the pharmacokinetics of tacrolimus attributes to individual genomic variation (*4*–*6*). As previously reported, PPIs are primarily metabolized by the cytochrome P450 subfamilies CYP2C19 and CYP3A4 (*7*, *8*). Tacrolimus is metabolized by CYP3A4 and CYP3A5 and pumped out of the cell membrane by multi-drug resistance 1 protein (MDR1/ABCB1) (Fig. 1) (*9*–*11*). Because CYP3A4 on the metabolic pathway is shared by both PPIs and tacrolimus, the competitive metabolism is influenced by the molecule activities on the collateral metabolic pathway, *CYP3A5*, *CYP2C19* and *MDR1* (Fig. 1). In clinical practice, the rapid and multiplex genotyping system is required in case such as the administration of tacrolimus.

Several technologies have been developed for the SNP detection system. The most accurate and commonly used SNP detection system is a direct sequencing of target gene regions; however, a sequencing technology needs long operation time over one working day and is not suitable for multiplex genotyping. Other systems are also commercially available such as SNPstream® by Beckman Coulter (*12*) and GeneChip® by Affimetrix (*13*), which are suitable for highly multiplexed analysis. On the other hand, we have previously developed an ultrasensitive DNA microarray based on three unique technologies: a black plastic substrate, a columnar array structure and a micro-bead agitation system. Due to these properties, the signal/noise ratio was markedly improved, achieving 100-fold higher sensitivity than that of conventional glass-supported microarrays (*14*).

In the present study, we focused on developing a new SNP detection assay in a compact system, more suitable for a clinical setting for personalized pharmacotherapy by the compact system. We developed multiplex and rapid genotyping system using an ultrasensitive DNA microarray with the SBE method for the analysis of six pharmacokinetically relevant SNPs (*MDR1*-C1236T, *MDR1*-G2677TA,

MDR1-C3435T, *CYP3A5*-A6986G, *CYP2C19*-G681A and *CYP2C19*-G636A). The entire workflow of this system completes within 5 h and specific with only 50 copies of genome. Using this system, we analyzed genomic DNA isolated from the blood of 83 liver transplant patients. The genotyping results were validated with the sequencing-based genotyping method.

Materials and Methods

Patients and DNA extraction

A total of 83 Japanese living-donor liver transplant patients, including donors and recipients were enrolled in this study (*6*). Prior to use, all DNA samples were anonymized using unlinked numbers. Written informed consent was obtained from all study participants prior to enrolment. This study was conducted in accordance with the Declaration of Helsinki and its amendments, and was approved by the Kyoto University Graduate School of Medicine Ethics Committee, as well as Human Genome Ethics Committee for R&D, Toray Industries, Inc.

Human genomic DNA was extracted from whole blood using a Wizard Genomic DNA Purification Kit (Promega Co., Madison, WI, USA), and the extracted DNA was dissolved in distilled water and stored at −30°C. The genomic DNA of cultured cells was extracted from HEK 293 cells using the QIAamp DNA Mini Kit (QIAGEN, Germantown, MD, USA), and the extracted DNA was dissolved in distilled water and stored at −30°C.

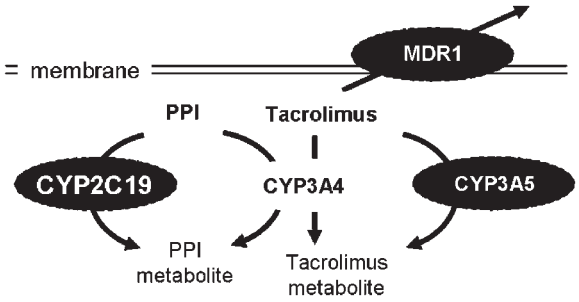

Fig. 1 Transport and metabolic pathway of Tacrolimus and PPI.

Oligonucleotides

Multiplex PCR primers were synthesized for the amplification of six regions including six SNPs. SBE primers designed to have each unique tag sequence were synthesized (Table I). Additionally, two pairs of control template (Control 1, 2) and SBE primers (Control 1SBE, 2SBE) were designed for the standardization of Cyanine (Cy) 3 and Cy5 fluorescence intensity, and another two pairs of control template (Control 3G, 4C) and SBE primers (Control 3SBE, 4SBE) were designed to monitor for dNTP contamination in SBE reaction (Table II). To hybridize SBE primers onto the DNA microarray, eleven unique tag-probes of 20 mer nucleotides, which were complementary to the SBE primer's tag sequence, were also synthesized. The 3' end of each tag-probe was aminated. All nucleotides were synthesized by Operon Biotechnologies (Tokyo, Japan).

Multiplex PCR and SBE reaction

Six regions of genomic DNA were simultaneously amplified by multiplex PCR, which include three SNPs of *MDR1* (C1236T, G2677TA and C3435T), one SNP of *CYP3A5* (A6986G) and two SNPs of *CYP2C19* (G681A and G636A). The amplification was performed in a reaction solution that consisted of 0.2 mM each deoxy-nucleotide (dNTP), 1.5 mM magnesium sulphate, 0.3 μM forward and reverse primers, 1.0 U KOD plus DNA polymerase (Toyobo Co. Ltd, Tokyo, Japan) and 10 pg to 100 ng of genomic DNA, adjusted to 50 μL with KOD-Plus-Vers.2 buffer. These mixtures were applied to a thermal cycler PCR system (ABI9700, Applied Biosystems, Foster City, CA, USA) and denatured at 94°C for 2 min followed by 30 PCR cycles (94°C for 30 s, 58°C for 30 s and 68°C for 1 min). Following the PCR reaction, free dNTPs were removed from the PCR products using GFX PCR DNA and a Gel Band Purification Kit (GE Healthcare UK Ltd, UK). The size of the amplicons was measured with a 2100 bioanalyzer system (Agilent Technologies, Inc., USA).

The SBE reaction was performed in 20 μL of reaction mixture containing 15 nM of each SBE primer, except in the case of control reactions, which contained Control 1SBE primer (7.5 nM) and control 3SBE primer (3.75 nM): 5 μL of reaction buffer, 2 μL of purified multiplex PCR product; 2.5 ng of each control template (except Control 1A (7.5 ng) and Control 1G (5.0 ng)); 500 nM each of Cy 5 labelled di-deoxy-UTP (ddUTP), Cy3-ddCTP, Cy5-ddGTP and Cy3-ddATP (PerkinElmer Inc., Boston, MA, USA); 500 nM of di-deoxy-nucleotides (ddNTPs); and 5 units of ThermoSequenase DNA Polymerase (GE Healthcare Life Science, USA). The assay was conducted in two separate tubes. The first tube, which was used for the detection of A or G SNP, contained purified multiplex PCR amplicons, SBE primers (MDR1-1236, MDR1-2677-1, MDR1-3435, CYP2C19-636, Control 1SBE primer and Control

Table I. Sequences of optimized primers for multiplex PCR amplification and for single-base extension assay at each SNP position.

SNP position	Primers	Sequences
MDR1-1236C/T	Forward	5′-AACAGTCAGTTCCTATATCCTGTGTC-3′
	Reverse	5′-CAGCTGGACTGTTGTGCTCTTCCC-3′
	SBE	5′-ACTCGTCCTGGTAGATCTTGAAGGG-3′
MDR1-2677G/T/A	Forward	5′-CCCATCATTGCAATAGCAGGAGT-3′
	Reverse	5′-GAGCATAGTAAGCAGTAGGGAG-3′
	SBE 1	5′-ATCATATTTAGTTTGACTCACCTTCCCAG-3′
	SBE 2	5′-ATCATATTTAGTTTGACTCACCTTCCCAG-3′
MDR1-3435C/T	Forward	5′-ACTGCAGCATTGCTGAGAACATTGCCT-3′
	Reverse	5′-CATTAGGCAGTGACTCGATGAAGGCAT-3′
	SBE	5′-AGCCGGGTGGTGTCACAGGAAGAGAT-3′
CYP3A5-6986A/G	Forward	5′-TGGCATAGGAGATACCCAC-3′
	Reverse	5′-TTCATATGATGAAGGGTAATGTG-3′
	SBE	5′-CTCTTTAAAGAGCTCTTTTGTCTTTCA-3′
CYP2C19-681G/A	Forward	5′-TGCAATAATTTTCCCACTATCATT-3′
	Reverse	5′-TACGCAAGCAGTCACATAACTAAG-3′
	SBE	5′-ATAATTTTCCCACTATCATTGATTATTTCCC-3′
CYP2C19-636G/A	Forward	5′-GAAAAATTGAATGAAAACATCAGG-3′
	Reverse	5′-TGCCATCTTTTCCAGATATTCACC-3′
	SBE	5′-GGAAGCAAAAAACTTGGCCTTACCTGGAT-3′

Forward: Forward primer for PCR amplification, Reverse: Reverse Primer for PCR amplification. SBE: Primer for single-base extension. The 5′ end of each primer was attached to an original tag sequence (not shown).

Table II. Sequence of control templates and control primers for single base extension assay.

Name	Sequences
Control 1	5′-TCTGCAGGTCAGGGGGGCAAGAAGGGGTAACCCAGGTGTCACTGAAGATTGTA"X" AGATTTGGGGAATGTTGCAGTATTTGAAAGTGCTCCACCAAAGCT-3′ X: A or G
Control 2	5′-GTAAGTGGAGCCTGATTTCCCTAAGGACTTCTGGTTTGCTCTT"X"AAGAAAGC TGTGCCCCAGAACACCAGAGACCTCAAATTACTTTACAAATAGAA-3′ X: T or C
Control 3G	5′-TACCATTTTAAAGGCTATCATTACTCTTTACCTGTGAAGAG"G"AGAACATG AAGAAATCTACTTTATTCAGATATTCTCCAGATTCCTAAAGATTAGAGAT-3′
Control 4C	5′-GTATAGCTCTGTGAAACCATTTGCAAATTTTTTGAATAGG"C"TAAGTTATAG CAGATACAACAGGTTGGGCACTGGATATATAAAGATAAACAAAATATAG-3′
Control 1 SBE	5′-TCAAATACTGCAACATTCCCCAAATCT-3′
Control 2 SBE	5′-TGGTGTTCTGGGGCACAGCTTTCTT-3′
Control 3 SBE	5′-TATTAATAAAGTAGATTTCTTCATGTTCT-3'
Control 4 SBE	5′-AACCTGTTGTATCTGCTATAACTTA-3′

Control: control SNPs template. "X" is the detection position of each SBE primer. SBE: Primer for single-base extention. The 5′ end of each primer is attached to an original tag sequence (not shown).

3SBE primer), control templates (1A, 1G and 3G), Cy3-ddCTP, Cy5-ddUTP, ddGTP, ddATP, reaction buffer and thermo sequenase polymerase. The second tube, which was used for the detection of T or C SNP, contained purified multiplex PCR amplicons, SBE primers (MDR1-2677-2, CYP3A5-6986, CYP2C19-681, Control 2SBE primer and Control 4SBE primer), control templates (2C, 2T and 4C), Cy3-ddATP, Cy5-ddGTP, ddCTP, ddUTP, reaction buffer and thermo sequenase polymerase. Heat denaturation at 95°C for 2 min was followed by 30 cycles of PCR reaction (95°C for 20 s and 60°C for 15 s).

The specificity of the SBE reaction was confirmed by the single signal intensity of Control 3SBE primer (Cy3) or Control 4SBE primer (Cy5). Control 1SBE primer with Cy5-ddUTP, Control 1SBE primer with Cy3-ddCTP, Control 2SBE primer with Cy5-ddGTP, and Control 2SBE primer with Cy3-ddATP were used to standardize the signal intensities of each nucleotide. These controls calibrated the enzyme reaction efficiency and the scanner's PMT gain.

Determination of SNPs with the microarray

The DNA microarray platform for 256 spots was provided by Toray Industries, Inc. Eleven unique oligonucleotide sequences that were not complementary to the human genome were selected as tag probes to capture the SNPs specific tagged detection primer. These tagged probes were covalently immobilized to the surface of the microarray as previously reported (*6*). 5 μL of each SBE reaction tubes was mixed with 30 μL of 3D-Gene™ hybridization buffer (Toray Industries, Inc., Tokyo, Japan) and was applied onto the DNA microarray. Then, the DNA microarray was placed in a hybridization chamber (TaKaRa Hybridization Chamber 5 No.TX711, TaKaRa Bio, Japan), and rotated horizontally at 42°C for 1.5 h at 250 rpm. After hybridization, the microarrays were washed and dried. The hybridized microarrays were scanned on a microarray scanner (ScanArray Express, PerkinElmer, Inc., USA) to measure fluorescence intensity. At a laser power setting of 60%, the photomultiplier sensitivity was adjusted so that the fluorescence signal intensities from the two fluorophores, Cy3 and Cy5, at the gain control spots on the microarray were almost comparable.

The ratio of Cy3 and Cy5 signal intensity (Cy3/Cy5, R) was log translated and used as a calibration factor (Log_2(R)) to standardize the signal intensities of each SNP. We used two reaction tubes and two sets of control template and SBE primers. For reaction tube 1, R was calculated as $R_1 = (Scy3/SCon1cy3)/(Scy5/SCon1cy5)$ where Scy3 and SCon1cy3 are the Cy3 signal intensity of the sample and of Control 1, and Scy5 and SCon1cy5 are the Cy5 signal intensity of the sample and of Control 1, respectively. The Cy3 signal intensity of Control 1SBE primer with Cy3-ddCTP was used for the calibration of the Cy3 signal intensity of MDR1-1236, MDR1-2677-1, MDR1-3435 and CYP2C19-636, and the Cy5 of Control 1SBE primer with Cy5-ddUTP was used for the calibration of the Cy5 signal intensity of MDR1-1236, MDR1-2677, MDR1-3435 and CYP2C19-636, respectively.

For reaction tube 2, R was calculated as $R_2 = (Scy3/SCon2cy3)/(Scy5/SCon2cy5)$ where Scy3 and SCon2cy3 are the Cy3 signal intensity of the sample and of Control 2, and Scy5 and SCon2cy5 are the Cy5 signal intensity of the sample and of Control 2, respectively. The Cy3 signal intensity of the Control 2_SBE primer with Cy3-ddATP was used for the calibration of the Cy3 signal intensity of MDR1-2677-2, CYP3A5-6986, and CYP2C19-681, and the Cy5 signal of Control 2SBE primer with Cy5-ddGTP was used for the calibration of the Cy5 signal intensity of MDR1-2677-2, CYP3A5-6986, and CYP2C19-681, respectively. The whole procedure, consisting of genome extraction, multiplexed PCR, SBE and detection by DNA microarray, is illustrated in Fig. 2.

Classification

As reported previously (*15*), genotype calls were made according to their Mahalanobis distance (D). All of $\log_2(R)$ values obtained from the training group (63 patients) were plotted and were separated into clusters (Fig. 3). The clusters were statistically validated by Dunnett test as well-defined clusters. The group centre (m) and the dispersion parameter (s) of each cluster were calculated. D was calculated using the $\log_2(R)$ value of test sample (x), as follows: $D^2 = (x - m)^2/s^2$. The genotypes of the samples were assigned to the cluster that provided the minimum D.

Direct sequencing using DNA sequencer

The nucleotide sequences of the PCR products were also measured by direct DNA sequencing using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). The PCR conditions outlined above were used for the amplification of SNP-containing DNA fragments in an ABI 2720 thermal cycler (Applied Biosystems), before being sequenced using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Results

Multiplex PCR condition was examined using the genomic DNA of HEK293 cells, which provide six DNA fragments including pharmacokinetically relevant SNPs (*MDR1*-C1236T, *MDR1*-G2677TA, *MDR1*-C3435T, *CYP3A5*-A6986G, *CYP2C19*-G681A and *CYP2C19*-G636A). The multiplex PCR conditions and primer sequences were optimized to obtain a similar amplification yield of each amplicon and to distinguish molecular length of each amplicon, which ranged from 120 to 218 bp in length (Table I) by electrophoretic separation.

For the multiplex SBE reaction, several combinations of detection primer as well as labelled ddNTPs were tested to obtain the optimal conditions for

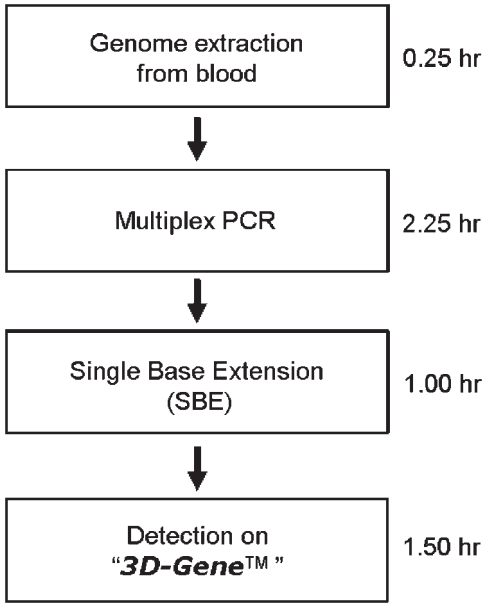

Fig. 2 Workflow and time course of the multiplex SNP detection by SBE reaction and 3D-Gene™ DNA microarray substrate.

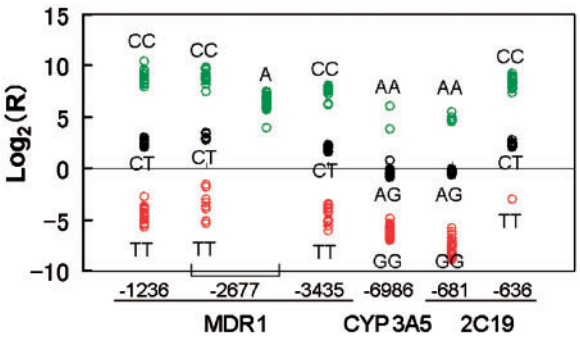

Fig. 3 Clusters based on $\log_2$(R) values. Each SNP in a multiplex assay results in three clusters. The plot shows 476 data points from 68 patient genomes. The green circles represent CC(*MDR1*-1236, -2677 and -3435, *CYP2C19*-636) or AA(*MDR1*-2677, *CYP3A5*-6986 and *CYP2C19*-681) homozygous genotype, and the red circles represent TT(*MDR1*-1236, -2677 and -3435, *CYP2C19*-636) or GG(*CYP3A5*-6986 and *CYP2C19*-681) homozygous genotype. The black circles represent the heterozygous genotype CT(*MDR1*-1236, -2677 and -3435, *CYP2C19*-636) or GA(*CYP3A5*-6986 and *CYP2C19*-681).

distinguishing the six different SNPs. As a result, seven primers were selected and are presented as Table I.

The total handling time from collection of blood samples to analysis all six SNPs was minimized to 5 h, which includes 0.25 h for extraction, 2.25 h for multiplex PCR and 1 h for SBE reaction, followed by 1.5 h hybridization (Fig. 2).

For the standardization of fluorescence intensities, two pairs of control templates (Control 1 and 2) and two SBE primers (control 1SBE and 2SBE) were used (Table II). Each pair of control templates was identical in sequence except one nucleotide which is the artificial SNP. Following the SBE reaction and DNA microarray hybridization, the Cy3 and Cy5 fluorescence intensities of each artificial SNP standardized that of each sample SNP, as described under 'Determination of SNPs with microarray' in the 'Materials and Methods' section.

To validate whether SBE reactions were contaminated with free dNTPs, the control template (Control 3G or 4C) and a SBE primer (control 3SBE or 4SBE) were used. For example, if Cy5 fluorescence was detected when Control 3G, control 3SBE primer, Cy3-ddCTP and Cy5-ddUTP were assayed in a single tube, the SBE reaction was determined to be erroneous.

The genomic DNA of 63 Japanese living-donor liver transplant pairs, including donors and recipients, were used to investigate genomic polymorphisms in *CYP3A5*, *CYP2C19* and *MDR1*. All of the 63 genomic DNA samples were amplified by multiplex PCR and were detected for all six SNPs by our detection system.

These 63 samples were used as training data to construct a cluster map. As shown in Fig. 3, the log transformed signal intensity ratio ($\log_2(R)$) of each sample was clearly separated into three clusters. These clusters corresponded to the genotypes (Fig. 3). In the case of *MDR1*-2677, four clusters were separated, because three types of SNPs——C, T and A might be available. The SBE primer MDR1-2677-1 discriminates CC or CT or TT, and MDR1-2677-2 discriminates AA or no signal. If MDR1-2677-1 detects TT and MDR1-2677-2 detects AA, the genotype would be TA.

Twenty additional genomic DNA samples were measured as a test group to confirm the accuracy of our SNP detection system. The 20 samples were measured by the SNP detection microarray and each Log_2(R) value and its Mahalanobis distance were calculated. These genotyping results were also measured by a capillary electrophoresis sequencer (ABI PRISM 3100), using the same PCR amplicons for 120 SNPs (six SNPs in 20 samples). Due to indistinct peaks in chromatogram by the direct sequencing (Fig. 4B), SNPs were detected in only 97 runs (81%) (Fig. 5). Failure of genotyping by direct sequencing may have been attributable to difficulties in multiplex PCR, as the amplicon yields were lower in the failed samples than those of successfully determined samples. These 23 problematic SNPs were identified by re-sequencing with PCR products amplified separately for each SNP. Conversely, the SNP detection microarray provided clear clusters and 119 data points (99%) were perfectly in accordance with sequencing results (Fig. 5). The one failed call for *MDR1*-3435 (sample ID:44) was identified following a fresh genomic DNA extraction.

To ascertain the sensitivity of our SNP detection system, 2 genomic DNA samples were randomly selected from samples of known genotype. The 2 samples of 1 μg each were subjected to 10-fold serial dilution and their genetic polymorphisms were measured at 100, 10, 1 ng and 100 pg amounts of genomic DNA. All six SNPs were detected from the samples containing only 100 pg of genomic DNA, which corresponds to approximately 50 genomic copies (Table III). Direct sequencing failed to genotype samples containing 100 pg of genomic DNA because of weak fluorescence signal intensity.

Discussion

In the clinical setting, the detection system for SNPs should be quick, precise and multiplex. We developed a multiplex genotype detection system based upon the ultrasensitive DNA microarray technology, and evaluated its performance using six SNPs relating to drug metabolism.

We first optimized the reaction conditions of multiple SNP analysis for both amplification and SBE, using genomic DNA derived from HEK293 cells. As a result, we identified an optimal combination of PCR primer sequences and SBE primer sequences as well as labelled ddNTPs. When a SNP could not be detected with the sense strand sequence, the anti-sense strand sequence was used for the SBE reaction, and this process was effective at distinguishing all six SNPs analysed. After optimizing these conditions, six SNPs were measured in 63 clinical samples by DNA microarrays. The genotyping result was validated with the sequencing-based genotyping method. We regarded these 63 clinical samples results as a training set. For the genotypic discrimination, we used the training set and adopted Mahalanobis distance for statistical calling of the genotypes. The genotype of new sample was assigned to the group by the shortest Mahalanobis distance. We evaluated our detection system using six SNPs of new 20 samples. The concordance rate of genotyping 120 SNPs by our detection system was 99%. Although one sample failed the detection of a SNP in *MDR1*-3435, re-purification of genomic DNA resulted in the correct detection of the SNPs in this sample, suggesting DNA degradation as a cause of incorrect genotyping. Over the course of this study, some genomic DNA samples were stored for longer than one year. In a clinical diagnostic setting, most of the blood sample would be treated within a few days of sampling and the sample quality should be ensured by the appropriate handling. Therefore, degradation of genomic DNA would not likely occur in the clinical setting.

In this study, we focused on developing a new SNP detection assay in a compact system, more suitable for a clinical setting for personalized pharmacotherapy by the compact system. As a primary step to develop an automated bed-side SNP-detection system constructed with the DNA microarray and the preprocessing lab-on-a-chip, we tried to find the minimum amount of DNA required for SNP detection by our system. In this study, the six SNPs were detected using only 100 pg of genomic DNA, which corresponds to 50 copies of genomic DNA obtained from 0.1 µL of blood. The required quantity of genomic DNA for this analysis is even less than SNPstream® (1~2 ng) and Infinium® HD BeadChip (200~400 ng).

Employing the highly sensitive DNA microarray substrate also enabled significant timesaving in SNP

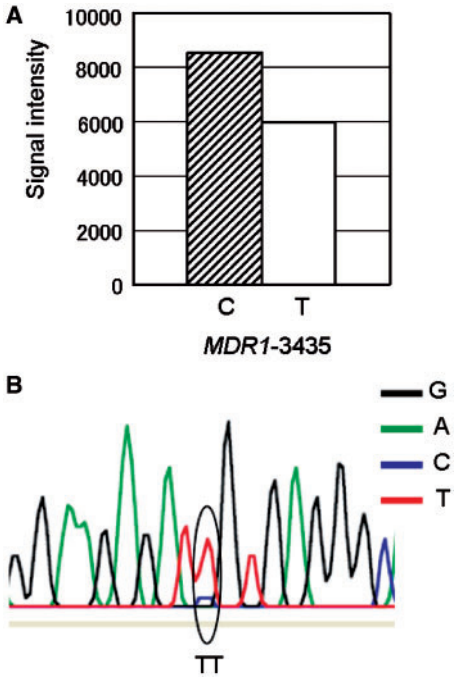

Fig. 4 A typical mismatched result of genotyping results obtained by DNA microarray and direct sequencing (sample ID:31, *MDR1*-3435). (A) Signal intensity of DNA microarray. The signal for Cy3 (striped bar-Cystein) shows 'C'. and Cy5 (open bar-Thymine) shows 'T', which show that this site is CT. (B) Electropherogram of the direct sequencing. The automated sequencing call was homozygotes TT.

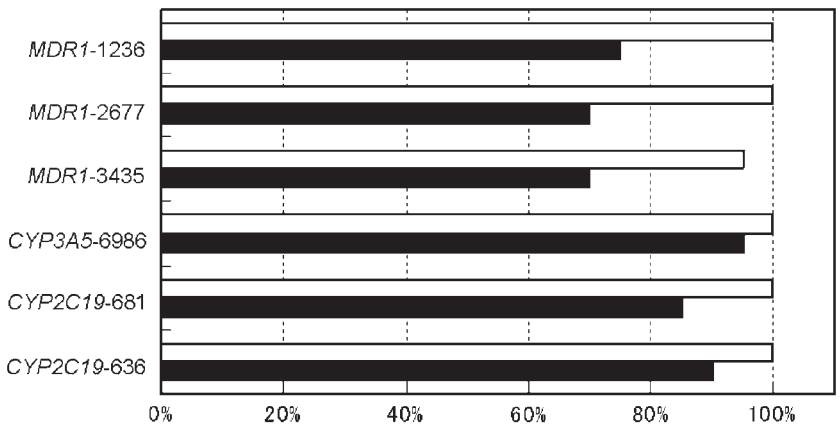

Fig. 5 Genotyping results by DNA microarray (open bar) or sequencing (closed bar) of the same amplicons amplified from 20 patients genome. The DNA microarray correctly distinguishes 100% of genotypes except for one SNP in *MDR1*-3435. The concordance rates by Sequencing are 70~95%, depending on the SNPs.

Table III. Representative results of the clusterization.

SNP position	$Log_2(R)$	Calculated by Mahalanobis distance			Judge
MDR1-1236	2.7	CC:79.0	CT:0.4	TT:132.4	CT
MDR1-2677-1	3.5	CC:69.1	CT:0.5	TT:41.4	CT
MDR1-3435	2.6	CC:64.1	CT:13.6	TT:62.4	CT
CYP3A5-6986	−0.7	AA:12.3	AG:0.0	GG:34.9	AG
CYP2C19-681	−10.8	AA:2637.6	AG:5073.0	GG:15.4	GG
CYP2C19-636	2.6	CC:314.0	CT:1.9	NC	CT

Genotyping judgements of six SNPs calculated by Mahalanobis distance. The $log_2(R)$ was calculated as shown in the 'Materials and methods' section. For the 'judge' calls, each SNP was assigned to the cluster with the smallest Mahalanobis distance. NC: No samples classified to the group.

detection. The hybridization reaction with the microarray is completed within 1.5 h, resulting in a total detection time of 5 h (Fig. 2). The short handling time is particularly suitable to use at the bedside. The substrate of this DNA microarray has been introduced by Nagino *et al.* (*14*) for the detection of the gene expression profiling. Nagino *et al.* showed that it takes 16 h for the hybridization of labelled cDNA to microarray probes. On the other hand, in our study it takes only 1.5 h for the hybridization. We suggest that one of the reasons for shortening of the hybridization is the difference of the length of labelled target nucleotides. The length of labelled cDNA used in Nagino's trial might range from several hundreds to several thousands mer; however, the tag-probe used in this study include polynucleotides of only 20 mer.

The genotypes of the *CYP3A5*, *CYP2C19* and *MDR1* genes were found to affect the pharmacokinetics of tacrolimus after liver transplantation (*4–6*, *9–11*). As the genotypes between the graft liver (donor) and native intestine (recipients) may be very different, it is necessary to detect the SNPs in DNA samples from both donor and recipients. As in the case of co-administration of tacrolimus and other drugs, risk evaluation by the point of care testing (POCT) is needed to prevent unexpected side-effects. Our SNP detection system which is sensitive and timesaving is suitable to the POCT.

On the other hand, SNPs related to various drug metabolism events are not limited to those shown in this study. The advantage of the SBE system on DNA microarray is its flexibility. By designing the sequence of PCR primers and SBE tagged primers, the number of detectable SNPs can be easily expanded. The system evaluated in this study could be increased to 256 tags for a sample.

In conclusion, the newly developed ultrasensitive DNA microarray achieved high sensitivity and accuracy for multiplex SNP genotyping. For further study, we are developing a lab-on-a-chip that enables automatic DNA isolation, labelling and hybridization to shorten the measurement time to within 3 h, avoid human-error and make it available at the clinical bedside.

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Conflict of interest

M.I., K.M., T.Y., I.N. and S.T. are employees of Toray Industries, Inc. Other authors have declared no conflict of interest.

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