Rapid and sensitive detection of UGT1A1 polymorphisms associated with irinotecan toxicity by a novel DNA microarray

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Recent progress in human genome analysis has paved the way for a new approach in disease treatment called precision or personalized medicine, which is tailored to the patient’s distinction. Genotyping methods that assist precision medicine by determining the direction of treatment are selected on the basis of rapid availability, accuracy, and low cost.

Presently, irinotecan treatment dosages are decided based on the presence of UGT1A1 polymorphisms. Irinotecan, a camptothecin derivative, is approved for the treatment of metastatic colorectal and other cancers. Carboxylesterases catalyzed irinotecan to 7-ethyl-10-hydroxycamptothecin (SN-38), which is a potent topoisomerase I inhibitor leading to cell death. SN-38 is then further catalyzed by hepatic uridine 5’-diphospho-glucuronosyltransferase (UGT) 1A (UGT1A) enzymes to form the inactive compound SN-38 glucuronide (SN-38G). In Japan, genotyping by the Invader assay has been approved for in vitro detection of two UGT1A1 polymorphisms, UGT1A1*28 and UGT1A1*6, known to be significantly associated with severe irinotecan toxicity, resulting from irinotecan-based chemotherapy for several carcinomas.

The polymorphism UGT1A1*28 contains an additional TA repeat in the UGT1A1 promoter region, giving seven rather than six TA repeats, while UGT1A1*6 has a G to A substitution at position +211 relative to the UGT1A1 translation start site, which results in impaired irinotecan metabolism. The relative frequency of UGT1A variants varies between Caucasian and Asian populations, and UGT1A1*6 is reportedly strongly associated with severe neutropenia in Asian patients in particular.

In this study, using a DNA array technique, we accurately and simultaneously detected both the 2-bp repeated sequence insertion and single nucleotide polymorphism (SNP) in UGT1A.
of 0.000–0.613, 0.916–1.340, and 1.472–2.000 were designated (TA)0/(TA)n, (TA)0/(TA)7, and (TA)7/(TA)7, respectively. Similarly, values of 0.000–0.332, 0.629–1.051, and 1.865–2.000 were designated homozygous of wild-type (G/G), heterozygous (G/A), and homozygous of UGT1A1*6 (A/A), respectively. If the FI from both probes were more than twofold lower than the BG, the genotype was not determined.

**DNA microarray for the detection of seven polymorphisms at the UGT1A locus.** We developed an additional DNA microarray capable of simultaneously detecting UGT1A polymorphisms at seven loci: UGT1A1*6 (211G > A, rs4148323), UGT1A1*27 (686C > A, rs3530960), UGT1A1*28 (T>A, rs8175347), UGT1A1*60 (–3279T > G, rs4124874), UGT1A7 (–57T > G, rs7586110), UGT1A7 (387T > G, rs178632), and UGT1A9*1b (–118T > T, rs3832043), also called UGT1A9*22 (26,29,30). For this, seven sets of primers and probes were used (Table 2). In the first PCR reaction, genomic DNA was amplified with Cy5-labeled dCTP (GE Healthcare, Tokyo, Japan). Multiplex PCR was performed in a 20 μL volume with 0.5 U FastStart Taq DNA polymerase (Roche diagnostics) and 10 ng DNA, using the following cycle procedure: 35 cycles of denaturation at 95°C for 30 s, 95°C for 30 s, annealing at 64°C for 30 s, and elongation at 72°C for 30 s were performed. Second, IC5-labeled DNA were hybridized to probes on the microarray at 56°C for 60 min. Third, the fluorescence intensities of the IC5-labeled PCR products hybridized to the microarray were measured using a Bioshot chip coupled device camera (Toyko Kohan, Tokyo, Japan).

The fluorescence intensity (FI) measured for each spot was subtracted from the background intensity (BG), and the discrimination values were calculated using the following equation: Discrimination value = FI of minor allele/average FIs of both alleles. To discriminate the UGT1A1*28 genotype, values

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**Table 2. Sequences of the primers and probes used to detect seven UGT1A polymorphisms in the DNA microarray**

| Name | Sequence (5’ to 3’) |
|------|---------------------|
| UGT1A1*6-S | CATCTGTCTCTCTCTGATG |
| UGT1A1*6-AS | GACCTGCTCTCTGAGAAG |
| UGT1A1*27-S | TGCTGCTCTCTCTCTCTCT |
| UGT1A1*27-AS | TCTCTGCTCTCTCTCTCT |
| UGT1A1*28-S | TCTCTGCTCTCTCTCTCT |
| UGT1A1*28-AS | TCTCTGCTCTCTCTCTCT |
| UGT1A1*60-S | AAACCGAGCTTGAC |
| UGT1A1*60-AS | TCACTGCTCTCTC |
| UGT1A7_-57-S | ACAACGAGCTTGAC |
| UGT1A7_-57-AS | ACAACGAGCTTGAC |
| UGT1A7-S | GCAGAGCTTGAC |
| UGT1A7-AS | GCAGAGCTTGAC |
| UGT1A9-S | GCAGAGCTTGAC |
| UGT1A9-AS | GCAGAGCTTGAC |

**Primers**

| Name | Sequence (5’ to 3’) |
|------|---------------------|
| UGT1A1*6-S | CATCTGTCTCTCTCTGATG |
| UGT1A1*6-AS | GACCTGCTCTCTGAGAAG |
| UGT1A1*27-S | TGCTGCTCTCTCTCTCTCT |
| UGT1A1*27-AS | TCTCTGCTCTCTCTCTCT |
| UGT1A1*28-S | TCTCTGCTCTCTCTCTCT |
| UGT1A1*28-AS | TCTCTGCTCTCTCTCTCT |
| UGT1A1*60-S | AAACCGAGCTTGAC |
| UGT1A1*60-AS | TCACTGCTCTCTC |
| UGT1A7_-57-S | ACAACGAGCTTGAC |
| UGT1A7_-57-AS | ACAACGAGCTTGAC |
| UGT1A7-S | GCAGAGCTTGAC |
| UGT1A7-AS | GCAGAGCTTGAC |
| UGT1A9-S | GCAGAGCTTGAC |
| UGT1A9-AS | GCAGAGCTTGAC |

**Probes**

| Name | Sequence (5’ to 3’) |
|------|---------------------|
| UGT1A1*6-211G | TAAAGGTCGCTCTGTGAG |
| UGT1A1*6-211A | TAAAGGTCGCTCTGTGAG |
| UGT1A1*27-686C | GATTTAACGCTGTAGAC |
| UGT1A1*27-686A | GATTTAACGCTGTAGAC |
| UGT1A1*28_T6 | TTTTTTCGAAATAAGACTGGA |
| UGT1A1*28_TA7 | TTTTTTCGAAATAAGACTGGA |
| UGT1A1*60_-3279T | GCTCTGCTCTCTCTCT |
| UGT1A1*60_-3279G | GCTCTGCTCTCTCTCT |
| UGT1A7_-57T | GTACTGCTCTCTCTCT |
| UGT1A7_-57G | GTACTGCTCTCTCTCT |
| UGT1A7_387G | TAAATCTTTTTTTCGAAATAAGACTGGA |
| UGT1A7_387T | TAAATCTTTTTTTCGAAATAAGACTGGA |
| UGT1A9_1b_T9 | AGTCTGAGTCTGTGAGAAG |
| UGT1A9_1b_T11 | AGTCTGAGTCTGTGAGAAG |

†These primers are used in multiplexed PCR reactions at final concentrations of 70 nM (UGT1A1*27, UGT1A1*60), 300 nM (UGT1A1*6, UGT1A1*28), 400 nM (UGT1A7_-57, UGT1A7_-3279), and 600 nM (UGT1A9_1b) primer sets. These reactions were performed using FastStart Taq DNA Polymerase (Roche Diagnostics). †The positions within probes that correspond to UGT1A1 polymorphisms are underlined.

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annealing at 58°C for 5 s, and elongation at 72°C for 30 s. Cy5-labeled DNAs were then hybridized to probes on the microarray in 3 × saline sodium citrate (SSC) buffer with 0.3% sodium dodecyl sulfate (SDS) at 55°C for 60 min. Hybridized microarrays were then washed sequentially with 1 × SSC with 0.1% SDS, 1 × SSC, and 0.5 × SSC. Fluorescence intensities of the hybridized PCR products were measured using the GenePix 4000B Microarray Scanner (Molecular Devices, Sunnyvale, CA, USA), and the discrimination value was calculated as described above using these fluorescence intensities.

Results

The total required time for our DNA microarray system. In our procedure, the fluorescent labeling PCR reaction took approximately 90 min, and hybridization of the fluorescent-labeled PCR products took approximately 60 min (Table 3). Following hybridization, it took approximately 15 min to obtain the resulting genotypes, giving a total of approximately 165 min to obtain genotype information from extracted genomic DNA using our DNA microarray assay system. Using the Invader \textit{UGT1A1} Molecular Assay system according to the manufacturer’s recommendations, it takes approximately 250 min to obtain genotype information from extracted genomic DNA, meaning that our DNA microarray system reduced the required time by approximately 1.5-fold.

Accuracy and Sensitivity of focused DNA microarray. Results obtained from LDTs were compared with those obtained from the DNA microarray and Invader assays (Table 4). DNA microarray assay results for both \textit{UGT1A1}*28 and \textit{UGT1A1}*6 were consistent with LDT results of the 111 samples. However, the genotype of one heterozygous for \textit{UGT1A1}*28 sample could not be determined by the Invader assay because of low sample quantity (0.2 ng/assay was used for the DNA microarray while 85 ng/assay was used for the Invader assay). The quantities of the remaining 110 samples ranged from 5.3 to 60.8 ng/assay, with a mean of 12.4 ng/assay, for the DNA microarray assay and 420 to 4870 ng/assay, with a mean of 994 ng/assay, for the Invader assay. Of the 111 samples tested, we found that seven were homozygous for \textit{UGT1A1}*28 [(TA)\textsubscript{6}/(TA)\textsubscript{7}] and 19 were heterozygous for \textit{UGT1A1}*28 [(TA)\textsubscript{6}/(TA)\textsubscript{7}]; the remaining 85 samples were homozygous for the reference allele \textit{UGT1A1}*1 [(TA)\textsubscript{6}/(TA)\textsubscript{6}]. Among the patients, six with A/A and 72 with G/G genotypes were homozygous for \textit{UGT1A1}, while 33 patients with the G/A genotype were heterozygous for \textit{UGT1A1} at the +211 position.

DNA microarray for the simultaneous detection of seven \textit{UGT1A} polymorphisms. We also developed a DNA microarray to simultaneously detect several types of polymorphisms, including single nucleotide substitutions, single nucleotide insertion/deletion, and repeated TA sequences. Using the genotypes determined by established methods as a baseline, the discrimination values from the DNA microarray showed that the genotypes were fully resolved by this system (Fig. 1).

Table 3. Comparison of the total required time for each system

| Process                  | Our focused DNA microarray | The Invader assay |
|--------------------------|----------------------------|-------------------|
| Time (min)               | Time (min)                 |                   |
| PCR                      | 90                         | 5                 |
| Hybridization at 56°C    | 60                         | 240               |
| Wash & Detection         | 15                         | 5                 |
| Total†                   | 165                        | 250               |

†Our focused DNA microarray was able to obtain genotype results approximately 1.5-times faster than the Invader assay.

Table 4. Correlation between the \textit{UGT1A1}*28 and \textit{UGT1A1}*6 genotyping results from the DNA microarray system and conventional assay systems

| \textit{UGT1A1}*28 by the fragment size analysis | \textit{UGT1A1}*28 by the Invader assay |
|----------------------------------------------|---------------------------------------|
| TA\textsubscript{6}/TA\textsubscript{6}      | TA\textsubscript{6}/TA\textsubscript{6} |
| TA\textsubscript{6}/TA\textsubscript{7}      | TA\textsubscript{6}/TA\textsubscript{7} |
| TA\textsubscript{7}/TA\textsubscript{7}      | TA\textsubscript{7}/TA\textsubscript{7} |
| nd.†                                         | nd.†                                  |
| Total                                        | Total†                                |
| UGT1A1*28 by our DNA microarray‡             | UGT1A1*28 by the Invader assay         |
| TA\textsubscript{6}/TA\textsubscript{6}      | TA\textsubscript{6}/TA\textsubscript{6} |
| TA\textsubscript{6}/TA\textsubscript{7}      | TA\textsubscript{6}/TA\textsubscript{7} |
| TA\textsubscript{7}/TA\textsubscript{7}      | TA\textsubscript{7}/TA\textsubscript{7} |
| nd.†                                         | nd.†                                  |
| Total                                        | Total†                                |

‡, nd., not determined. †Probes were spotted in duplicates. The fluorescence intensity (FI) of each spot was subtracted from the background intensity, and the discrimination values were calculated as follows: (FI of minor allele)/average FIs of both alleles.

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Fig. 1. Simultaneous identification of seven UGT1A polymorphisms using the DNA microarray system. Single nucleotide substitutions (a–e), a single nucleotide insertion/deletion (f), and a TA-repeat microsatellite (g) were examined simultaneously using our novel DNA microarray. The discrimination values shown on the y-axis were calculated as described in Materials and Methods. Full separation of each UGT1A genotype is shown. In addition to 133 patients recruited for this analysis, two, seven, and four patients were added for UGT1A1*6, UGT1A1*28, and UGT1A1*27 polymorphisms, respectively, due to the low minor allele frequencies of these polymorphisms. No patients harbored a homozygous UGT1A1*27 polymorphism.
**Discussion**

While LDTs and the Invader assay detect one SNP at a time, we could detect multiple polymorphisms in a single DNA microarray assay. The assay detects polymorphisms by fluorescent labeling sample DNA, with specific probes for each polymorphism. In this study, we demonstrated that genotyping results of UGT1A*28 and UGT1A*6 by the newly developed DNA microarray assay were in almost complete agreement with those obtained by established methods. Additionally, this DNA microarray assay requires only 10 ng/assay (optimal) for accuracy, which is approximately 20 times less than that required for the Invader assay (optimal: 200–700 ng/assay for detection of a single polymorphism), and genotyping results of multiple polymorphisms can be obtained simultaneously in a single assay. Furthermore, unlike with comprehensive SNP arrays, both single-nucleotide and TA-repeat polymorphisms can be detected simultaneously using this method. While the simultaneous detection of polymorphisms can also be achieved using next-generation sequencing (NGS) platforms, this technique is less practical because it is much more expensive and involves complicated data handling procedures.

The DNA array developed has a diamond-like carbon (DLC) coated base (Gene Silicon; Toyo Kohan) measuring 3 mm² in size with increased signal to background ratio. This DLC-coated DNA array took 1 h for hybridization (Table 3), and fluorescence could be detected not only by a high-resolution scanner but also by a compact instrument with charge coupled device camera. Condensing the focused microarray onto a small chip helped to reduce reaction times and running costs.

Determination of the UGT1A polymorphisms before irinotecan treatment has been known to be clinically useful and important for predicting and preventing related toxicities. Therefore, we also successfully developed a DNA microarray assay system wherein several types of polymorphisms, including worldwide results of UGT1A polymorphisms, could be detected on the same 3-mm² chip. The TA-repeat polymorphism UGT1A*28, the SNP UGT1A*6, and the single nucleotide insertion/deletion polymorphism UGT1A9*1b could all be distinguished simultaneously on the same chip (Fig. 1), which will allow the system to be applied in other situations. As well as the germline mutations, such as SNPs, that were considered in this study, somatic mutations and gene expression in cancer cells are used as in vitro diagnostics based on pharmacogenomics in clinical decision-making. For example, KRAS mutations have been linked to a lack of response to anti-EGFR therapy. Our DNA microarray system can distinguish several types of nucleotide sequence changes at once, and we have preliminary data showing that our DNA microarray system can be applied to KRAS mutations in carcinomas (data not shown). While the sequence-dependent hybridization used in our system could be seen as a limitation in comparison to more comprehensive techniques such as clinical sequencing using NGS, our system also has benefits, including convenience and cost effectiveness.

In conclusion, our newly developed method for detecting UGT1A polymorphisms is feasible and has the potential for wide usage alongside the Invader assay for its rapid and accurate genotyping of UGT1A polymorphisms prior to irinotecan treatment.

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**Disclosure Statement**

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