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

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Recent developments in the field of human genomics have greatly enhanced the potential for precision and personalized medicine. We have developed a novel DNA microarray, using a 3-mm square chip coated with diamond-like carbon to enhance the signal-to-background ratio, for use as an *in vitro* diagnostic tool in precision medicine. To verify the genotyping effectiveness of this newly developed DNA microarray we examined UDP-glucuronosyltransferase 1A1 (*UGT1A1*) polymorphisms in DNA extracted from patients with metastatic colorectal cancer. It is established that the polymorphisms of *UGT1A1*28* and *UGT1A1*6* are significantly associated with severe toxicity induced by the anti-cancer drug irinotecan. For each sample, the results obtained with the novel microarray platform were compared with those obtained using other, more established, methods, including direct sequencing and the Invader assay. The polymorphisms tested included a single nucleotide substitution (*UGT1A1*6*) and a TA-repeat polymorphism (*UGT1A1*28*), both of which were detected simultaneously and accurately using our method. Moreover, our method required 1.5-fold less time to assay and 20-fold less sample than those required by the Invader assay. In summary, our newly developed DNA microarray is more practical than established methods, and is at least as accurate; this will increase the efficiency of polymorphism detection prior to diagnosis and the commencement of treatment, and can feasibly be applied in precision medicine.

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.[1–5] 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.[6–14] Carboxylesterases catabolized irinotecan to 7-ethyl-10-hydroxycamptothecin (SN-38), which is a potent topoisomerase I inhibitor leading to cell death.[15–17] SN-38 is then further catabolized by hepatic uridine 5′-diphospho-glucuronosyltransferase (UGT) 1A (UGT1A) enzymes to form the inactive compound SN-38 glucuronide (SN-38G).[18] In Japan, genotyping by the Invader assay has been approved for *in vitro* detection of two *UGT1A1* polymorphisms,[19] *UGT1A1*28* and *UGT1A1*6*, known to be significantly associated with severe irinotecan toxicity, resulting from irinotecan-based chemotherapy for several carcinomas.[6–8,12,20,21] The polymorphism *UGT1A1*28* contains an additional TA repeat in the *UGT1A1* promoter region, giving seven rather than six TA repeats,[22,23] 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.[24] 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.[10,25,26]

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*.

Materials and Methods

Patients. We recruited 251 patients with metastatic colorectal cancer from the Department of Gastroenterological, Breast and Endocrine Surgery, Yamaguchi University Graduate School of Medicine, Japan for this study. Written informed consent was obtained from each study participant.

Genotyping of *UGT1A*. Genomic DNA was extracted from peripheral blood samples by the conventional sodium iodide

(NaI) method.[27] As a baseline reference, *UGT1A* genotyping was performed using the following established laboratory developed tests (LDTs). The number of TA repeats in the *UGT1A1* promoter region was determined by the fragment size analysis followed by direct sequencing as described previously.[3,4] *Taq*Man hydrolysis probe assay were used to determine the *UGT1A1*6* genotype, as described previously.[28] Additionally, *Taq*Man hydrolysis probes were used to determine the *UGT1A1*60* genotype, and direct sequencing was used to determine the genotypes at the *UGT1A7* (387T > G and 622T > C), and *UGT1A9*1b* loci.

In addition to the tests described above, the Invader *UGT1A1* Molecular Assay (Sekisui Medical, Tokyo, Japan) was performed to assess the *UGT1A1* genotypes, according to the manufacturer's recommendations.

DNA microarray for *UGT1A1*28* and *UGT1A1*6* polymorphisms. The focused DNA microarray was developed on a small chip measuring 3-mm^2 in size. Two sets of primers, each labeled with IC5-OSu (N-Ethyl-N'-[5-(N"-succinimidyloxycarbonyl)pentyl]-3,3,3′,3′-tetramethyl-2,2′-indodicarbocyanine iodide; λ_{ex} = 640 nm and λ_{em} = 660 nm; Dojindo Laboratories, Kumamoto, Japan), and probes were designed for detecting *UGT1A1*28* and **6* (Table 1). The *UGT1A1*28* and **6* target sites differ by the number of TA repeats, $(TA)_6 > (TA)_7$, in the promoter region and an SNP, 211G > A. All Probes were spotted in duplicates. Genomic DNA was extracted, and then the following steps were performed. First, specific DNA sequences were amplified by PCR using IC5-labeled primers and a FastStart *Taq* DNA polymerase (Roche diagnostics, Tokyo, Japan). In PCR procedure, 37 cycles of denaturation at 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 charge coupled device camera (Toyo 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 of 0.000–0.613, 0.916–1.340, and 1.472–2.000 were designated $(TA)_6/(TA)_6$, $(TA)_6/(TA)_7$, 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, rs35350960), *UGT1A1*28* (TA_6 > TA7, rs8175347), *UGT1A1*60* (−3279T > G, rs4124874), *UGT1A7* (−57T > G, rs7586110), *UGT1A7* (387T > G, rs17868323), and *UGT1A9*1b* ($-118T_9 > T_{10}$, 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 Japan, 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,

Table 1. Sequences of the primers and probes used to detect the *UGT1A1*28* and *UGT1A1*6* polymorphisms in the DNA microarray

Name	Sequence (5′ to 3′)
Primers	
UGT1A1*28-S	TAGTCGTCCTTCTTCCTCTCTGGT
UGT1A1*28-AS†	IC5-ATGGCGCCTTTGCTCCT
UGT1A1*6-S†	IC5-CCATGCTGGGAAGATACTGTTGA
UGT1A1*6-AS	GATCACACGCTGCAGGAAAG
Probes	
UGT1A1*28-TA5	TTTTTGCCATATATATATATAAGTAGGA
UGT1A1*28-TA6	TTTTTGCCATATATATATATATAAGTAGGA
UGT1A1*28-TA7	GTTTTTGCCATATATATATATATATAAGTAGG
UGT1A1*28-TA8	GTTTTTGCCATATATATATATATATATAAGTAGGA
UGT1A1*6-211G	TAAAATGCTCCGTCTCTGATG
UGT1A1*6-211A	TAAAATGCTCTGTCTCTGATGT

†The 5′ end of primers were labeled with IC5-OSu, *N*-Ethyl-*N*'-[5-(*N*"-succinimidyloxycarbonyl)pentyl]-3,3,3′,3′-tetramethyl-2,2′-indodicarbocyanine iodide; λ_{ex} = 640 nm and λ_{em} = 660 nm. The positions within probes that correspond to *UGT1A1* polymorphisms are underlined.

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

Name	Sequence (5′ to 3′)
Primers†	
UGT1A1*6-S	CCATGCTGGGAAGATACTGTTGA
UGT1A1*6-AS	GATCACACGCTGCAGGAAAG
UGT1A1*27-S	GACGGACCCTTTCCTTCCTT
UGT1A1*27-AS	TCCTGGACAGTCACCTCTCTCT
UGT1A1*28-S	TAGTCGTCCTTCTTCCTCTCTGGT
UGT1A1*28-AS	ATGGCGCCTTTGCTCCT
UGT1A1*60-S	AAACCCGGACTTGGCACTT
UGT1A1*60-AS	CACCTGTCCAAGCTCATTCCT
UGT1A7_-57-S	TCAATGTCGTCAAGGCCAAAA
UGT1A7_-57-AS	GCAAAGCCACAGGTCAGCA
UGT1A7-S	GATCAGGACCGGGAGTTCA
UGT1A7-AS	AAAGTCAGTTCGCAACAACCAA
UGT1A9-S	TGCTCTGGGACAAATTCCAA
UGT1A9-AS	AGCAGACACACACATAGAGGAAGG
Probes‡	
UGT1A1*6_211G	TAAAATGCTCCGTCTCTGATG
UGT1A1*6_211A	TAAAATGCTCTGTCTCTGATGT
UGT1A1*27_686C	GTTTATTCCCCGTATGCAAC
UGT1A1*27_686A	GTTTATTCCCAGTATGCAAC
UGT1A1*28_TA6	TTTTTGCCATATATATATATATAAGTAGGA
UGT1A1*28_TA7	GTTTTTGCCATATATATATATATATAAGTAGG
UGT1A1*60_-3279T	GCTTTGTTCAAACTGAACTCT
UGT1A1*60_-3279G	GCTTTGTTCACACTGAACT
UGT1A7_-57T	GTACTTCTTCCACTTACTATATTATAG
UGT1A7_-57G	TACTTCTTCCACGTACTATATTATA
UGT1A7_387T	CTACTAATTTTCGGTCATTAAACA
UGT1A7_387G	TACTAATTTTTTGTCCTTAAACAAAC
UGT1A9*1b_T9	AGTGACTGATTTTTTTTTATGAAAG
UGT1A9*1b_T11	GTGACTGATTTTTTTTTTTATGAAAG

†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_322), 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 *UGT1A* polymorphisms are underlined.

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 *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.

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

Our focused DNA microarray		The Invader assay	
Process	Time (min)	Process	Time (min)
PCR	90	Denaturation at 95°C	5
Hybridization at 56°C	60	Incubation at 63°C	240
Wash & Detection	15	Cooling & Detection	5
Total†	165	Total†	250

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

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 *UGT1A1*28* and *UGT1A1*6* were consistent with LDT results of the 111 samples. However, the genotype of one heterozygous for *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 *UGT1A1*28* [$(TA)_7/(TA)_7$] and 19 were heterozygous for *UGT1A1*28* [$(TA)_6/(TA)_7$]; the remaining 85 samples were homozygous for the reference allele *UGT1A1*1* [$(TA)_6/(TA)_6$]. Among the patients, six with A/A and 72 with G/G genotypes were homozygous for *UGT1A1*, while 33 patients with the G/A genotype were heterozygous for *UGT1A1* at the +211 position.

DNA microarray for the simultaneous detection of seven *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 4. Correlation between the *UGT1A1*28* and *UGT1A1*6* genotyping results from the DNA microarray system and conventional assay systems

	*UGT1A1*28* by the fragment size analysis					*UGT1A1*28* by the Invader assay				
	TA_6/TA_6	TA_6/TA_7	TA_7/TA_7	nd.†	Total	TA_6/TA_6	TA_6/TA_7	TA_7/TA_7	nd.†	Total
*UGT1A1*28* by our DNA microarray assay‡										
TA_6/TA_6	85	–	–	–	85	84	–	–	1	85
TA_6/TA_7	–	19	–	–	19	–	19	–	–	19
TA_7/TA_7	–	–	7	–	7	–	–	7	–	7
nd.†	–	–	–	–	0	–	–	–	–	0
Total	85	19	7	0	111	84	19	7	1	111

	*UGT1A1*6* by using the hydrolysis probes					*UGT1A1*6* by the Invader assay				
	211G/G	211G/A	211A/A	nd.†	Total	211G/G	211G/A	211A/A	nd.†	Total
*UGT1A1*6* by our DNA microarray assay‡										
211G/G	72	–	–	–	72	72	–	–	–	72
211G/A	–	33	–	–	33	–	33	–	–	33
211A/A	–	–	6	–	6	–	–	6	–	6
nd.†	–	–	–	–	0	–	–	–	0	–
Total	72	33	6	0	111	72	33	6	0	111

†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).

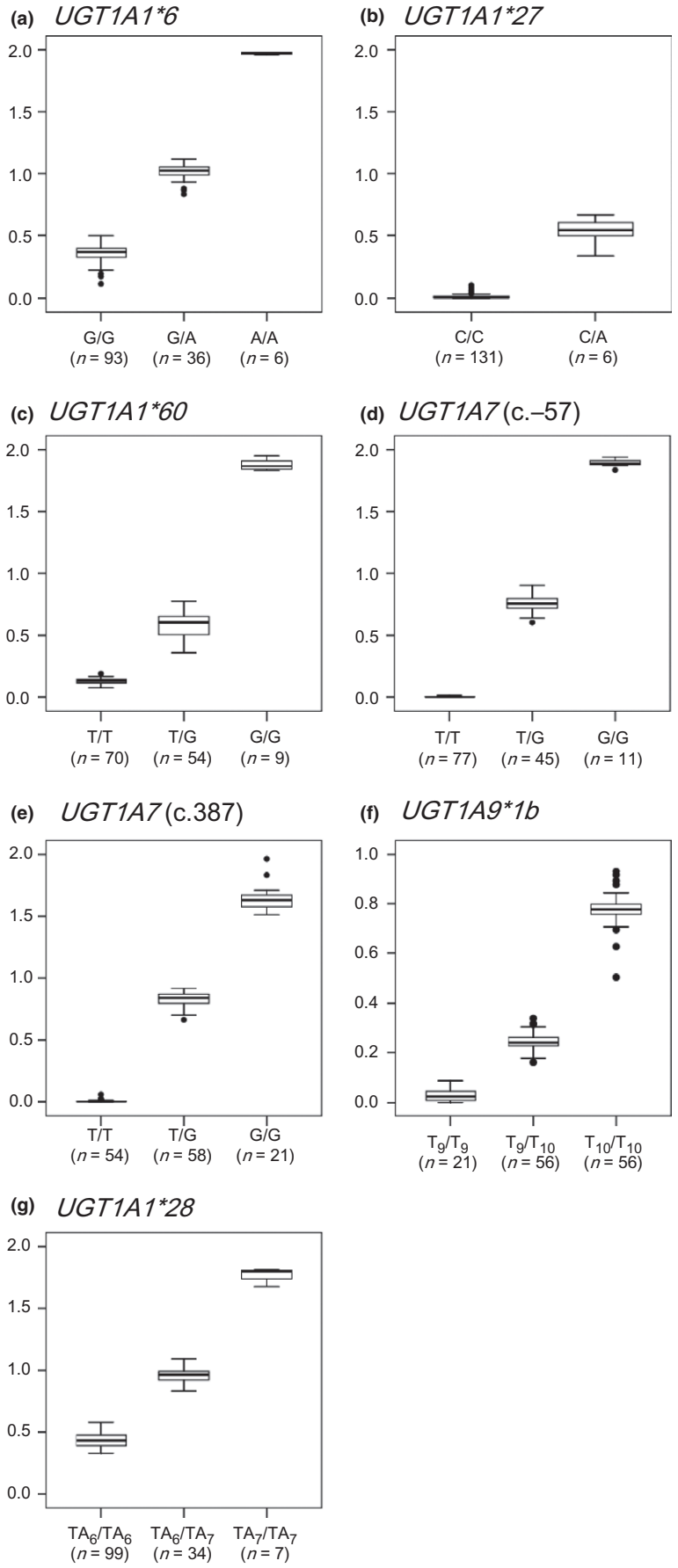

Fig. 1. Simultaneous identification of seven *UGT1A* polymorphisms using the DNA microarray system. Single nucleotide substitutions (a–e), a single nucleotide insertion/deletion (f), and a TA-repeat microsatellite (g) were examined simultaneously using our novel DNA microarray. The discrimination values 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 *UGT1A1*28* and *UGT1A1*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 mm^2 in size with increased signal to background ratio.[31] 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 *UGT1A1* polymorphisms before irinotecan treatment has been known to be clinically useful and important for predicting and preventing related toxicities.[13,25,30] Therefore, we also successfully developed a DNA microarray assay system wherein several types of polymorphisms, including worldwide results of *UGT1A1* polymorphisms,[26,29] could be detected on the same 3-mm^2 chip. The TA-repeat polymorphism *UGT1A1*28*, the SNP *UGT1A1*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.[32–41] 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 *UGT1A1* polymorphisms is feasible and has the potential for wide usage alongside the Invader assay for its rapid and accurate genotyping of *UGT1A1* polymorphisms prior to irinotecan treatment.

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Disclosure Statement

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