Development of Pyrosequencing Method for Detection of *UGT1A1* Polymorphisms in Thai Colorectal Cancers

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Background: *UGT1A1* is a polymorphic enzyme that has been associated with irinotecan drug metabolisms. We developed a pyrosequencing method to detect allele frequency and genotype of *UGT1A1* polymorphisms (*UGT1A1**28 and *UGT1A1**6) in Thai colorectal cancer patients. **Method:** A pyrosequencing method was designed to determine *UGT1A1* genetic polymorphisms including *UGT1A1**28 (A[TA]7TAA) and *UGT1A1**6 (211G>A) in 91 Thai colorectal cancers. **Result:** Genotyping by the pyrosequencing technique was 100% concordant with capillary electrophoresis sequencing.

Key words: *UGT1A1*; genetic polymorphisms; pyrosequencing; SNP; Thai

The allele frequencies for *UGT1A1* genetic polymorphisms were *1/*1 (54.95%), *1/*6 (13.19%), *1/*28 (25.27%), *28/*6 (4.40%), and *28/*28 (2.20%). No homozygous mutation *UGT1A1**6 was found in our population. **Conclusions:** We developed a rapid, reliable, more cost-effective, and simple assay to detect *UGT1A1* genetic polymorphisms in routine practice before initiating irinotecan therapy. The *UGT1A1**28 and *UGT1A1**6 alleles were found to be similar in the Asian populations. J. Clin. Lab. Anal. 30:84–89, 2016. © 2014 Wiley Periodicals, Inc.

INTRODUCTION

The Uridine diphosphate (UDP)-glucuronosyltransferase (UGT), an important class of phase II metabolic enzyme, catalyzes the glucuronidation step and facilitates the excretion of hydrophobic endobiotic and xenobiotic compounds (1, 2). The UGT enzymes are generally divided into three subfamilies, UGT1A, UGT2A, and UGT2B (3). The *UGT1A1* is mediated primarily by the *UGT1* family polypeptide A1, which is located on chromosome 2q37 (4).

The *UGT1A1**1 [A(TA)₆TAA] is a wild-type allele that includes six TA repeats in the TATA box of promoter region (5, 6), whereas *UGT1A1**28 [A(TA)₇TAA] is the most common variant allele that contains seven TA repeats. *UGT1A1**28 has a decreased UGT enzymatic activity accounting 60–80% of the normal level (7–9). The allele frequency of *UGT1A1**28 is 33.4–36.5% in the Caucasian population and 39.0–40.4% in Africans, whereas in Asians the allele frequency is less (13.9%) (10).

The *UGT1A1**6 (211G>A, G71R), polymorphism on exon 1 of the *UGT1A1* gene (11), has 50% less activity than wild-type *UGT1A1* (8). *UGT1A1**6 is most frequent in Asians (13.0%), while it is missing or very few in Caucasians or African-Americans (12). Previous studies have elucidated the *UGT1A1* polymorphisms in Thai population, and has been reported in Gilbert syndrome, Crigler–Najjar syndrome, and HIV patients who were treated with indinavir (13–15).

Irinotecan (camptothecin-11, CPT-11, 7-ethyl-10-[4-(1-(piperidino)-1-piperidino) carbonylcamptothecin] is a

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TABLE 1. Oligonucleotide Primers Used for PCR and Pyrosequencing

Allele	PCR primers	Sequence primer	Size (bp)
<i>UGT1A1</i> *28			
Forward	5'-AGGTTTCGCCTCTCCTACTTATA-3'	5'-CGCCTCTCCTACTTATATAT-3'	82
Reverse	B 5'-CACGTGACAAGTCAAACATTAAC-3'		
<i>UGT1A1</i> *6			
Forward	5'-ACCTACGCCTCGTTGTACAT-3'	5'-CTCGTGATCATCAGAGA-3'	108
Reverse	B 5'-TGCCGAGACTAACAAAAGACT-3'		

B, biotinylated on 5'-end of primer.

widely used anticancer drug approved for the treatment of solid tumors, especially, colorectal cancer (16–18). Irinotecan is a prodrug of topoisomerase 1 inhibitor SN-38, which prevents the DNA strand reannealing, interrupts DNA replication, and leads to subsequent cell death. Irinotecan is metabolized by carboxylesterases to an active 7-ethyl-10-hydroxycamptothecin (SN-38; (18–20)), which is 100- to 1,000-fold more cytotoxic than the parent drug. SN-38 is glucuronidated by UGTs to an inactive SN-38 glucuronide (SN-38G) (21, 22). The major toxicities of irinotecan are febrile neutropenia and diarrhea (23). The polymorphism of *UGT1A1* has been associated with reduced enzymatic activity and SN-38 glucuronidation, which increases toxicity of irinotecan. The polymorphism of *UGT1A1**28 and *UGT1A1**6 alleles has a significantly decreased SN-38G correlated with irinotecan toxicities (12, 19, 24).

Pyrosequencing is a recently developed technique to detect polymorphisms in the *UGT1A1* gene and its promoter region. It involves the hybridization of a primer to a single-stranded PCR template, and the sequencing analysis is started by adding nucleotides. This method is based on the formation of pyrophosphate and can monitor light peak in a pyrogram (25). Pyrosequencing is a high-throughput technique with high accuracy (26). Thus, the objective of this study was to develop and evaluate a method based on pyrosequencing for detecting *UGT1A1* polymorphisms (*UGT1A1**28 and *UGT1A1**6) in Thai colorectal cancer patients.

MATERIALS AND METHODS

Clinical Subjects

Ninety-one Thai colorectal cancer patients were recruited from Division of Medical Oncology, Department of Medicine Ramathibodi Hospital, Mahidol University. DNA analyses of *UGT1A1* polymorphisms were investigated by Division of Pharmacogenomics and Personalized Medicine, Department of Pathology Ramathibodi Hospital, Mahidol University. Written and informed consent was obtained from all participants. The study was

approved by the Ethics Committee, Ramathibodi Hospital, Mahidol University, Thailand.

Human Genomic DNA Samples and Pyrosequencing Technique

Genomic DNA was extracted from 1 ml EDTA whole blood by DNA extraction automated MagNA Pure Compact (Roche Applied Science, Penzberg, Germany). The quality of genomic DNA was assessed by using NanoDrop ND-1000. Table 1 shows the different sets of primers used for the PCR reactions and pyrosequencing, including TA insertion in TATA box of the promoter, and SNPs on the exon 1 (211G>A) of the *UGT1A1*. The PCR and sequencing primers were designed using PyroMark Assay Design 2.0 program (Qiagen, Singapore). The *UGT1A1**28 and *UGT1A1**6 were amplified using biotin tagged on 5'-end of reverse primer.

PCR conditions

PCRs were performed with an initial denaturation for 15 min at 95°C, followed by 42 cycles of denaturation for 20 s at 95°C, primer annealing for 30 s at 53°C, and extension for 20 s at 72°C followed by a final extension for 5 min at 72°C. All amplification reactions were performed with the Thermal cycler (Applied Biosystem, Gene Amp PCR System 9700 Foster City, California, USA) and then PCR products were checked by running on a 2% agarose gel. PCR conditions were the same for two mutations tested.

Pyrosequencing conditions

Fifteen microliters of PCR template was immobilized with 2 µL sepharose beads and 40 µL pyromark-binding buffer by incubator (Shaker 1,400 rpm, 15 min, room temperature). For sequence primer annealing, 21 µL PyroMark annealing buffer and 4 µL sequencing primer were incorporated into each well of 24-well pyrosequencing plate. For strand separation, all liquid was removed by the Vacuum Prep Workstation and then the bead containing immobilized PCR template was captured. The cap-

TABLE 2. Characteristics of Patients

Clinical characteristics	No. of patients	Percentage or range
Age (years), mean	63	27–94
Sex		
Male	52	57.14
Female	39	42.86
Site of disease		
Ascending	11	10.68
Transverse	4	3.88
Descending	6	5.83
Sigmoid	36	34.95
Rectum	46	44.66
Metastatic focus		
Liver	38	57.57
Lung	23	34.85
Other	5	7.58
Histopathology type		
Well differentiated	34	37.36
Moderately differentiated	52	57.14
Poorly differentiated	5	5.49

tured beads on probes were transferred to 70% ethanol for 5 s, denaturing solution for 5 s and wash buffer for 10 s, respectively. All liquid was totally drained from the filter probes. The sepharose beads with the biotinylated single-stranded templates attached were released into 24-well pyrosequencing plate containing sequence primer annealing. The 24-well pyrosequencing plate was heated at 80°C for 2 min and then allowed to cool to a room temperature (25°C) for 10 min. The reaction was analyzed on PyroMark Q24 software.

Statistical Analysis

Genetic equilibrium was tested by Haploview 4.2 according to the formula of Hardy–Weinberg. The frequencies of all genotypes in this study were observed within the 95% confidence interval. Chi-squared test or Fisher’s exact test was used to compare genotype frequencies in different population. A *P* value of less than 0.05 was considered to be statistically significant. All analyses were performed using SPSS version 18.0 (SPSS Inc., Chicago, IL).

RESULTS

Characteristics of Patients

We recruited 91 Thai colorectal cancer patients include 52 males (57.14%) and 39 females (42.86%). The median age was 63 years (range, 27–94 years). Clinical characteristics are described in Table 2. The most common sites of disease were rectum (44.66%) and sigmoid (34.95%). The predominant metastatic organs were the liver (57.57%) and lung (34.85%).

Sensitivity and Specificity of Pyrosequencing

To analyze the frequencies of the *UGT1A1* polymorphisms (*UGT1A1**28 and *UGT1A1**6) included in the research, we developed genotyping assays based on the pyrosequencing method. A representative pyrogram for each genotype is shown in Fig. 1. The assay was designed

Fig. 1. Pyrosequencing histogram for *UGT1A1* by pyrosequencing software. (A) Wild-type TA6/TA6, (B) heterozygous TA6/TA7, (C) homozygous TA7/TA7, (D) wild-type G/G, (E) heterozygous G/A.

TABLE 3. The Comparison of Result *UGT1A1* Polymorphisms Between 2 Methods

Genotypes	Capillary electrophoresis sequencing (gold standard) <i>n</i> = 91 (%)	Pyrosequencing <i>n</i> = 91 (%)
*1/*1	50 (54.95)	50 (54.95)
*1/*6	12 (13.19)	12 (13.19)
*1/*28	23 (25.27)	23 (25.27)
*6/*28	4 (4.40)	4 (4.40)
*28/*28	2 (2.20)	2 (2.20)

TABLE 4. Genotype and Allele Frequencies of *UGT1A1* in Thai Colorectal Cancer

<i>UGT1A1</i>	Frequency	95% CI
Alleles	<i>n</i> = 182 (%)	
*1	135 (74.18)	67.63–80.37
*6	16 (8.79)	4.84–13.16
*28	31 (17.03)	11.54–22.46
Genotypes	<i>n</i> = 91 (%)	
*1/*1	50 (54.95)	44.78–65.22
*1/*6	12 (13.19)	6.09–19.91
*1/*28	23 (25.27)	16.37–34.23
*6/*28	4 (4.40)	0.19–8.61
*28/*28	2 (2.20)	0–5.21

to generate unique peak for *UGT1A1* polymorphisms by setting a suitable nucleotide additional order. The sequence data identified from pyrosequencing were validated by capillary electrophoresis sequencing. The result showed that genotypes were 100% concordant between pyrosequencing and capillary electrophoresis sequencing method (Table 3).

Frequencies of *UGT1A1**28 and *UGT1A1**6 in Thai Colorectal Cancer

The frequencies of *UGT1A1**28 (A(TA)7TAA) and *UGT1A1**6 (211G>A) were determined in individual samples (*n* = 91) by PSQ 24 in Thai colorectal cancer patients and all were in Hardy–Weinberg equilibrium.

The results showed the allele frequencies of *1/*1 (50/91, 54.95%), *1/*6 (12/91, 13.19%), *1/*28 (23/91, 25.27%), *6/*28 (4/91, 4.40%), *28/*28 (2/91, 2.20%). No homozygous *UGT1A1**6 was found in our population. The allele frequencies of *UGT1A1**1, *UGT1A1**6, and *UGT1A1**28 were 0.74, 0.09, and 0.17, respectively (Table 4). Each pairwise comparison of Thai population with Chinese, Japanese, and Caucasian populations is shown in Table 5. The allele frequencies of *UGT1A1**28

variant are similar among Thai, Chinese, and Japanese, whereas the homozygous *UGT1A1**28 (TA7/TA7) was found lower in our population (3.0%) than Caucasian population (11.50%; *P* = 0.006). On the other hand, the homozygous *UGT1A1**6 variant is absent in Caucasian population, while *UGT1A1**6 variant is more commonly found in Thai, Chinese, and Japanese populations (27,28).

DISCUSSION

The designed pyrosequencing was validated and confirmed by capillary electrophoresis sequencing (Gold standard) (29). The results of pyrograms (patterns of peaks) showed clear distinction between mutation genotypes, and each genotype exhibited a unique peak. Therefore, interpretation will be easier and accurate. The throughput of the pyrosequencing instrument was 24 genotypes (24-well plate) in 15 min.

In this study, the designed pyrosequencing method has analyzed *UGT1A1**28 and *UGT1A1**6. The allele frequency of *UGT1A1**28 was higher than *UGT1A1**6 in our population. Among Thai, Chinese, and Japanese populations, the genotype distributions of the *UGT1A1**28 variant are not different, whereas among the Caucasian population these are much higher. On the other hand, the *UGT1A1**6 variant is absent in Caucasian population, while *UGT1A1**6 variant is more commonly found in Thai, Chinese, and Japanese populations.

Several genetic polymorphisms of *UGT1A1* gene have been associated with the occurrence of severe neutropenia and diarrhea toxicities during treatment with irinotecan (30). Severe neutropenia or diarrhea is associated with *UGT1A1**28 (31). In 2005, the US Food and Drug Administration (FDA) recommended for *UGT1A1**28 testing in patients who receive irinotecan (22). In Asian population, *UGT1A1**6 is the most common allele correlated with reduced SN-38 glucuronidation activity and drug toxicity (32). Therefore, *UGT1A1* genotyping must be performed before initiating treatment with irinotecan. Moreover, the *UGT1A1* polymorphisms are currently known to affect enzyme activity, leading to constitutional unconjugated jaundice (Crigler–Najjar types I and II or Gilbert's syndromes) (9). Therefore, we can study Crigler–Najjar and Gilbert's syndromes associated with *UGT1A1* polymorphisms in Thai populations. However, this is the first study in Thai colorectal cancer associated with *UGT1A1* polymorphisms.

CONCLUSION

In conclusion, the pyrosequencing method for detecting *UGT1A1**28 and *UGT1A1**6 seems to be rapid, reliable, and more cost-effective. Thus, we developed a pyrosequencing method to detect *UGT1A1**28 and

TABLE 5. Genotype Frequencies of *UGT1A128 and *UGT1A1**6 in Four Populations**

Gene	Thai ^a	Chinese ^b	Japanese ^b	Caucasian ^c	<i>P</i> value		
<i>UGT1A1</i> *28	(A, %)	(B, %)	(C, %)	(D, %)	A vs. B	A vs. C	A vs. D
TA6/TA6	68.2	70.6	82.7	37.2	0.645	0.014	0.000
TA6/TA7	28.8	24.5	15.3	51.4	0.871	0.054	0.000
TA7/TA7	3.0	4.9	2.0	11.5	0.470	1.00	0.006
<i>UGT1A1</i> *6							
G/G	81.3	74	73.7	100	0.236	0.236	0.000
G/A	18.7	18	22.4	0	0.856	0.599	0.000
A/A	0	3	3.8	0	0.081	0.043	-

^aPresent study.^b(27).^c(28).

*UGT1A1**6 for a routine clinical practice. Our results suggested that genotyping of *UGT1A1**28 and *UGT1A1**6 should be considered before colorectal cancer patients are prescribed with irinotecan. Pyrosequencing technique can be used for further additional study of association between *UGT1A1* genetic polymorphisms and pharmacokinetics drug plasma concentration and clinical outcome in Thai colorectal cancer patients in large sample population.

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