การพัฒนาเทคนิคไพโรซีเควนซิ่งสำหรับการตรวจความหลากหลายทางพันธุกรรมของยีน *UGT1A1* ในผู้ป่วยไทยที่เป็นมะเร็งลำไส้ใหญ่

Development of Pyrosequencing Method for *UGT1A1* Polymorphisms in Thai Colorectal Cancer.

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บทคัดย่อ

วัตถุประสงค์ของการศึกษานี้เพื่อหาความถี่อัลลีลและจีโนไทป์ของยีน *UGT1A1* (*UGT1A1*28* and *UGT1A1*6*) ในผู้ป่วยมะเร็งลำไส้ใหญ่ที่ได้รับการรักษาด้วยยาเออริโนทีแคน และพัฒนาวิธีตรวจหาความหลากหลายทางพันธุกรรมของยีน *UGT1A1* ด้วยเทคนิคไพโรซีเควนซิ่ง โดยใช้ผู้ป่วยจำนวน 91 ราย ผลการศึกษาพบความถี่จีโนไทป์ ดังนี้ 54.95% (**1/*1*), 13.19% (**1/*6*), 25.27% (**1/*28*), 4.40% (**28/*6*) และ 2.20% (**28/*28*) แต่ไม่พบ *UGT1A1*6/*6* การทดสอบยืนยันผลเทคนิคไพโรซีเควนซิ่งให้ผลจีโนไทป์เป็น 100% ตรงกับวิธีแคปปิลลารี อิเล็กโตรโฟรีซิส ดังนั้นเทคนิคนี้จึงเป็นเทคนิคที่มีความน่าเชื่อถือ รวดเร็ว และมีประสิทธิภาพ ในการตรวจหาความหลากหลายทางพันธุกรรมของยีน *UGT1A1* ในห้องปฏิบัติการ ก่อนผู้ป่วยจะได้รับยาเออริโนทีแคน

ABSTRACT

The objective of this study was to determine allele frequency and genotyping of *UGT1A1* polymorphisms (*UGT1A1*28* and *UGT1A1*6*) in Thai colorectal cancer patients who received irinotecan treatment and develop pyrosequencing technique for *UGT1A1* genetic polymorphisms detection. The Ninety-one patients entered the study. The results showed the allele frequencies for *UGT1A1* genetic polymorphisms were 54.95% (**1/*1*), 13.19% (**1/*6*), 25.27% (**1/*28*), 4.40% (**28/*6*) และ 2.20% (**28/*28*). No homozygous mutation *UGT1A1*6/*6* was found in our population. Genotyping by pyrosequencing were 100% concordant with capillary electrophoresis sequencing direct sequencing for validation. The technique we set up is appropriate for the detection of *UGT1A1* genetic polymorphisms in routine practice before irinotecan therapy. And this technique is rapid, reliable, and more cost-effective.

คำสำคัญ: ยีน *UGT1A1*, ความหลากหลายทางพันธุกรรม, ไพโรซีเควนซิ่ง, ยาเออริโนทีแคน

Keywords: *UGT1A1*, genetic polymorphisms, pyrosequencing, irinotecan drug

INTRODUCTION

Irinotecan (Camptothecin-11, CPT-11), a widely used anticancer drug, is approved for the treatment of solid tumors, including colorectal cancer which either alone or in combination with 5-fluorouracil (5-FU) and leucovorin (LV)(Hu Z Y , et al.,2010). The major toxicities of irinotecan are febrile neutropenia and diarrhea (Huang S H , et al.,2011). Irinotecan, an anticancer prodrug, is hydrolyzed by carboxylesterases to the active metabolite SN-38. The SN-38 is 100-1,000 folds more cytotoxic than the parent drug as a topoisomerase I inhibitor and eliminated predominantly by UDP-glucuronosyltransferase (UGT) 1A1 enzyme to SN-38 glucuronide (Hoskins J M , et al.,2007). This UGT1A1 is mediated primarily by the UGT1 family polypeptide A1 that is encoded by the *UGT1A1* gene. The *UGT1A1*1* [A(TA)6TAA] is wild type allele that includes six TA repeats in the TATA box of the promoter (Guillemette C , et al.,2001). Whereas *UGTA1A1*28* [A(TA)7TAA] is the most common variant allele, contains seven TA repeats, has decreasing expression of UGT enzymatic activity having the result that increase serum bilirubin levels (Rodriguez-Novoa S , et al.,2007). 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 lower (13.9%).(Kobayashi E and Satoh N,2012). The *UGT1A1*6* (211G>A, G71R) is polymorphisms on exon 1 of the *UGT1A1* gene and correlated with reduced SN-38 glucuronidation activity. In Asians, *UGT1A1*6* is the most frequently in Asians (13.0%), while it is missing or very few in Caucasians or African-Americans (Takano M , et al.,2009). The polymorphism of Uridine-diphosphoglucuronosyl transferase 1A (*UGT1A*), in the promoter and coding regions, has been associated with reduce enzyme activity and SN-38 glucuronidation, which increased toxicity of irinotecan. The polymorphism of *UGT1A1 * 28* and *UGT1A1 * 6* allele have significant decreased SN-38G that correlated with irinotecan toxicities (Takano M , et al.,2009). And the previous studied has been reported that colorectal cancer patients who received irinotecan with have genetic polymorphism of *UGT1A1* (*UGT1A1*28* and *UGT1A1*6*) result in increased SN-38 active. The high SN-38 active leads to high adverse drugs which has diarrhea and febrile neutropenia. The objective of this study was developed and evaluated a technique based on pyrosequencing for detect *UGT1A1* polymorphisms (*UGT1A1*28* and *UGT1A1*6*) in Thai colorectal cancer patients.

MATERIALS AND METHODS

1. Subjects

91 Thai colorectal cancer patients (53 males and 38 females) were recruited from division of medical oncology, department of medicine Ramathibodi Hospital, Mahidol University. Written and informed consent was obtained from all participants.

Genomic DNA was extracted from 1 ml. of 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 the *UGT1A1* polymorphisms to be analyzed, TA insertion in TATA box of the promoter, and SNPs on the exon 1 (211G> A).

2. 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 in DNA the Applied Biosystems GeneAmp PCR system 9700. And then PCR products were checked by running on a 2 % agarose gel. PCR conditions were the same for two mutations tested.

3. Pyrosequencing conditions

15 µL PCR template was immobilized with 2 µL sepharose beads and 40 µL pyromark binding buffer by incubator (Shaker 1400 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 the PSQ 24. For strand separation, all liquid was removed by the Vacuum Prep Workstation and then captured the bead that containing PCR template immobilized. The captured 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 release into a PSQ 24 plate containing sequence primer annealing. The PSQ 24 plate was heated at 80 °C for 2 min and then allowing it to cool to room temperature (25 °C) for 10 min. The reaction was analyzed on a PyroMark Q24 automated (pyrosequencing).

Table 1 Oligonucleotide primers used for PCR and pyrosequencing

Allele	PCR primers	Sequence primer	Size (bp)
*UGT1A1*28*			
Forward	5'-AGGTTCGCCTCTCCTACTTATA-3'	5'-CGCCTCTCCTACTTATATAT-3'	82
Reverse	B 5'-CACGTGACAAGTCAAACATTAAC-3'		
*UGT1A1*6*			
Forward	5'-ACCTACGCCTCGTTGTACAT-3'	5'-CTCGTGTACATCAGAGA-3'	108
Reverse	B 5'-TGCCGAGACTAACAAAAGACT-3'		

B, biotinylated on 5'-end of primer.

Statistical analysis: Genetic equilibrium was tested according to the formula of Hardy–Weinberg by Haploview 4.2. The frequencies of all the genotypes observed in the present study were within the 95% confidence interval. Chi-squared test or Fisher exact test was use to compare the genotype frequencies in different population. A p value of < 0.05 was considered to be statistically significant. All analyses were performed using SPSS version 16.0 (SPSS Inc., Chicago, Illinois, USA).

RESULTS AND DISCUSSION

The assay was designed to generate a specific sequence for *UGT1A1* polymorphisms by setting a suitable nucleotide additional order. The sequence data identified from pyrosequencing was validated by Capillary electrophoresis sequencing. The result showed genotypes obtained were 100% concordance between pyrosequencing and capillary electrophoresis sequencing method (Table 3). When we analyzed *UGT1A1* genetic polymorphisms with pyrosequencing method in 91 Thai colorectal cancer patients, the results show that **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*, **6*, and **28* were 0.74, 0.09, and 0.17 respectively (Table 2). Each pairwise comparison of Thai population with Chinese, Japanese and Caucasian populations are shown in Table 4. Among the Thai, Chinese and Japanese populations, the genotype distributions of the *UGT1A1*28* variant are similar, whereas among the Caucasian population is 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.

Table 2 Genotype and allele frequencies of *UGT1A1* in Thai colorectal cancer

Gene	Frequency	95% CI
UGT1A1 genotype	(n) (%)	
**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
**6/*6*	0 (0.0)	0
**28/*28*	2 (2.20)	0-5.21
UGT1A1 allele (%)		
**1*	74.18	67.63-80.37
**6*	8.79	4.84-13.16
**28*	17.03	11.54-22.46

Table 3 The comparison of result UGT1A1 polymorphisms by Pyrosequencing and Capillary electrophoresis method

Method		Capillary electrophoresis method		
		Positive (mutant)	Negative (wild type)	Total (n=91)
Pyrosequencing method	Positive (mutant)	41	0	41
	Negative (wild type)	0	50	50
	Total (n=91)	41	50	91

In this study, the designed pyrosequencing technique have been able to analyse *UGT1A1*28* and *UGT1A1*6*. The published *UGT1A1* pyrosequencing technique have analysed only *UGT1A1*28* and *UGT1A1*6*, separately. This technique reduces running cost compared with commercial kit. The allele frequency of *UGT1A1*28* is higher than *UGT1A1*6* in our population.

CONCLUSION

The developed pyrosequencing method for detecting *UGT1A1*6* and *UGT1A1*28* seems to be rapid, reliable, and more cost-effective. Thus we developed the pyrosequencing method to detect *UGT1A1*6* and *UGT1A1*28* for become a routine practice. The further study, sample size will be more collected for this study.

Table 4 Genotype frequencies of *UGT1A1*28* and **6* in four populations.

	Thai[a]	Chinese[b]	Japanese[b]	Caucasian[c]	*P* value		
*UGT1A1*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
*UGT1A1*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	-

[a]Current study, [b](Teh L K , et al.,2012), [c](Sung C , et al.,2011)

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