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Characteristic CYP2A6 genetic polymorphisms detected by TA cloning-based sequencing in Chinese digestive system cancer patients with S-1 based chemotherapy

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Abstract. S-1 is an oral antitumor agent that contains tegafur, which is converted to fluorouracil (5-FU) in the human body. Cytochrome P450 2A6 (CYP2A6) is the principal enzyme responsible for bioconversion of tegafur to 5-FU. A number of CYP2A6 polymorphisms have been associated with variations in enzyme activity in several ethnic populations. The CYP2A6*4C allele leads to deletion of the entire CYP2A6 gene, and is the main finding in patients with reduced CYP2A6 enzymatic activity. Thus, the aim of our study was to evaluate the allele frequencies of CYP2A6 polymorphisms in a population with cancer of the digestive system. We developed a simple screening method, which combined TA cloning and direct-sequencing, to detect CYP2A6 genetic polymorphisms in Chinese patients with cancers of the digestive system. A total of 77 patients with various types of digestive system cancers were screened for CYP2A6 genetic polymorphisms. The allele frequencies of CYP2A6*1A, CYP2A6*1B and CYP2A6*4C in the 77 patients screened were 62, 42 and 13%, respectively. Frequencies of the homozygous genotypes for CYP2A6*1A and CYP2A6*4C were 27 and 12%, respectively. As expected, patients that were determined to be homozygous for CYP2A6*4C exhibited the characteristic chemotherapy efficacy and toxicity profiles. The TA cloning-based direct sequencing method facilitated allele frequency and genotyping determination for CYP2A6*1A, 1B and 4C of cancer patients. The findings indicated that the population carries a high frequency of the CYP2A6*4C homozygous genotype. Thus, the reduced efficacy of standard chemotherapy dosage in Chinese cancer patients may be explained by the lack of CYP2A6-mediated S-1 bioconversion to 5-FU.

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Introduction

S-1 is an oral prodrug of fluorouracil (5-FU) that is composed of tegafur, 5-chloro-2,4-dihydroxypyridine (CDHP; gimestat), and potassium oxonate (otastat) that shows clinical efficacy in patients diagnosed with various solid tumors, especially those of the digestive system (1-3). Cytochrome P450 2A6 (CYP2A6) is the principal enzyme responsible for the bioconversion of tegafur to 5-FU (4). Polymorphisms of CYP2A6 have been associated with variations in enzymatic activity in several ethnic groups (5). The most intensively investigated genetic polymorphisms of CYP2A6 are CYP2A6*1A, CYP2A6*1B and CYP2A6*4C. CYP2A6*1A is recognized as the wild-type allele, while CYP2A6*1B is believed to be a gene conversion with CYP2A7 in the 3'-untranslated region (UTR). CYP2A6*4C, on the other hand, leads to complete deletion of the CYP2A6 gene, which results in overall reduced enzymatic activity. The reduced enzymatic activity subsequently inhibits the bioconversion of tegafur to 5-FU and impacts the efficacy of 5-FU-based chemotherapies in cancer patients (6).

Clearly, CYP2A6 polymorphisms play an important role in S-1-based chemotherapy treatment of cancer patients. However, few studies to date have evaluated the CYP2A6 polymorphism status in the Chinese population. Thus, we developed a method of TA-based cloning using direct-sequencing to detect CYP2A6 genetic polymorphisms in Chinese patients with various digestive system cancers.

Materials and methods

Participants. From May 2010 to May 2011, peripheral blood samples were collected from patients with digestive system cancer in The First Affiliated Hospital, College of Medicine at Zhejiang University. All patients provided written informed consent, and the study protocol was approved by the hospital's Institutional Ethics Committee. Patients were administered

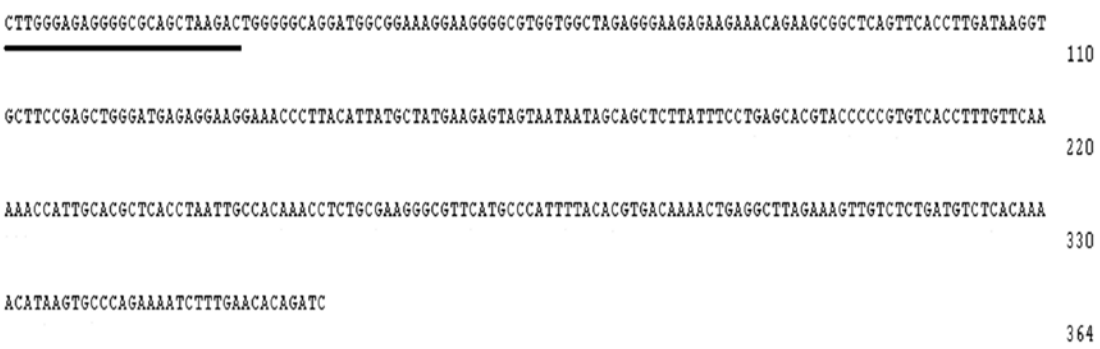

Figure 1. Typical sequence of a 364 bp length PCR product with initiation sequences that are identical to the 5' of the CYP2A6 gene. The underlined section is the specific sequence distinguishing CYP2A6 from CYP2A7.

Figure 2. Typical sequence of a 379 bp length PCR product with initiation sequences that are identical to the 5' of the CYP2A7 gene. The underlined section is the specific sequence distinguishing CYP2A6 from CYP2A7.

S-1 orally at a dose of 40-60 mg twice daily for 7-14 days, followed by a rest period of seven days. Nineteen patients were treated with monochemotherapy, while the remaining patients were treated with combinations of chemotherapeutics according to the regimens designed for their specific cancer diagnosis. Chemotherapy efficacy evaluation was performed according to the Response Evaluation Criteria in Solid Tumors (RECIST) guidelines version 1.1. The National Cancer Institute of Common Toxicity Criteria (CTC) version 3.0 was used to evaluate toxicity.

Reagents and equipment. PCR reagents were purchased from TransGen Biotech (Beijing, China). dNTPs were purchased from Biochemical Service Science and Technology Co., Ltd. (Beijing, China). The DNA Gel Extraction kit was purchased from Axygen (Union City, CA, USA). The pGEM-T vector was purchased from Promega (Madison, WI, USA). The pMD-18T vector was purchased from Takara (Dalian, China). A 2720 thermal cycler (Applied Biosystems, Inc., San Diego, CA, USA) was used to perform PCR.

DNA isolation and PCR procedure. Genomic DNA was extracted from whole blood with the QIAamp DNA Mini kit (Qiagen, Germany) according to the manufacturer's protocol. Primer pairs were designed to correspond to sequences in the 3'-UTR of the CYP2A6 sequence (7) and were as follows: P1 (sense), 5'-CCTCAAGTCCTCCCAGTCAC-3'; P2 (antisense), 5'-AGCAAGCACAGATTACACCC-3'. The primers were synthesized and purified by the Huirui Biotechnology Co., Ltd. (Shanghai, China). The PCR reaction included 0.5 μmol/l each of P1 and P2, 1.5 mmol/l $MgCl_2$, 200 μmol/l dNTPs, 0.2 units HS DNA polymerase, and 50 ng genomic DNA template; deionized water was added to adjust the total volume to 50 μl. The PCR reaction conditions were as follows: denaturation at 94˚C for 30 sec, 30 cycles of annealing at 52˚C for 60 sec and 72˚C for 60 sec, and elongation at 72˚C for 5 min.

Direct-sequencing and TA cloning. The PCR products were subjected to direct-sequencing following treatment with ExoSAP-IT® (Affymetrix, Shanghai, China). The PCR products that were not suitable for direct-sequencing were subcloned into the pMD-18T vector and transformed into JM109 competent cells. Positive clones were selected by blue-white screening and then subjected to direct-sequencing. Differences between the wild-type sequence (reference) and the PCR products were determined.

Criteria for the determination of polymorphisms. Based on the previous structural characterization of CYP2A6*1A, 1B, and 4C, we focused on the analysis of the deleted position of the 3'-UTR in CYP2A6 (7). According to the lengths of the cloning products, we selected two sequences at the bilateral site of the 3'-UTR 15 bp deletion and selected a specific sequence in the 5'-UTR useful for distinguishing CYP2A6 from CYP2A7. The sequences were as follows: CYP2A6, CTTGGGAGAGGGG CGCAGCTAAGA and CYP2A7, TTCGCGGGAAGAGGCG GGTATAAGAA. The 3'-CAGAAAATCTTTGAACACAG ATC-5' sequence was conserved between the two genes. We evaluated the similarity of the gene sequences by using a specific position of CYP2A6 and CYP2A7 as the anchor for

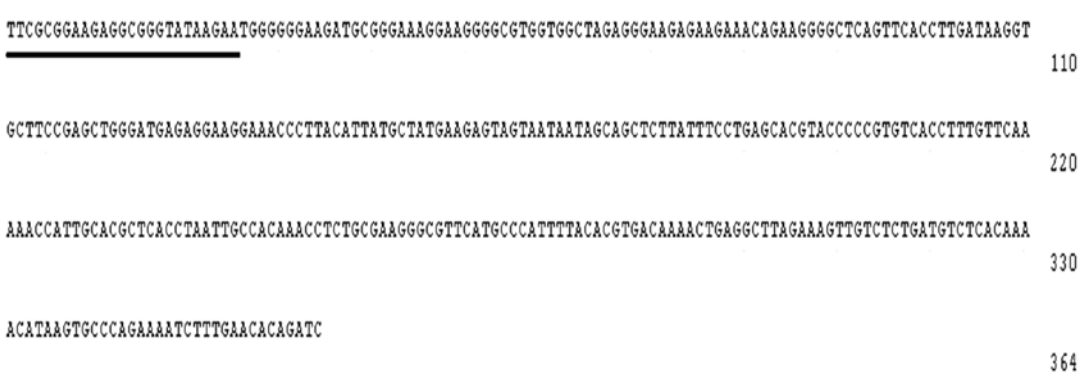

Figure 3. Typical sequence of a 364-bp length PCR product with initiation sequences that are identical to the 5' of the CYP2A7 gene. The underlined section is the specific sequence distinguishing CYP2A6 from CYP2A7.

comparison, as described in three typical circumstances below: i) for the homozygotes of CYP2A6*1A: two PCR products were produced, one of 364 bp and another of 379 bp. The 364 bp product had 100% similarity to CYP2A6 in the 5' region (Fig. 1). The 379 bp product had 100% similarity to CYP2A7 in the 5' region (Fig. 2); ii) for the CYP2A6*1B allele: the PCR product that possessed 58 bp sequences from the 3'-UTR had sequences substitution from the corresponding region of the CYP2A7; and iii) for the homozygotes for CYP2A6*4C: the products were all 364 bp in length with 100% similarity to CYP2A7 in the 5' region (Fig. 3).

Results

Patient characteristics. A total of 77 patients with clinically confirmed digestive system cancer were enrolled in the study (Table I). The median patient age was 58 years old (range, 16-74 years), and 65% of the patients (n=50) were male. Gastric cancer accounted for the majority of diagnoses (n=50) Each patient was treated with S-1-based chemotherapy, including single agent or combination regimens. All participants were evaluated for CYP2A6 genetic polymorphisms (CYP2A6*1A, 1B and 4C). A subset of patients with the genotype of CYP2A6*4C/CYP2A6*4C were assessed for S-1-therapy efficacy and toxicity.

Frequencies of CYP2A6 genotypes and alleles. PCR products of approximately 740 bp in length were obtained. However, a majority of the PCR products were not suitable for direct-sequencing and were characterized by multiple mixed peaks, which made analysis impossible. Accordingly, TA cloning was performed and the resulting cloned PCR products were sequenced. The genotype and allele frequencies of CYP2A6 are shown in Table II. Homozygotes for CYP2A6*1A and CYP2A6*4C were detected in 27% (n=21) and 12% (n=9) of the patients, respectively. The allele frequencies of CYP2A6*1A, CYP2A6*1B and CYP2A6*4C in the patients were 62, 42 and 13%, respectively.

We were unable to determine the exact allele (either 1B or 4C) in five patients. In addition, 21% (n=16) of the patients characterized by PCR produced unknown lengths (of 363, 368, 374, 375, 378 and 437 bp, respectively), which implied that these patients may harbor unknown CYP2A6 alleles.

Table I. Patient characteristics (n=77).

Characteristics	n (%)
Median age, years (range)	58 (16-74)
Gender	
Male	50 (65)
Female	27 (35)
Location of primary tumor	
Esophagus	1 (1.3)
Gastric	50 (65)
Bile duct	1 (1.3)
Pancreatic	18 (23.3)
Colorectal	7 (9.1)
Disease status	
R0 resected	6
Metastatic	71

Table II. Frequencies of CYP2A6 genotypes and alleles in the patients (n=77).

	Frequency	
	n	%
Genotype		
CYP2A6*1A/CYP2A6*1A	21	27
CYP2A6*1A/CYP2A6*1B	19	25
CYP2A6*1A/CYP2A6*4C+Unknown	0	0
CYP2A6*1A/Unknown	8	10
CYP2A6*1B/Unknown	8	10
CYP2A6*1B/Unknown	5	6
CYP2A6*4C/CYP2A6*4C	9	12
CYP2A6*4C/Unknown	1	1
Allele		
1A	48	62
1B	32	42
4C	10	13

Table III. Clinical efficacy and toxicity in patients homozygous for CYP2A6*4C.

	Clinical efficacy				Distinctive S-1-related toxicity (grade)			
Case no.	CR	PR	SD	PD	Abdominal pain	Diarrhea	Stomatitis	Pigmentation
1				√	0	0	II	II
2			√		0	II	II	0
3	NA				0	0	0	0
4	NA				0	0	0	0
5			√		0	0	0	II
6				√	0	0	0	0
7			√		0	0	0	0
8			√		0	0	0	0
9				√	0	0	0	0

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NA, not applicable.

*Clinical efficacy and toxicity in patients homozygous for CYP2A6*4C*. There were nine patients with the homozygous genotype of CYP2A6*4C/CYP2A6*4C. Two of those patients could not be assessed for treatment efficacy because they had no detectable lesions prior to chemotherapy. However, none of the remaining seven patients experienced a complete response (CR) or partial response (PR), regardless of chemotherapy regimen type. Nearly half of these patients (n=3) were characterized as progressive disease (PD) after receiving the S-1-based chemotherapy. In addition, none of the nine patients presented Grade III or IV distinctive S-1-related toxicity (Table III).

Discussion

The S-1 chemotherapeutic drug is currently used in Korea and Japan for digestive system cancer patients and is now available for use in mainland China. The ability of the human body to biotransform this orally administered drug inevitably affects the chemotherapeutic efficacy and toxicity. Polymorphisms of the enzyme responsible for the bioconversion of tegafur to 5-FU, CYP2A6, have been associated with variations of enzymatic activity in several ethnic groups. We used a TA cloning-based sequencing strategy to determine the frequencies of CYP2A6 genetic polymorphisms for certain known important alleles of CYP2A6*1A, 1B and 4C in Chinese digestive system cancer patients.

Several methods exist to detect polymorphisms of the CYP2A6 gene. The most widely utilized technique is PCR combined with the restriction enzyme fragment length polymorphism analysis (7-9). The shortcoming of this method lies in the precision of the designed digestive site. Therefore, we sought to determine if direct-sequencing was a better method for detecting CYP2A6 polymorphisms. However, the direct-sequencing method that was utilized resulted in multiple mixed peaks, which were not acceptable for analysis. In order to avoid this issue, TA cloning was performed to generate plasmid vectors containing the gene fragments that would be suitable for direct-sequencing. Fragment lengths of 364 and 379 bp were obtained, which corresponded to lengths reported previously (7).

During the study, we found that it was difficult to distinguish homozygotes of CYP2A6*1B, which suggested the need for a more specific method. However, since previous studies have demonstrated that the allele of CYP2A6*1B does not lead to decreased enzyme activity, the identification of this polymorphism was not as critical for the patients in our study (10).

Since new methods are known for their ability to detect previously unrecognized genetic polymorphisms of CYP2A6 (11,12), we were not surprised to find several novel sequences, including previously unreported lengths of 363, 368 and 374 bp. The TA cloning method is considered to have relatively dependable precision for direct-sequencing, suggesting that those novel sequence fragments may represent new polymorphisms of CYP2A6. This theory warrants evaluation in future studies. In the current study, the genotype frequencies of CYP2A6*1A/CYP2A6*1A, as well as the allele frequency of CYP2A6*1A, were similar to those previously reported in other Asian populations (7,13). However, the frequencies of the whole gene deletion genotype CYP2A6*4C/CYP2A6*4C (12%), as well as the allele itself (13%), were different from previous reports. Two studies have specifically reported on the allele frequencies of CYP2A6*4C in Chinese populations. One determined the frequency to be much lower (5%) (13), while the other (15%) was comparable to our results (14). A previous study indicated that the phenomenon of whole CYP2A6 gene deletion (CYP2A6*4C) was more common in Asians than in Caucasian populations (15). Besides, it was reported that the Japanese populations had a much lower CYP2A6*4 genotype frequency (2.2-3.4%) as compared to the current study (12%) (7,8), and similar results were found in Malays (7%) and Indians (2%) (13). Another study also reported that the frequencies of the CYP2A6*4 allele are different between Koreans and Japanese (16). Taken together, these findings strongly imply that there are ethnic differences in CYP2A6 gene polymorphisms, even between Asian populations. Thus, it is likely that the Chinese population possesses a distinct whole gene deletion polymorphism CYP2A6*4C.

Unfortunately, we could not make a direct comparison of clinical efficacy between the different CYP2A6 polymorphism groups in this study, due to the variety of chemotherapy regimens and different cancer types. However, it was noted that patients homozygous for CYP2A6*4C had no CR or PR, and experienced no grade III or IV distinctive S-1-related toxicity. Similarly to previously published findings (8), one of our female patients with wild-type homozygous CYP2A6*1A endured severe adverse effects following monotherapy of S-1, including grade IV diarrhea, IV stomatitis and IV pigmentation, which are all characteristic S-1-related toxicities.

In conclusion, although the TA cloning method utilized in this study did not reveal additional polymorphisms of CYP2A6 that are also related to reduced enzymatic activity (16-18) (such as CYP2A6, 7, 9, 10), it was able to precisely determine the CYP2A6*1A, 1B and 4C alleles by sequencing of cloned PCR fragments. It has been demonstrated that the frequency of CYP2A6*4C/CYP2A6*4C was higher than that reported for other Asian and Western populations. Moreover, the lower efficacy and toxicity of S-1 in Chinese patients was likely due to the impaired bioconversion of tegafur to 5-FU caused by homozygosity for the CYP2A6*4C whole gene deletion polymorphism. Collectively, our results warrant further controlled studies to determine whether there is a critical need to modify the standard dose of S-1 in Chinese cancer patients to improve the efficacy. Screening for polymorphisms of CYP2A6 in the Chinese population would help to improve patient outcomes and allow for personalized adjustment of S-1 dosages depending on the polymorphism status.

Acknowledgements

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