Clin. Lab. 2019;65:XXX-XXX

ORIGINAL ARTICLE

Comparing PyroMark Q24 Pyrosequencing and MALDI-TOF MS for Identification of CYP2D6*10

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SUMMARY

Background: **CYP2D6*10 is mainly responsible for the large pharmacokinetic variability of routinely administered metoprolol in middle-aged and elderly Asian patients. Utilizing an efficient method for identifying the CYP2D6*10 genotypes is clinically important for evaluating the pharmacokinetic effect of administration of metoprolol. This study attempted to evaluate the effectiveness of the two methods used to detect the rs1065852 and rs1135840 SNPs of the CYP2D6*10 gene.**

Methods: **Blood samples were processed for the collection of genomic DNA from 198 subjects across Chinese population, and detection of CYP2D6*10 (rs1065852 and rs1135840) was performed using the PyroMark Q24 pyrosequencing and matrix-assisted laser desorption/ionization time-of-flight mass-spectrometry (MALDI-TOF MS). The discordant results were further validated with Sanger sequencing. We eventually attempted to assess some features of these two methods including reliability, rapidness, being appropriate, and cost-effectiveness.**

Results: **Genotyping of rs1065852 and rs1135840 detected by MALDI-TOF MS were concordant with those identified by PyroMark Q24 pyrosequencing in all 198 (100%) individuals. The hands-on-time and the turnaround time were shorter in the PyroMark Q24 pyrosequencing method than in the MALDI-TOF MS method for SNP of CYP2D6*10. In terms of being cost-effective and high-throughput, the MALDI-TOF MS method outperformed the PyroMark Q24 pyrosequencing method.**

Conclusions: **CYP2D6*10 genotypes detected by PyroMark Q24 pyrosequencing and MALDI-TOF-MS showed that both methods were reliable, rapid, appropriate, and cost-effective methods. These methods are valuable for clinical applications.**

(Clin. Lab. 2019;65:xx-xx. DOI: 10.7754/Clin.Lab.2018.180909)

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Manuscript accepted November 14, 2018

KEY WORDS

CYP2D6 gene, polymorphism, PyroMark Q24 pyrosequencing, MALDI-TOF MS, cost-effective

INTRODUCTION

Cytochrome P450 2D6 (CYP2D6) in humans mediates oxidative metabolism of a variety of clinical medicines [1]. CYP2D6 gene polymorphism has become a hot issue for the metabolism of clinically used drugs (e.g., Metoprolol) metabolized by this enzyme in pharmacogenomics [2]. Polymorphisms in CYP2D6 result from single-nucleotide polymorphisms (SNP) as well as from insertion/deletions of nucleotide bases. Based on the CYP2D6 activity score system, each allelic variant of

an individual's CYP2D6 metabolizer phenotype is classified into one of the four predicted groups: poor metabolizers (PMs), intermediate metabolizers (IMs), extensive metabolizers (EMs), and ultra-rapid metabolizers (UMs) [3].

Metoprolol is one of the drugs frequently used to treat hypertension and is extensively used in middle-aged and elderly populations with cardiovascular disease in clinic [4]. Taguchi reported that CYP2D6*10 was the major factor affecting the plasma concentrations of routinely administered metoprolol even in middle-aged and elderly Asian patients and may be clinically important [5]. However, response to metoprolol therapy is highly variable. Despite its widespread use, understanding the genotyping of CYP2D6*10 is of great importance from a clinical standpoint because the enzyme is responsible for metabolizing > 30% of drugs belonging to different therapeutic classes [6].

Interindividual variability in drug response is a major clinical problem. As a first step toward identifying poor drug metabolizers on metoprolol in the clinical setting, it was imperative to select an appropriate method for identifying the CYP2D6*10 genotypes simultaneously and rapidly in a cost-effective manner.

Genotyping methods, such as restriction fragment length polymorphism (RFLP) analysis extended for CYP2D6 genotyping [7], have been replaced in recent years by several novel techniques. Real-time polymerase chain reaction (PCR)-based methods are also available for CYP2D6 SNP genotyping [8]. TaqMan assays are used to assure specificity of the genotypes obtained [9]. These methods have a disadvantage of being non-scalable, e.g., low-throughput; however, they possess a number of advantages, including the detection of gene deletions and multiplications with clinical relevance. At present, there are some simple and quick SNP analysis techniques. Pyrosequencing is the first alternative to the conventional Sanger method for de novo DNA sequencing, facilitating short-read sequencing, and renders it easier and more rapid than conventional Sanger sequencing. Pyrosequencing has been used for the identification of CYP2D6*3 genotyping [10]. Matrix-assisted laser desorption/ionization time-of-flight mass-spectrometry (MALDI-TOF MS) has been also used for genotype testing of CYP2D6 with its high-throughput advantage [11].

Subjects with T188 or C4268 in CYP2D6*10 showed a significantly higher log (MR) than subjects with homozygous C188 and G4268, where polymorphisms may explain the interracial variations between Chinese and Caucasian subjects, as well as genetic variations among Chinese subjects [12,13]. In the current study, we evaluated the performance of PyroMark Q24 and MALDI-TOF MS in the identification of CYP2D6*10 (rs1065852 and rs1135840) genotypes. We compared the accuracy of results achieved to assess the potential usefulness in a routine clinical practice.

MATERIALS AND METHODS

Study population

A total of 198 hypertension patients' blood samples (58 females and 140 males, mean age 66 ± 11 years, age range 46 - 78 years) used for the evaluation and were obtained from the Chinese People's Liberation Army General Hospital (Beijing, China). The samples were collected and anticoagulated with EDTA-K_2. All experiments were carried out according to the relevant laws and guidelines in accordance with the ethical standards of the Declaration of Helsinki. This study was approved by the Ethics Committee of Chinese People's Liberation Army General Hospital. An informed consent was obtained from each patient as well.

DNA extraction

All of the 198 blood samples (2 mL) were collected in Vacutte® (Greiner) tubes containing EDTA as an anticoagulant. DNA was extracted from 200 µL of blood using the blood genomic DNA Mini Kit (Changsha Sanji Biotech Co. Ltd., Changsha, Hunan province, China) according to the manufacturer's protocol, and the final elution volume was 30 µL. The extracted DNA was quantified using NanoDrop One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and the concentrations were in the range of 35 - 50 ng/µL. The DNA extraction was used to conduct pyrosequencing and MALDI-TOF MS analyses.

MALDI-TOF MS analysis SNP for rs1065852 and rs1135840 genotype

First, the primers for rs1065852 and rs1135840 with a perfect match were designed using the MassARRAY Assay Design software (Sequenom Inc., San Diego, CA, USA). There are three primers for each SNP, including a pair of PCR primers and a single-base extension primer (Table 1). In addition, a tag (5'-ACGTTGGATG) was included in the primer sequence to equalize extreme relative percentage-GC contents [15].

The primers were synthesized by Shanghai Biological Engineering Technology Co. Ltd. (Shanghai, China). Then, the PCRs were performed in a total volume of 5 µL, which contained 1 µL DNA, 0.1 µL HotStarTaq DNA Polymerase (5 U/µL), 0.325 µL $MgCl_2$ (25 mmol/L), 0.625 µL PCR buffer (10 x) (Agena BioScience Inc., Shanghai branch), 1 µL dNTPs (2.5 mmol/L), and 0.95 µL H_2O and 1 µL of the designed primers at optimum concentrations. The PCR conditions were as follows: at 94°C for 15 minutes, followed by 45 cycles at 94°C for 20 seconds, 56°C for 30 seconds, 72°C for 1 minute, and a final step at 72°C for 3 minutes. The samples were kept at 4°C until conducting further analysis.

After the PCRs were performed, the products were treated with Shrimp Alkaline Phosphatase (SAP) to remove excess dNTPs, and the reaction conditions were

37°C for 40 minutes and 85°C for 15 minutes. The products were used for the primer extension reactions using iPLEX Gold Reagent Kit (Agena BioScience Inc., Shanghai, China) with the reaction conditions of 94°C for 30 seconds, 40 cycles at 94°C for 5 seconds, 5 cycles at 52°C for 5 seconds, 80°C for 5 seconds, and a final step at 72°C for 3 minutes. The extension products were treated with a cationic exchange resin (AG 50W-X8 Resin; Bio-Rad Laboratories Inc., Hercules, CA, USA) for 30 minutes to remove salts. Then, the products were spotted onto 384-well microtiter plates and air dried. The target plate was then inserted into the MassARRAY Compact System (Sequenom Inc., San Diego, CA, USA), and the analysis was performed using 1,800 nitrogen laser shots for each sample. The mass range of the MS instrument was set at 3,920 - 12,023 Da.

Pyrosequencing analysis rs1065852 and rs1135840 genotypes

Pyrosequencing PCR and sequencing primers for rs1065852 and rs1135840 are shown in Table 2. High performance liquid chromatography (HPLC) purified primers were used for real-time PCR and biotinylated at their 5' end. Copy DNA was generated with the PyroMark PCR Kit (Qiagen, Germany). 25 µL reactions contained 1 x PyroMark PCR Master Mix, 1 x CoralLoad (Qiagen, Germany), 0.2 µM forward and reverse primers, and either 100 ng gDNA or 10 ng copy DNA. Cycling conditions were 95°C for 15 minutes followed by 40 cycles at 94°C for 30 seconds, 61°C for 30 seconds, 72°C for 30 seconds, followed by a final extension step at 72°C for 7 minutes. All pyrosequencing reagents, except for Streptavidin Sepharose High Performance beads (GE Healthcare, Chicago, IL, USA), belonged to the PyroMark Q24 Advanced Reagents Kit (Qiagen, Germany). Then, 20 µL double-stranded DNA was bound to 1 µL Streptavidin Sepharose High Performance beads in 40 µL binding buffer. Beads carrying the double-stranded and biotinylated amplicons were captured on filter probes with aspiration. While maintaining aspiration, filter probes were immersed in 70% ethanol for 7 seconds, in denaturation buffer for 7 seconds, and eventually in Wash Buffer 1 for 10 seconds. Single-stranded and biotinylated amplicons were released into wells containing 1 µM sequencing primer in a total of 22 µL annealing buffer, and annealed for 2 minutes at 80°C.

Pyrosequencing reactions were performed on the PyroMark Q24 (Qiagen, Germany). dATP was provided as dATPaS in order to avoid interference with the luciferase enzyme. Pyrograms were analyzed with the PyroMark Q24 Analysis Software (version 3.0.0; Qiagen, Germany) in allele quantification mode using default settings.

Statistical analysis

All demographic numerical data were expressed as mean ± standard deviation (SD), whereas categorical data were expressed as percentages. All statistical analyses were performed using SPSS 22.0 software (IBM, Armonk, NY, USA).

RESULTS

Primes design used for SNP genotyping in MALDI-TOF-MS and pyrosequencing methods

For MALDI-TOF MS, PCR primers were designed by MassARRAY Assay 2.0 and synthesized by the Shanghai Biological Engineering Technology Corporation (Shanghai, China). The primers' names and sequences are listed in Table 1. PCR primers for pyrosequencing were designed by PyroMark Assay 2.0 and synthesized by the Shanghai Biological Engineering Technology Corporation (Shanghai, China). The primers' names and sequences are listed in Table 2.

Genotyping results by two methods

Genotyping of rs1065852 and rs1135840 by MALDI-TOF MS were concordant with PyroMark Q24 pyrosequencing in all 198 (100%) individuals. The genotype and allele frequencies of both SNPs are presented in Table 3. Figure 1 shows the representative examples of SNP results by MALDI-TOF MS method. Figure 2 shows the representative examples of SNP results by pyrosequencing method.

Duration of detection and cost-effectiveness of two methods

Approximate time required to perform each step of the two methods is summarized in Table 4. The hands-on time and the turnaround time were shorter in the PyroMark Q24 pyrosequencing method than that in the MALDI-TOF MS method for SNP of CYP2D6*10. In terms of the cost-effectiveness and the high-throughput, the MALDI-TOF MS method outperformed the pyrosequencing method.

DISCUSSION

The genotyping of CYP2D6 is widely used for pharmacogenetics/pharmacogenomics studies. There is ample evidence in the literature suggesting that the CYP2D6 polymorphisms can affect the pharmacokinetics of metoprolol as well as other β blockers [14-16]. Accurate genotyping is essential because all pharmacogenomics-based treatment decisions are centered on genotype-phenotype correlation [17]. However, genotype testing for CYP2D6 is not routinely performed in clinical practice.

In the present study, we compared PyroMark Q24 pyrosequencing and MALDI-TOF MS methods to analyze the SNP in CYP2D6*10. PyroMark Q24 pyrosequencing was designed to detect changes in specified variable positions of the DNA, on the basis of detection of released pyrophosphate during DNA elongation. MALDI-TOF MS was used to detect single nucleotide differ-

Table 1. Primers used for SNP genotyping by MALDI-TOF MS method.

SNP-ID	Forward primer (5'-3')	Reverse primer (5'-3')	Primer for extension reaction
rs1065852	ACGTTGGATGTGGTGGACCTGATGCACCG	ACGTTGGATGAGTCCACATGCAGCAGGTTG	TGGGCTGCACGCTACC
rs1135840	ACGTTGGATGCCATGGTGTCTTTGCTTTCC	ACGTTGGATGACTAGGTACCCCATTCTAGC	CTTTGCTTTCCTGGTGAC

Table 2. Primes used for SNP genotyping by Pyrosequencing method.

SNP-ID	Forward primer (5'-3')	Reverse primer (5'-3')	Sequence to be analyzed
rs1065852	TCAACACAGCAGGTTCA	CTGTGGTTTCACCCACC	A/GGTAGCGTGCAGCCCAGCGTTGGCGC
rs1135840	CATGGAGCTCTTCCTCTTCT	CAAGGGTAACTGACATCTGC	GC/GTCACCAGGAAAGC

Table 3. Distribution of genotypes in CYP2D6*10 in 196 patients with hypertension according to two presented methods in this study.

Method	SNPrs1065852			SNPrs1135840		
	Genotype (%)			Genotype (%)		
	CC	CT	TT	GG	GC	CC
MALDI-TOF MS	57 (26.15)	86 (39.45)	75 (34.40)	106 (48.62)	87 (40.27)	25 (11.46)
Pyrosequencing	57 (26.15)	86 (39.45)	75 (34.40)	106 (48.62)	87 (40.27)	25 (11.46)

Table 4. Comparing the characteristics of each technique.

Item	Method	
	MALDI-TOF MS	Pyrosequencing
Spent time (min)	430	360
Hands-on time (min)	45	40
DNA concentration (ng)	10	10
Cost per sample ($)	10	15
Test (well)	384	24

ences according to a single complementary mass-modified base. By comparing the results of the two methods, concordant results were observed for all the samples (Table 3). The results are consistent with other studies, reflecting that MALDI-TOF MS method is highly concordant with PyroMark Q24 pyrosequencing [18-20]. We concluded that these two methods are indeed appropriate for genotyping the CYP2D6*10.

According to Edenberg et al.'s study, a crucial point in high-throughput genotyping is the selection of an appropriate method for the aim and the stage of the investigation, taking into account the number of samples and the available infrastructure. Each method has its particular advantages and disadvantages; however, the main differences are DNA concentration, hands-on time, reagents' cost, and time spent on conducting the analysis (Table 4). In this study, the two genotyping assays only required 10 ng of DNA compared with other methods (e.g., Sanger sequencing and RFLP) [21,22]. In the present study, DNA quality was sufficient in all cases and none of the MALDI MS or PyroMark Q24 pyrosequencing methods had to be repeated. It is very important to collect high quality DNA from patients' blood by CYP2D6 polymorphism testing. The quality of individ-

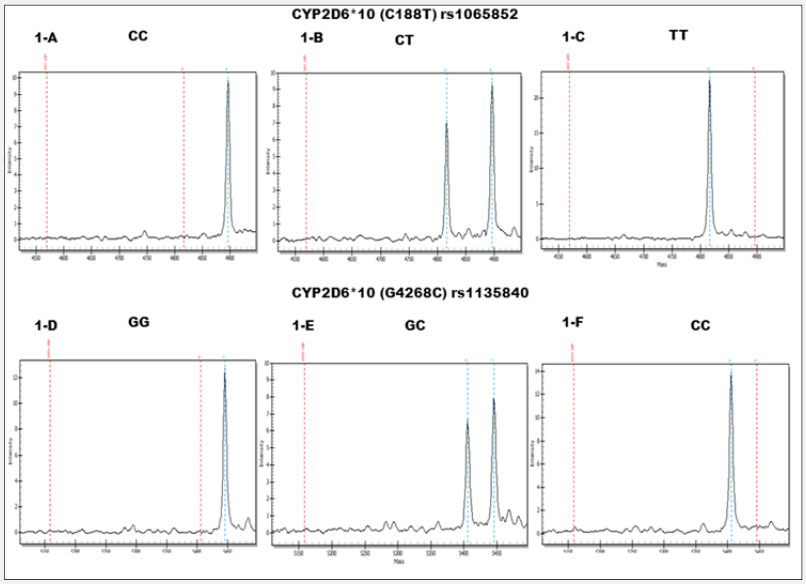

Figure 1. Representative examples of MALDI-TOF MS results on CYP2D6*10.

The SNP of CYP2D6*10 (C188T) rs1065852 was detected by the MALDI-TOF MS method (CC genotype in 1-A, CT genotype in 1-B, TT genotype in 1-C). The SNP of CYP2D6*10 (G4268C) rs1135840 was detected by the MALDI-TOF MS method (GG genotype in 1-D, GC genotype in 1-E, CC genotype in 1-F).

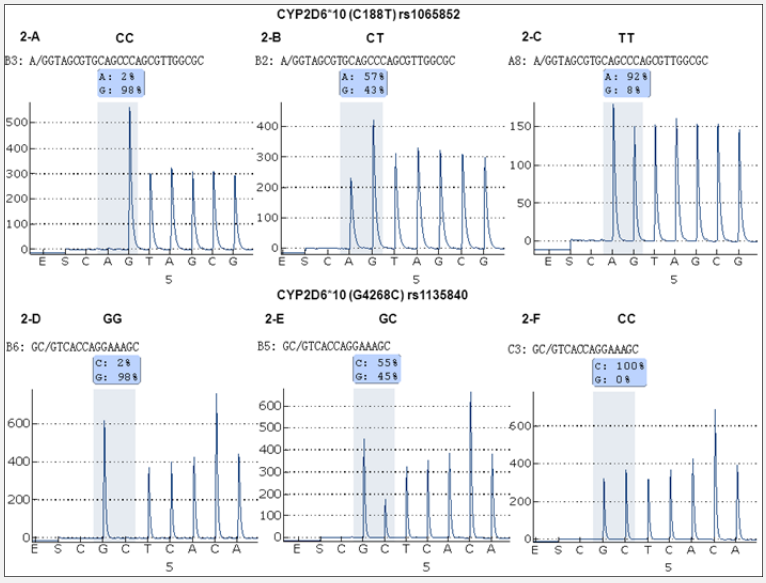

Figure 2. Typical pyrograms using the two sequencing primers of rs1065852 and rs1135840.

The SNP of CYP2D6*10 (C188T) rs1065852 was detected by the pyrosequencing method (CC genotype in 2-A, CT genotype in 2-B, TT genotype in 2-C). The SNP of CYP2D6*10 (G4268C) rs1135840 was detected by the pyrosequencing method (GG genotype in 2-D, GC genotype in 2-E, CC genotype in 2-F).

ual genomic DNA was indicated by low UV absorbance ratios and LongRange PCR Kit. Medical laboratories routinely isolate DNA from the patients' blood during the first intervention, thereby avoiding long-term storage, as well as reducing the likelihood of DNA degradation.

When the cost of a test is estimated, three parameters are mainly taken into account, including the cost of instrumentation, the cost of conducting each test, and hands-on-time. We believe that MALDI MS is a cost-effective method to detect clinically relevant SNPs. As it is an open platform, more mutational hotspots for testing may be easily added. However, this is only confirmed for high-throughput laboratories, as the cost of equipment is higher than the pyrosequencing analysis method. For low-throughput laboratories, some techniques with low expense of equipment may be more beneficial in short- and long-term studies.

In addition to importance of the results of hands-on-time and the turnaround time, which has been reported to be 2 working days for Sanger sequencing and 1.5 working days for pyrosequencing [20,22], which is in agreement with our results. We conducted MALDI-TOF MS within 1.5 working days and pyrosequencing within 1 day. We believe that a time-to-result of within 2 working days seems to be reasonable. Hands-on-time is an important factor, and that was around 45 minutes for performing the MALDI-TOF MS and 40 minutes for the pyrosequencing in this study.

CONCLUSION

Due to the high percentage of CYP2D6*10 alleles in the Asian population, the genotyping of this allele is of high medical value. It was revealed that the MALDI-TOF MS method provides reliable results and enables the genotyping of up to thousands of samples by taking advantage of the high-throughput and cost-effectiveness. Besides, the PyroMark Q24 pyrosequencing method presents reliable results for genotyping accompanied with high accuracy. These methods are both cost-effective, rapid, appropriate, and accurate for clinical applications.

Acknowledgment:
The present study was supported by National Natural Science Foundation of China (61827806) and the PLA General Hospital Clinical Support Fund Program (2016 FC- TSYS- 1041).

Declaration of Interest:
None.

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