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Comparison between MassARRAY and pyrosequencing for CYP2C19 and ABCB1 gene variants of clopidogrel efficiency genotyping

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ABSTRACT

Clopidogrel is one of the most frequently used drugs in patients to reduce cardiovascular events. Since patients with different genetic variations respond quite differently to clopidogrel therapy, the related genetic testing plays a vital role in its dosage and genetic testing related to clopidogrel therapy is currently considered as routine test worldwide. In this study, we aim to use two different methods MALDI-TOF mass spectrometry and pyrosequencing to detect gene variant of CYP2C19 and ABCB1. Six single nucleotides polymorphisms (SNP) within CYP2C19 (*2, *3, *4, *5, *17) and ABCB1 C3435T in 458 Chinese Han patients were determined using both MassARRAY and Pyrosequencing. Sanger sequencing was used for verification. Results of both methods were analyzed and compared. Allele frequencies of each SNP and distribution of different genotypes were calculated based on the MassARRAY and Sanger sequencing results. Both methods provided 100% call rates for gene variants, while results of six samples were different with two methods. With Sanger sequencing as the reference results, MassARRAY generated all the same results. The minor allele frequencies of the above six SNPs were 27.1% (CYP2C19*), 5.9% (CYP2C19*3), 0% (CYP2C19*4), 0% (CYP2C19*5), 1.1% (CYP2C19*17), 40.9% (ABCB1), respectively. MassARRAY provides accurate clopidogrel related genotyping with relatively high cost-efficiency, throughput and short time when compared with pyrosequencing.

List of abbreviation: SNP: single nucleotides polymorphisms; ACS: acute coronary syndromes; PCI: percutaneous coronary intervention; ABCB1: ATP-binding cassette subfamily B member 1; PGx: Pharmacogenetics; LOF: loss of function; dNTPs: deoxynucleotides; SBE: single base extension; ddNTP: dideoxynucleotide; HWE: Hardy-Weinberg equilibrium; MACE: major adverse cardiac events

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1. Introduction

Clopidogrel is considered as standard antiplatelet drug in medical care for patients with acute coronary syndromes (ACS) and percutaneous coronary intervention (PCI) undergoing stent implantation (Antman et al., 2008). Over 80% of ischemic events and post-PCI cardiovascular events could be prevented by clopidogrel therapy (Cayla et al., 2011). Through inhibiting the purinergic ADP receptor P2Y₁₂, clopidogrel reduces adenosine diphosphate-induced platelet aggregation and decreases the risk of cardiovascular events (Rytkin et al., 2017). However, a large number of patients continue to suffer recurrent ischemic events, and this clinical phenomenon has been correlated with lesser degrees of platelet inhibition (Udell et al., 2016). This failure of the antiplatelet drug to inhibit its target of action is called clopidogrel non-responsiveness or

clopidogrel resistance (Nguyen et al., 2005). Although novel drugs including prasugrel and ticagrelor have been shown to be more rapid and effective P2Y₁₂ receptor inhibitor, they tend to induce more reverse complications such as bleeding events (Kubica et al., 2014; Wiviott et al., 2007). Thus, clopidogrel remains to be one of the most extensively used antiplatelet drugs and research focused on how to diagnose the inter-individual variability in clopidogrel disposition and efficacy is of vital significance.

Clopidogrel is a prodrug that requires a two-step hepatic metabolism by several cytochrome P450 isoforms (Kazui et al., 2010). This procedure is firstly induced by CYP2C19 enzyme followed by further oxidation where the intermediate metabolite turns into an active metabolite by CYP3A4, CYP2C19, CYP2B6, and CYP2C9 isoenzymes. Accordingly, polymorphisms of CYP2C19 gene can partially explain variability

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in response to clopidogrel (Mega et al., 2009). In addition, clopidogrel is an oral drug, whose absorption in the duodenum requires P-glycoprotein involvement in the intestinal transport. The ATP-binding cassette sub-family B member 1 (ABCB1) gene encodes the enteric intestinal efflux transporter pump P-glycoprotein, which limits the oral bioavailability of clopidogrel and modulates its absorption in the intestine (Beitelshees et al., 2015). Currently, more than 50 SNPs residing in the coding region of this gene have been described (Fung & Gottesman, 2009). Among these, ABCB1 c.3435C > T (rs1045642) polymorphism has been extensively studied and some investigations have shown that this polymorphism influences the function of P-glycoprotein and, consequently, alter the absorption of clopidogrel (Simon et al., 2009). Results of systematic review also suggested that ABCB1 C3435T polymorphism may increase the risk of bleeding in Asian patients treated with clopidogrel (Zhai et al., 2017).

Pharmacogenetics (PGx)-study of variations in DNA sequences affecting drug response has been gradually applied in personalized patient care (Jani et al., 2015; Yip et al., 2015). CYP2C19 genotyping detection for clopidogrel therapy has been recommended by Clinical Pharmacogenetics Implementation Consortium (Scott et al., 2013). Various techniques involving fast and accurate detection of gene detection have been used in hospitals, such as TaqMan PCR, Sanger sequencing, gene-chip, high-melting curve and MALDI-TOF mass spectrometry. Since some of the patients may suffer from complications, clinical doctors hope that the genotype result is available at the time of prescription. In addition, they also require that the therapy be decided in a very short time (Kubica et al., 2014). The ability to identify more SNPs in a single test is also highly desirable in clinical practice.

Taking these factors into consideration, we aim to compare the most commonly used two methods: MassARRAY and pyrosequencing. MassARRAY technology is reported to shorten the turn-around-time to 12 h when compared with Sanger sequencing, the later of which uses about 24 h to provide clinicians with results. In addition, MassARRAY could obtain results of several SNPs per test. In our current study, we tested the loss of function (LOF) allele of CYP2C19 (*2, *3, *4, *5, *17) and ABCB1 (C3435T) with both MassARRAY and Pyrosequencing and compared the accuracy of these two techniques.

2. Methods and materials

2.1. Study volunteers and DNA sample preparation

This study included 458 unrelated Chinese Han volunteers who underwent routine examination of clopidogrel

related genotyping test in our department from February 2016 to October 2017. 2 mL peripheral blood was collected using EDTA anticoagulant tube without coagulation or hemolysis. All volunteers have signed the informed consent and the study was approved by Ethics Committee of our hospital.

Genome DNA was extracted based on protocol of DNA isolation kit (HiPure Blood DNA Mini Kit, Magen). DNA extraction must be completed within 4 hours of blood collection. Then DNA was quantified by NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA) and diluted to the concentration between 2.5–5.0 ng/μL and stored at –80 °C.

2.2. Genotyping of CYP2C19 and ABCB1 by MassARRAY and pyrosequencing

Genotyping was performed using MassARRAY platform integrating iPLEX and Mass ARRAY technology (Agena Bioscience, San Diego, CA, USA)- based MALDI-TOF MS assay and with the kit designed for gene loci (Bioyong Technology Ltd, Beijing, China). We performed MassARRAY on the DNA extraction from 458 samples and focused on the following SNPs: CYP2C19*1-*5, CYP2C19*17 and ABCB1 C3435T. Our MassARRAY procedure was based on the previously described study of Stacey G (Gabriel et al., 2009). Primer pairs for amplification and sequencing were designed and provided by Bioyong Technology Ltd (Beijing). This technique contained five major procedures: Firstly, initial locus-specific PCR amplification was performed. Then, shrimp alkaline phosphatase was used to neutralize the uncombined deoxynucleotides (dNTPs). Thirdly, single base extension (SBE) uses mass-modified dideoxynucleotide (ddNTP) terminators of an oligonucleotide primer that anneals immediately upstream of the target polymorphic site. Then we used MALDI-TOF mass spectrometry to analyze the distinct mass of extended primer, which could trace the alternative alleles. Positive and negative template control samples were included in each assay plate. Any assay found as positive in negative template control was removed from the study.

Pyrosequencing was performed strictly according to the protocol of Real time quantitative Pyrosequencing analyzer (PyroMark Q24 MDx, Germany, Qiagen) and the supporting kit (Qiagen, Germany). Figure 1 further illustrates the key steps of these two methods.

2.3. Sanger sequencing for verification

After comparing the genotyping results of MassARRAY and Pyrosequencing, inconsistent samples were

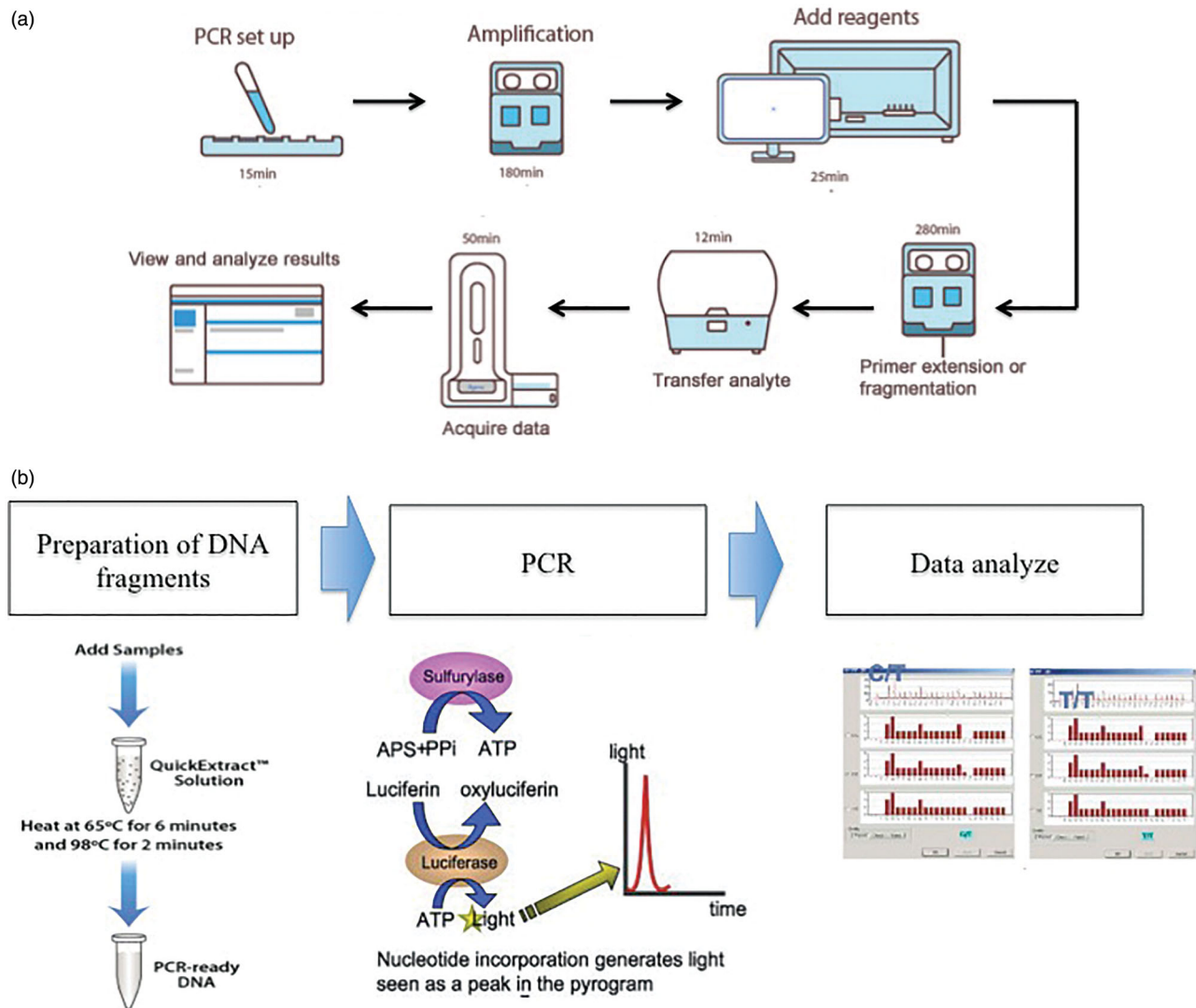


Figure 1. Key steps of MassARRAY (1a) and PyroMark Q24 (1b).

selected for Sanger sequencing verification (Shanghai Biological Engineering Inc, Shanghai).

2.4. Statistical analysis

SPSS 22.0 was used for statistical analysis. Samples that produce results in the first run were regarded as determinate while others as indeterminate. The call rates of MassARRAY and Pyrosequencing were assessed by the percentage of determinate samples (Call rate = the number of determinate samples / (the number of determinate + the number of indeterminate samples)). The Chi-square test was applied to assess the consistency of MassARRAY and Pyrosequencing. Hardy-Weinberg equilibrium (HWE) was also calculated. $p < 0.05$ (two-tailed) was considered as statistical significance. The genotyping results of each SNP were calculated.

3. Results

3.1. Call rate evaluation

We tested all 458 samples with both MassARRAY and Pyrosequencing. For all the SNPs in our study: CYP2C19*2 (rs4244285), CYP2C19*3 (rs4986893), CYP2C19*4 (rs28399504), CYP2C19*5 (rs56337013), CYP2C19*17 (rs12248560) and ABCB1 C3435T (rs1045642), the call rates of both these two methods were 100%. Thus, there was no significant difference in call rates between MassARRAY and Pyrosequencing ($p > 0.05$).

3.2. Distribution of genotyping in Chinese Han population

Among all the included samples, the distribution of genotypes and alleles of the CYP2C19 and ABCB1 variants in our sample is summarized in Table 1.

Table 1. Genotype and allele frequencies of CYP2C19 and ABCB1 variants in Chinese Han population and comparison with European and Asian countries in previous studies.

Allele	SNP	Genotype frequencies (n = 458)		Genotype frequencies (European)	Genotype frequencies (Asian)
CYP2C19*2	681 G > A rs4244285	GG	0.5239(247)	0.745	0.4445
		GA	0.380(174)	0.238	0.4851
		AA	0.081(37)	0.017	0.0693
CYP2C19*3	636 G > A rs4986893	GG	0.884(405)	1.000	0.9286
		GA	0.114(52)	0.000	0.0714
		AA	0.002(1)	0.000	0
CYP2C19*4	1 A > G rs28399504	AA	1.00(458)	1.00	1.00
		GG	0.00(0)	0.00	0.00
CYP2C19*5	1297 C > T rs56337013	CC	1.00(458)	1.00	1.00
		TT	0.00(0)	0.00	0.00
CYP2C19*17	806 C > T rs12248560	CC	0.978(448)	0.559	0.9777
		CT	0.022(10)	0.388	0.0223
		TT	0.00(0)	0.053	0
		CT	0.00(0)	0.053	0
ABCB1 C3435T	3435 C > T rs1045642	CC	0.358(164)	0.173	0.3616
		CT	0.177(81)	0.581	0.4643
		TT	0.465(213)	0.246	0.1741

The minor allele frequencies of the above six SNPs were 27.1% (CYP2C19*2, rs4244285), 5.9% (CYP2C19*3, rs4986893), 0% (CYP2C19*4, rs28399504), 0% (CYP2C19*5, rs56337013), 1.1% (CYP2C19*17, rs12248560), 40.9% (ABCB1, rs1045642), respectively. The genotype distributions of all SNPs (except rs28399504 and rs56337013) were in Hardy-Weinberg equilibrium ($p > 0.05$). Due to the absence of the variant for rs28399504 and rs56337013, X^2 value could not be defined. According to the results in our study, mutation rates of ABCB1 (rs1045642) is the highest in current population, followed by CYP2C19*2, *3 and *17. The observed CYP2C19 and ABCB1 variant frequencies in our study were also compared with those reported for both Chinese and European populations (Borecki et al., 2017; Santos et al., 2011; Zhuo et al., 2018).

Based on the MassARRAY results and Sanger sequencing verification, seven genotypes were found in our study: *1/*1(636GG, 681GG), *1/*2(636GG, 681GA), *2/*2(636GG, 681AA), *1/*3(636GA, 681GG), *2/*3(636GA, 681GA), *3/*3 (681GG, 636AA) and *1/*17 (-806CT). The frequencies of each genotype were: 43.01%, 35.15%, 8.07%, 8.51%, 2.83%, 0.21% and 2.18%, respectively (Table 2). Among the included 458 samples, we also divided genotypes according to their relevance for clopidogrel metabolism, which could help to predict risk of cardiovascular complications. The highest metabolic type is intermediate metabolism (*1/*2, *1/*3), followed by extensive metabolism (*1/*1), poor metabolism (*2/*2, *2/*3, *3/*3) and ultra metabolism (*1/*17, *17/*17). The frequencies of each type were 43.67%, 43.01%, 11.13% and 2.18%, respectively.

3.3. Performance assessment of MassARRAY and pyrosequencing

Then we evaluated performance of MassARRAY and Pyrosequencing, considering Sanger sequencing as the reference test. We found that concerning CYP2C19*2 and CYP2C19*3, 6 samples have different results (Table 3). Then we performed Sanger sequencing on these 6 samples, all results were the same as MassARRAY, which indicated that the accuracy of MassARRAY is higher than that of Pyrosequencing. Then we analyzed the difference between MassARRAY and Pyrosequencing. Kappa values of CYP2C19*2 and CYP2C19*3 were 0.948 and 0.931, respectively, indicating a concordance between these two methods.

3.4. Operational characteristics

We compared the operational characteristics of these two methods (Table 4). Turnaround time of each sample depends on the throughput and working time per run of the equipment. For both MassARRAY iPLEX and PyroMark Q24, the turnaround times of genotyping of clopidogrel were similar (average 7–8 h). The highest throughput of MassARRAY iPLEX is 384, which means 384 samples are allowed per plate. For each plate, multiplex PCR is available, thus allowing for multiple SNPs detection. As for PyroMark Q24, 24 samples are allowed each run and for each plate only one locus can be tested. Thus, compared with PyroMark Q24, MassARRAY iPLEX can be used for larger number of samples with more genotypes identified and less turnaround time. For both methods, the sensitivity is the same and both require 2ul DNA extraction. In addition, MassARRAY iPLEX can directly analyze the molecular

Table 2. Distribution of CYP2C19 genotypes according to their relevance for clopidogrel metabolism phenotypes in Chinese Han population.

Clopidogrel metabolism phenotype	Genotype (SNP)	Population (frequency %) (n = 458)
Poor metabolizer	*2/*2 (681AA, 636GG)	8.07% (37)
	*2/*3 (681GA, 636GA)	2.83% (13)
	*3/*3 (681GG, 636AA)	0.21% (1)
Intermediate metabolizer	*1/*2 (681GA, 636GG)	35.15% (161)
	*1/*3 (681GG, 636GA)	8.51% (39)
Ultra metabolizer	*1/*1 (681GG, 636GG)	43.01% (197)
Ultra metabolizer	*1/*17 (–806CT)	2.18% (10)
	*17/*17 (–806TT)	0 (0)

Table 3. Comparison of MassARRAY iPLEX and PyroMark Q24 on CYP2C19 genotypes.

Sample number	MassARRAY iPLEX	PyroMark Q24 (CYP2C19*2)	Sanger sequencing
1	GG	GA	GG
2	GG	GA	GG
3	GG	GA	GG
5	GA	GG	GA
6	GG	GA	GG
7	GG	GA	GG

Table 4. Characteristics of the two methods and the gold standard (Sanger sequencing) Available for Clopidogrel Efficacy Genotyping.

	MassARRAY iPLEX	PyroMark Q24	Sanger sequencing
Number of samples per plate	384	24	1
Turnaround time, hours	7–8	7–8	12–24
Reagent cost per plate	\$ 5	\$ 12.3	\$ 20
Number of SNP per run	6 or more	1	1
Operation complexity	Low	Low	Low

weight of amplification product, which is rarely influenced by outside factors, thus generating more precise results. However, PyroMark Q24 analyze through capturing fluorescent signal, which might be affected by efficiency of fluorescence combination. Reagent cost per plate of MassARRAY iPLEX is much lower than PyroMark Q24 since the former method does not require fluorescent reagent and has higher throughput. According to recent studies (Svidnicki et al., 2015), cost of each sample with MassARRAY iPLEX for six SNPs (CYP2C19*2, *3, *4, *5, *17 and ABCB1-3435) is \$5, while cost of each sample with PyroMark Q24 for 2 SNPs (CYP2C19*2 and *3) is about \$12.3.

4. Discussion

Aspirin and clopidogrel are the standard care for patients experiencing acute coronary syndrome (ACS) and/or percutaneous coronary intervention (PCI). Despite the unambiguous clinical benefit of aspirin plus clopidogrel, a number of patients continue to experience major adverse cardiac events (MACE). In order to promote the prediction and accuracy of clopidogrel efficiency in clinical practice, several rapid and precise gene-testing methods have been applied in clinics. However, each method has its defect: Sanger

sequencing requires complex operation and longer time to generate results (Zhang et al., 2013); TaqMan requires expensive probes and are vulnerable to lab contaminant (Carlquist et al., 2013; Cervinski et al., 2013); gene chip, with its low flexibility, needs specific equipment, which is difficult to realize in common clinical labs (Buchan et al., 2011; Chae et al., 2013; Choi et al., 2014). Since doctors require the detection time to be as short as possible, a technique with a high call rate and shorter turnaround time is required.

After the discovery of SNPs associated with a particular phenotype in a genome-wide association studies (GWAS) study, replication of findings will usually be required in a second population. Often only tens of SNPs require genotyping for this phase. MassARRAY is one such platform that allows for tens to hundreds of user-defined SNPs to be genotyped in hundreds to thousands of DNA samples in a high-throughput and cost-effective manner (Ellis & Ong, 2017). MassARRAY is based on MALDI-TOF mass spectrometry, with the soft-ionization technology that allows the analysis of biomolecules. The whole workflow includes mainly the following procedures. Firstly, PCR is employed to amplify the regions of the genome containing each SNP. An 'extension' PCR reaction is then performed, in which an extension primer anneals just proximal to

the polymorphic base, and a single ‘terminator’ nucleotide base extends the DNA fragment by one additional base that is specifically complementary to the polymorphic base. The terminator bases are ‘mass-modified’, whose masses are different between fragments, which is distinguished by a single base are detectable by mass spectrometry (Oeth et al., 2009). Its advantages include the wide range of molecular weight, the fast speed of scanning, the simple steps of operation, and particularly higher sequencing flux when compared with Sanger sequencing.

In the last several years, the range of applications for this platform was significantly expanded. In a multicenter study concerning patients with lung adenocarcinoma suspected of EGFR mutation, when comparing MassARRAY with pyrosequencing and PNAcl, it was indicated that MassARRAY has a higher accuracy for the detection of EGFR mutations in tissue specimens (Min et al., 2016). In another meta-analysis, Tania et al. (Fleitas et al., 2016) found that when used in combination with the OncoCarta™ panels and other customized panels, MassARRAY is cost-effective in terms of screening for somatic mutations in solid tumors. It would allow a rapid decision making, which would improve patient clinical trial inclusion. In addition, the initial cost of MassARRAY (Agena Bioscience, San Diego, CA, USA) ranges between \$300,000 and \$800,000 but it rarely requires routine maintenance. The cost of Pyrosequencing analyzer (PyroMark Q24 MDx, Germany, Qiagen) ranges between \$70,000 and \$100,000 and the routine maintenance costs about \$100 per month. Thus, mass spectrometry has the potential to become an applicable approach in clinical diagnosis and can be used in genetic individualized therapy. Based on MassARRAY platform and iPLEX GOLD technique, it can conveniently analyze as many as 40 genotypes, with flexible and cheap design.

In our study, Chinese Han population was chosen as subjects. Among them, 27.1% carried the loss-of-function allele CYP2C19*2 A, who were poor clopidogrel metabolizers. CYP2C19*2 is the first allele discovered and it contains a single base pair mutation on exon 5 (681G > A), leading to an aberrant splice site. Since the A allele is associated with reduced conversion of clopidogrel to active metabolite. Patients with this allele are more vulnerable for cardiovascular complications including stent thrombosis (Mega et al., 2010). CYP2C19*3 A was found to account for 5.9% of our sample, which is reported to be present only in Asians with frequency from 1 to 6% (Genomes Project et al., 2015). CYP2C19*4 and *5 were not observed in our current research. CYP2C19*17 (TT homozygote)

accounted for 1%; these “ultra-rapid metabolizers” may suffer from increased risk of bleeding events (Li et al., 2012). Almost one-third of our patients possessed CYP2C19 variants phenotypically expressed by alteration of clopidogrel metabolism. In addition, an unexpected 41.5% of patients presented with ABCB1 mutation, which is the gene encoding P-glycoprotein multidrug resistance spontaneous transporters. Therefore, identification of these variants at early stage would allow consideration of alternative drugs for treatment.

To date, at least 28 alleles of CYP2C19 have been identified, and there are ethnic and geographical differences in the distribution of CYP2C19 variants (Kurose et al., 2012). When compared with Caucasian and African populations, CYP2C19*1 allele (wild-type) frequency was significantly lower, while the frequency of CYP2C19*3 allele was higher in the Chinese Han subjects (Ganoci et al., 2017; Masimirembwa et al., 1995). Zhou et al. (2009) compared allele frequencies of Han samples (Beijing) with five other geographical subpopulations of Han Chinese (Hong Kong, Shanghai, Shantou, Shenyang and Xi’an) and observed that the frequencies of CYP2C19*1, *2 and *3 were significantly different from that of Shenyang and Xi’an subpopulations. However, Hu et al. (Hu et al., 2012) reported that the frequency of the CYP2C19*2 allele and the CYP2C19*3 allele found between southern and northern Chinese Han populations are not significantly different. When compared with European countries, the allele frequencies of CYP2C19*4, CYP2C19*5, and CYP2C19*17 in our cohort were much lower (Ferguson et al., 1998; Ghasemi et al., 2016). Three common genotypes that cause the poor metabolizer (PM) phenotype include CYP2C19*2/*2, CYP2C19*3/*3 and one heterozygous genotype CYP2C19*2/*3. The frequencies of CYP2C19*1/*1, *1/*2, *1/*3, *2/*2, *3/*3 and CYP2C19*2/*3 were not significantly different between our current study and previous studies (Dai et al., 2014; Zhou et al., 2009). In addition to the common allele, CYP2C19*6, *18, *23, *24, *25, *27, *29, *33, *34 although quite rare, have also been identified in Chinese Han population (Dai et al., 2014; Zhou et al., 2009).

5. Conclusion

In this study, we investigated two commonly used methods for CYP2C19 and ABCB1 polymorphisms in clinical laboratories among clopidogrel-treated Chinese patients. We found that with MALDI-TOF MS as the detection method, there are opportunities for providing genotyping results in a reasonable time

frame that can facilitate their application. Thus, MassARRAY based on mass spectrometry is an excellent tool for the study of SNPs in patients who are potential candidates for targeted therapies, which should facilitate in the near future its approval for clinical testing. In the future, we aim to collect the clinical data to investigate the association between CYP2C19 and ABCB1 polymorphisms and the antiplatelet effect of clopidogrel.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

Authors' contribution

guarantor of integrity of the entire study: Juan Liu
study concepts: Zesheng Xu
study design: Ya Li
definition of intellectual content: Shipeng Dai
literature research: Junying Liu
clinical studies: Junjun Pan
experimental studies: Yang Jiang
data acquisition: Yang Jiang
data analysis: Junjun Pan
statistical analysis: Junying Liu
manuscript preparation: Juan Liu
manuscript editing: Ya Li
manuscript review: Shipeng Dai

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