Jian-Min Xiao, Jie Xiong, Guan-Yang Kang, Qiang Li, Zhong-Yong Jiang, Jiu-Hao Chen, Li-Ling Wang, Fu-De Yao and Jia-Wu Song*

A novel biosensor-based microarray assay for the visualized detection of *CYP2C19* *2, *3, *4 and *5 polymorphisms

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Abstract

Background: A number of studies have indicated that the conversion of clopidogrel to its active metabolite is reduced in patients who carry the CYP2C19 *2, *3, *4 or *5 loss-of-function allele, resulting in decreased response of platelet to clopidogrel treatment and worse cardiovascular outcome. The aim of this study was to develop a novel biosensor-based microarray to visually detect CYP2C19 polymorphisms.

Methods: The target DNA was amplified from regions flanking the respective alleles using 5′-biotinylated reverse primer, and plasmids were prepared for the respective alleles. High stringency reversed hybridization, horseradish peroxidase-labeled streptavidin reaction, and color development, with multiple washes in different steps, were carried out and the results were recorded with an optical camera. The gene chips were tested for specificity, detection limit, intra- and inter-batch variations using the constructed plasmids. Finally, 88 clinical samples were assayed with this microarray as well as direct sequencing.

Results: The results could be seen with the naked eye. Concordance tests indicated that for alleles *2, *3, *4, and *5, the κ values between this assay and plasmids all reached 1.000. The detection limit was 5×10^2 cells/mL. Concordance test between direct sequencing and the microarray assay using 88 clinical samples gave rise to the κ value of 0.983, and $p<0.01$, indicating very high concordance.

Conclusions: This novel biosensor-based microarray assay can amplify the signal in situ so that it can be detected by simple instruments or even the naked eyes. It is promising for clinical application in hospital laboratories.

Keywords: biosensor; clopidogrel; CYP2C19; microarray; polymorphism.

***Corresponding author: Jia-Wu Song,** PhD, Department of Gastroenterology, the Fifth Affiliated Hospital of Sun Yat-Sen University; Zhuhai Sinochips Bioscience Co. Ltd., 4F, Building #2, 24 Jinfeng Road, West, Zhuhai City, Guangdong 519085, P.R. China, Phone: +86 756 3318449, Fax: +86 756 3318499, E-mail: songjw@mail.sysu.edu.cn

Jian-Min Xiao and Qiang Li: Department of Cardiology, the Dongguan Affiliated Hospital of Medical College of Jinan University, the Fifth People's Hospital of Dongguan (also called Taiping People's Hospital of Dongguan), Guangdong, P.R. China

Jiu-Hao Chen: Department of Pediatrics, the Dongguan Affiliated Hospital of Medical College of Jinan University, the Fifth People's Hospital of Dongguan (also called Taiping People's Hospital of Dongguan), Guangdong, P.R. China

Jie Xiong and Zhong-Yong Jiang: Department of Clinical Laboratory, Chengdu Military General Hospital, Chengdu Military District, Sichuan, P.R. China

Guan-Yang Kang: Department of Cardiology, the Dongguan Affiliated Hospital of Medical College of Jinan University, the Fifth People's Hospital of Dongguan (also called Taiping People's Hospital of Dongguan), Guangdong, P.R. China; and Medical College of Shantou University, Guangdong, P.R. China

Li-Ling Wang and Fu-De Yao: Zhuhai Sinochips Bioscience Co. Ltd., Guangdong, P.R. China

Introduction

Dual antiplatelet therapy with acetylsalicylic acid (ASA) and clopidogrel has revolutionized the treatment of patients with acute coronary syndrome and those undergoing percutaneous coronary intervention (PCI) [1, 2]. However, the occurrence of acute or subacute thrombosis after a coronary event or implantation of coronary stents remains a serious clinical problem. Recent studies have demonstrated that there is distinct response variability to clopidogrel antiplatelet therapy interindividually which can lead to the increased risk of recurrent cardiovascular events [3–5].

Clopidogrel is a prodrug which is converted into active metabolite capable of inhibiting platelet function by *CYP2C19* enzyme, a member of the hepatic cytochrome P450 family [6]. Numerous polymorphisms in *CYP2C19* have been identified that can result in increased or decreased activity of this enzyme and individuals can be classified as the phenotype of ultrarapid metabolizers (UMs), extensive

metabolizers (EMs), intermediate metabolizers (IMs) and poor metabolizers (PMs) according to the polymorphisms of *CYP2C19*. A number of studies have indicated that the conversion of clopidogrel to its active metabolite is reduced in patients who carry the *CYP2C19* *2, *3, *4 or *5 loss-of-function allele, resulting in decreased response of platelet to clopidogrel treatment and worse cardiovascular outcome [7, 8]. Besides, it has been observed that increasing the dose of clopidogrel in patients carrying the loss-of-function polymorphisms can increase antiplatelet response, and a second or third clopidogrel loading dose was demonstrated to reduce platelet reactivity during the therapy in these patients [9, 10]. Therefore, a *CYP2C19* polymorphisms genotyping assay with a convenient and accurate method could be beneficial in the appropriate dosing of clopidogrel based on the genotype of the patients or allowing selection of an alternative antiplatelet drug.

Genotyping assays for CYP2C19 polymorphisms can be performed with numerous molecular methods [11–16], such as restriction fragment length polymorphism (RFLP), direct sequencing of PCR products, real-time PCR, single-strand conformation polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE), fluorescence-based high-resolution melting analysis (HRMA), amplification refractory mutant system (ARMS) technology, flow cytometric assay, and DNA microarray chip-based method. Although the comparison of different genotyping methods is beyond the scope of this paper, some of these tests are not suitable for applications in routine clinical diagnosis in terms of simplicity, performance, sensitivity, overall testing time, and price. For example, direct sequencing is technologically complicated, time-consuming and needs special expertise. RFLP is almost impossible to be used for the detection of all significant polymorphisms since it requires a specific cutting site and restriction enzyme for the particular site. There is a real need for a highly sensitive and specific assay for the genotyping of *CYP2C19* polymorphisms that is easy to perform and compatible with clinical practice.

The aim of this study was to develop a novel biosensor-based microarray (BBM) which can provide a direct visual readout in a convenient and accurate manner for genotyping of the *CYP2C19* polymorphisms, and to assess the performance of this BBM assay in the clinical practice.

Materials and methods

BBM chip preparation

Eight probes, highly specific for wild-type and variant forms of *CYP2C19**2, *3, *4, and *5 polymorphisms, plus positive and negative probes (10 mM Tris·HCl, 1 mM EDTA buffer, pH 8.0), and quality control probes were designed and synthesized (Invitrogen, Shanghai, China). The positive control probes were used to monitor the assay process. The negative probes were used to monitor contamination and the quality control probes were used to monitor the gene chip production quality. All of the probe sequences included in this study were from public database (http://www.ncbi.nlm.nih.gov/) and were based on the known human genome in the *CYP2C19* region (GenBank accession number NG_008384.2). After optimization, the probes (approximately 5 nL at 5 μM concentration) were arrayed on the BBM chip using an AD1500 workstation (BioDot, Irvine, CA, USA). The probes were then covalently cross-linked to the pre-treated silicon chips which have amino radicals on its surface by UV irradiation, the dimension of each chip was 6.5 mm×6.5 mm×0.7 mm. The probe sequences are summarized in Table 1. Three gene chips were mounted in place in three square grooves (about 3 mm in depth and slightly larger than the chips) of a plastic plate (76.2 mm×25.4 mm×4 mm). The grooves served as the mini containers (the holding capacity of each container is 100 μL liquid) for subsequent processes, such as hybridization, washing, horseradish peroxidase-labeled streptavidin reaction, and color development.

DNA extraction and PCR amplification

Human genomic DNA was extracted from 200 μL of patient blood samples using 200 μL commercially available DNA extraction kit (Qiagen, Dusseldorf, Germany) according to the manufacturer's instructions. The final elution volume was 50 μL.

To determine the *CYP2C19* genotypes, DNA fragments in the *CYP2C19**2, *3, *4, and *5 regions were amplified by polymerase chain reaction (PCR) using the primers as listed in Table 1. The

Table 1 Sequences of primers and probes.

PCR primers and probes	Sequences (5′–3′)
CYP2C19*2-Fwd	AATTACAACCAGAGCTTGGC
CYP2C19*2-Rev	Bio-TATCACTTTCCATAAAAGCAAG
CYP2C19*3-Fwd	AAATTGTTTCCAATCATTTAGCT
CYP2C19*3-Rev	Bio-ACTTCAGGGCTTGGTCAATA
CYP2C19*4-Fwd	GGAGTGCAAGCTCATGGTTG
CYP2C19*4-Rev	Bio-TTGGTTAAGGATTTGCTGACA
CYP2C19*5-Fwd	TCCCTATGTTTGTTATTTTCAGG
CYP2C19*5-Rev	Bio-GAGCAGCCAGACCATCTGTG
CYP2C19*2-G681G	Ald-tttttttttGATTATTTCCCGGGAACCCA
CYP2C19*2-G681A	Ald-tttttttttGATTATTTCCCAGGAACCCA
CYP2C19*3-G636G	Ald-tttttttttCACCCCCTGGATCCAGGTA
CYP2C19*3-G636A	Ald-tttttttttCACCCCCTGAATCCAGGTA
CYP2C19*4-A1A	Ald-tatatatataGAAGGCTTCAATGGATCCTTT
CYP2C19*4-A1G	Ald-AtatatatatGAAGGCTTCAGTGGATCCTTT
CYP2C19*5-C1297C	Ald-ttttttttaaTTCAGGAAAACGGATTTGTGTG
CYP2C19*5-C1297T	Ald-ttttttttaaTTCAGGAAAATGGATTTGTGTG
Positive control probe	Ald-tttttttttttGATTATTTCCCRGGAACCCA

Ald, aldehyde; Bio, biotin; Fwd, forward primer; Rev, reverse primer. Underlines indicate the points of mutation.

reaction mixture (25 μL) contained 2.5 μL DNA as template, 2.5 μL of 10 μmol/L buffer (Juntan, Shanghai, China), 0.5 μL deoxynucleoside triphosphates (Roche, Basel, Switzerland), 0.75 μL each of forward and reverse primers (Invitrogen), 1.25 U (1.25 μL) hot Taq polymerase (Juntan), and 16.75 μL high-performance liquid chromatography-grade water. PCR amplification was carried out under the following conditions: an initial denaturation at 95 °C for 4 min, 40 cycles at a denaturation temperature of 94 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 30 s, with a final prolonged extension at 72 °C for 10 min. The PCR products (5 μL) were visualized on 1.5% agarose gels.

Preparations of allele-specific plasmids and clinical samples

DNA sequences containing the wild-type forms of *CYP2C19**2, *3, *4, and *5 alleles were first obtained by PCR amplification using primers listed in Table 1 and genomic DNA preparation from a healthy volunteer effective metabolizer of clopidogrel as template, then plasmids containing the desired sequences were constructed by TOPO TA cloning kit (Invitrogen), and the cloned sequences were further confirmed by direct sequencing. Plasmids containing variant forms of *2, *3, *4, and *5 were obtained by site-specific mutagenesis.

Blood samples obtained at Taiping People's Hospital of Dongguan (Dongguan City, Guangdong, China) were used to evaluate the detection specificity and detection limit of the BBM assay in clinical applications. A survey of 88 samples was carried out with both BBM assay and direct sequencing. The study protocol was approved by the Ethics Committee of the Dongguan Taiping People's Hospital and all patients enrolled in this study provided written informed consent.

Detection of *CYP2C19* genotypes

PCR amplicons were subjected to reversed hybridization and Sanger's dideoxy-sequencing method. A 10 μL volume of PCR-amplified product was denatured at 99 °C for 5 min, then immediately cooled for 5 min on ice. The amplified product was placed on the surface of the BBM chip and the procedures from hybridization, washing, horseradish peroxidase-labeled streptavidin reaction, color development to photographing was carried out using an SLQ-108A automated BBM chip processor (Sinochips Bioscience Co., Zhuhai, China). The hybridizations were carried out at 50 °C for 60 min by adding 50–60 μL hybridization buffer [20×SSC (3 M NaCl and 0.3 M trisodium citrate), 5% sodium dodecylsulfate (SDS), and 0.1% Triton X-100, pH 7.6]. The BBM chips were soaked in wash buffer A (0.2×SSC, 0.05% SDS) pre-warmed to 45 °C for 1 min, the wash buffer was discarded and the chip surface was air dried, then incubated with 1:5000 to 1:8000 diluted horseradish peroxidase-labeled streptavidin (Vectorlabs, Shanghai, China) in 20×SSC at room temperature (20–30 °C) for 10 min, then rinsed three times with wash buffer B (0.1×SSC), then incubated with tetramethylbenzidine (TMB) for 2 min in the dark. Finally, the residues on the BBM were washed in 0.1×standard saline citrate and distilled water at room temperature to boost signal/noise ratio. The remaining amplified PCR product was sent for direct sequencing (GenScript, Nanjing, China).

Assay specificity

Eight synthetic plasmids, each incorporating wild-type and variant alleles (*CYP2C19**2, *3, *4, and *5, i.e., 681G, 681A, 636G, 636A, 1A, 1G, 1297C, and 1297T) and 88 general survey blood samples were used to validate the specificity of the assay to detect the four alleles. The specificity of the four alleles was tested individually. The plasmids diluted to 10^4 copies/mL were used to determine the assay specificity of the BBM. Blindly arranged plasmid DNA was tested by the BBM assay, and the results obtained were compared with the variation represented by the known plasmids. We also tested the variations within and between batches of the BBM assay by carrying out 10 tests for each batch. For the clinical evaluation, PCR amplicons obtained from patients' blood samples were tested under the same conditions as the plasmid DNA. The percent agreement of the results of BBM assay with the results of direct sequencing was determined.

Detection limit of the BBM assay

To estimate the detection limit of the genotyping assay in clinical practice, 10 blood samples were used. The white blood cell (WBC) counts were determined in each sample and then diluted to 10^4 cells/mL, serial dilutions were made between 10^2 and 10^4 cells/mL. The DNA was extracted from 200 μL of each sample and was amplified. The lowest level of WBC counts in which all 10 detections were successful was determined as the detection limit.

Statistical analysis

The κ coefficient was used to measure the agreement between direct sequencing and the BBM assay for the detection of alleles and genotypes. A κ value >0.75 was defined as substantial agreement, and a κ value >0.95 as perfect agreement. A $p<0.05$ was considered statistically significant.

Results

PCR amplification of DNA fragments spanning CYP2C19*2, *3, *4, and *5

Figure 1 shows the result of gel electrophoresis for PCR-amplified products of DNA fragments spanning CYP2C19*2, *3, *4, and *5, respectively. There was only one clear band for each PCR reaction which was consistent with the length of the desired target fragment.

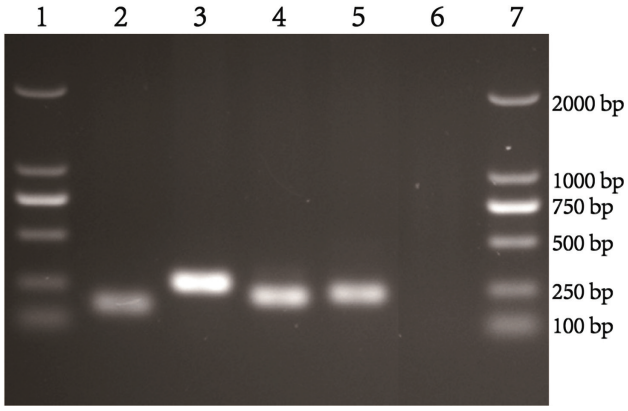

Figure 1 PCR amplification products of DNA regions containing CYP2C19*2, *3, *4, and *5 SNPs visualized on polyacrylamide gel electrophoresis.
Lanes 1 and 7 MW markers; lane 2: *2 region; lane 3: *3 region; lane 4: *4 region; lane 5: *5 region; lane 6: negative control.

BBM assay specificity

To demonstrate that the CYP2C19*2 allele was correctly identified from its wild-type counterpart, plasmids containing 681G and 681A were used to mimic the *1/*1 and *2/*2 genotypes, and the 1:1 mixture of 681G/681A plasmid DNA was use to mimic the *1/*2 genotype. Blind tests were carried out by the BBM assay to evaluate the concordance. The results are summarized in Table 2, and the κ value was 1.000. Similar tests were conducted for CYP2C19*3, *4, and *5, and the κ values were also 1.000 (see Supplementary Material, Supplementary Tables 1–3, which summarize the test results).

Sensitivity and detection limit of BBM assay

Ten clinical samples were normalized to WBC counts of 10^4 cells/mL, further dilutions were made between 10^2 and 10^4 cells/mL. DNA was extracted from 200 μL of each sample and was amplified. The lowest level of WBC counts in which all 10 samples detected successfully was found to be 5×10^2 cells/mL (data not shown). Variations within and between batches of gene chips were also tested by carrying out 10 tests for each batch. The test results indicated that the gene chips produced in various batches had <5% variation (data not shown).

Table 2 Concordance of BBM assay with given plasmids for *CYP2C19*2*.

BBM assay	Plasmids with given genotype			Total
	*1/*1	*1/*2	*2/*2	
*1/*1	10	0	0	10
*1/*2	0	10	0	10
*2/*2	0	0	10	10
Total	10	10	10	30

κ=1.000. BBM, biosensor-based microarray.

Survey of poor and intermediate metabolizers of clopidogrel

Paralleled tests of BBM assay and direct sequencing were performed for 88 samples and the typical genotypes found are summarized in Figure 2. Only heterozygous genotypes including *1/*2, *1/*3, and homozygous genotype *1/*1 (at positions *2 and *3), *2/*2 were found in this experiment. Other genotypes were not detected in this sample set. We found the discordant result in one of 88 samples because of the unclear and ambiguous signal in both *1/*1 and *1/*2 positions. The sample had been retested which generated a definitive signal in *1/*2 position. Table 3 summarized the test results, and κ test indicated that the κ value was 0.983, with $p<0.01$.

We performed a small-scale survey (n=88) for EM, PM, and IM of clopidogrel, the results indicated that EM, IM, and PM accounted for 43.2% (*1/*1), 40.9% (the sum of *1/*2 and *1/*3 which accounted for 34.1% and 6.8%, respectively), and 15.9% (*2/*2), respectively (see Supplementary Material, Supplementary Figure 1).

Discussion

The combined therapy of ASA plus clopidogrel is currently the standard of care for patients with acute coronary syndrome and those undergoing PCI around the world. However, it has been reported that 4%–30% patients with coronary artery disease do not respond to the clopidogrel antiplatelet therapy adequately [17]. Several factors including inappropriate dosing, poor absorption of the prodrug, variable clearance of the active metabolite, potential drug-drug interactions, diabetes mellitus, elevated body mass index, genetic polymorphisms of cytochrome P450 isoenzymes and so on, have been proposed to influence individual response to clopidogrel therapy [18–22]. Since the transformation of the clopidogrel prodrug to its active metabolite is dependent on the CYP2C19 enzyme, plenty of studies had found that the *CYP2C19* loss-of-function polymorphisms are associated with the poor metabolism of clopidogrel and thus worse cardiovascular outcome [4, 23]. Therefore, the development of a convenient and

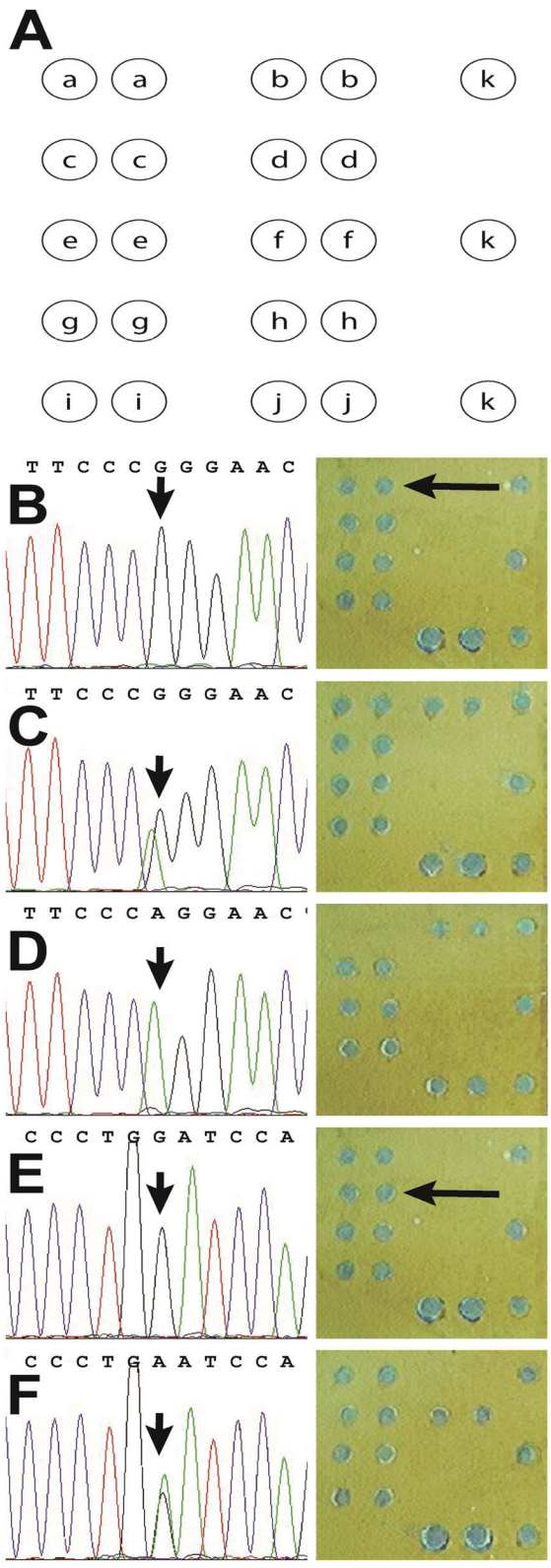

Figure 2 Comparison of direct sequencing and BBM assays for *CYP2C19* genotypes in clinical samples.
(A) the probes were arrayed on the gene chips in the following order: a. 681G (**2* w.t.); b. 681A (**2*); c. 636G (**3* w.t.); d. 636A (**3*); e. 1A (**4* w.t.); f. 1G (**4*); g. 1297C (**5* w.t).; h. 1297T (**5*); i. negative control; j. positive control; k. quality control. (B–F) Sequencing and BBM assay results for homozygous wild-type (**1/*1* at position **2*), **1/*2*, **2/*2*, homozygous wild-type (**1/*1* at position **3*), and **1/*3*, respectively. The vertical arrows indicated the points of variations. The left-ward arrows indicated the wild-type forms at positions **2* and **3* (note that their BBM assays had the same result but the positions were different), respectively.

accurate assay for the genotyping of *CYP2C19* polymorphisms will help both clinicians and patients choose the treatment regimen which can provide an appropriate dosing of clopidogrel or permit change to other antiplatelet agents.

Microarray-based tests have emerged as important approaches for genotyping [16, 24]. We have previously reported a rapid and simple biosensor-based microarray assay for detecting the genotypes of IFNL3 (formerly IL28B) [25]. The assay has high specificity, reproducibility and low detection limit, and has passed the certification of State Food and Drug Administration of China. This indicates that the assay is suitable for clinical application. Herein, as there is a real need for a highly sensitive and specific assay for the genotyping of *CYP2C19* polymorphisms that is easy to perform and compatible with clinical practice, the BBM described in this study may be an ideal clinical assay.

As depicted in Figure 2, the positive signals on the BBM chip were sufficiently distinct to be completely discriminated from negative results. The results with plasmid amplicons showed that all the polymorphism alleles could be detected successfully and that heterozygous and homozygous alleles could be distinguished. Of 120 plasmid amplicons employed to validate the specificity of the BBM assay, a complete agreement between the results of the BBM assay and known plasmid sequences was observed, confirming the high specificity of the BBM assay in determining the genotypes of *CYP2C19* polymorphisms.

Table 3 Concordance of BBM assay with PCR sequencing of the blood samples for *CYP2C19* genotypes.

BBM assay	Direct sequencing				Total
	**1/*1*	**1/*2*	**2/*2*	**1/*3*	
**1/*1*	38	1	0	0	39
**1/*2*	0	29	0	0	29
**2/*2*	0	0	14	0	14
**1/*3*	0	0	0	6	6
Total	38	30	14	6	88

κ=0.983. BBM, biosensor-based microarray.

Further tests indicated that the detection limit was the cell density of 5×10^2 cells/mL. As the WBC count of human peripheral blood is generally $>10^3$ WBC/μL, the detection limit of the BBM assay ensures that clinical blood samples would be tested accurately. This novel method thus met the sensitivity criteria for clinical diagnosis. Variation within and between batches of production is <5%. The above tested indicated that the gene chip met all of the design specifications.

Parallel tests by direct sequencing and BBM assay were carried out for 88 clinical samples. The BBM assay correctly identified nearly all of the genotypes, including heterozygous ones. The κ value was 0.983, and $p<0.01$, indicating the concordance between the BBM assay and direct sequencing is very high and the BBM would be a satisfactory novel assay when used for determination of the genotypes of *CYP2C19* polymorphisms in the clinical practice. In our initial paralleled tests of BBM assay and direct sequencing, we found the discordant result in one of 88 samples because of the unclear and ambiguous signal in both *1/*1 and *1/*2 positions. The sample had been retested which generated a definitive signal in *1/*2 position and revealed 100% concordance rate for all 88 samples that were analyzed between BBM assay and direct sequencing. A possible explanation for this would be the PCR amplification of non-specific target DNA hybrided with the probes which resulted in unclear signal in the gene chips. The calculated minor allele frequencies for *2 and *3 were 33.0% and 3.4%, respectively, which were very close to 32.0% (*2 allele for Chinese) and 5.8% (*3 allele for Chinese in Beijing and Japanese in Tokyo) in the HapMap project.

The results indicated that the BBM assay has the advantages of convenient operation as well as accurate detection. After a successful PCR amplification, the process from hybridization, washing, horseradish peroxidase-labeled streptavidin reaction, color development to image analysis have all been integrally automated and left unattended without any human intervention. More importantly, the signal on the chip was clear enough to be interpreted even by the naked eye, which meant that only an ordinary digital camera was needed to record the results. The assay is also suitable for clinical diagnostic and research laboratories where large numbers of samples are tested on a daily basis. In short, the BBM assay is a very clinically promising protocol.

In comparison with existing assays, this BBM assay has the advantages of simple operation and visual results. In an earlier study, we have demonstrated a BBM assay for the detection of two polymorphisms in IFNL3 gene responsible for the prognosis of the regimen of pegilated interferon α plus ribavirin for hepatitis C virus infection in humans [25]. This study further proved that the biosensor-based microarray assay for the *CYP2C19* *2, *3, *4 and *5 polymorphisms was also highly reliable.

The BBM assay has some disadvantages to overcome in its future development. The patient population benefiting from the BBM assay is small, meanwhile, the assay is only capable of detecting the presence of known polymorphisms. With the identification and detection demand of more polymorphisms associated with clopidogrel resistance, novel probes have to be designed and the BBM assay has to be constantly upgraded.

Conclusions

In conclusion, we have demonstrated that the BBM assay is a visual, accurate, and promising clinical application for hospital laboratories and clinics to aid in guiding antiplatelet therapy and improving patient outcome. The assay has highly sensitivity and specificity to detect the genotypes of *CYP2C19* polymorphisms and can facilitate the acquisition of genotyping data to better translate this knowledge into diagnostic and therapeutic applications.

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