Detection of the Polymorphism of 19 SNPs Associated with the Metabolism of Anti-CVD Drugs by Multiplex PCR-LDR in CVD Patients

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ABSTRACT Cardiovascular diseases (CVD) are worldwide threat to human health, resulting in the highest mortality among all causes of death. The effect of therapeutic medicines for CVD varies greatly among people because of their different genetic background. In order to evaluate the necessity of having genetic testing before taking medicines in CVD patients, 19 SNPs influencing the metabolism of frequently-used anti-CVD drugs were selected and detected in 237 CVD patients by PCR-LDR method. The results showed that the genotypic distribution of most SNPs met the Hardy–Weinberg principle. The allelic distribution of the SNPs in the tested samples was similar to that in Chinese population. 78 percent of these patients carried at least one allele that affected the efficacy of the medicines. The accuracy of the PCR-LDR detection for the clinical samples was comparable to that of Sanger sequencing, and with higher multiplexity and lower cost.

INTRODUCTION

Cardiovascular diseases (CVD) refer to ischemic or hemorrhagic diseases in the heart (sometimes brain) caused by hyperlipidemia, blood viscosity, atherosclerosis and hypertension etc. CVD are worldwide threat to human health, resulting in the highest mortality among all causes of death. In China, about 270 million people suffer from CVD, and the death rate was 309.33/100,000 in the rural and 265.11/100,000 in the urban area of China, representing above 43 percent of total mortality in 2016 (Hu et al. 2019). The common clinical medicines for CVD include Aspirin, Clopidogrel, Warfarin, Nitroglycerin and Statins. However, the same medicine may have distinct effects on different patients, partly because of their different genetic background. The genes of COX-1, GPIIb/IIIa, CYP2C19, CYP2C9, VKORC1, ALDH2 have been reported to play a role in the absorption, distribution, metabolism and excretion of these medicines, and the single nucleotide polymorphisms (SNPs) on these genes affecting their functions have been disclosed.

Warfarin is the most widely-used anticoagulants in clinic, while it causes bleeding and embolism in some people (Cooper et al. 2008). The polymorphisms on the gene of VKORC1 and CYP2C9 are the main genetic factors that influence the individual differences in treatment dose of warfarin (Rost et al. 2005). CYP2C9 is highly genetic polymorphic, its two main mutant versions are CYP2C9*2 and CYP2C9*3, which code the enzymes with activities 30 and 80 percent lower than the wild type CYP2C9*1, respectively. Several SNPs on VKORC1 were found to be related to individual difference in dose, two common versions are 1173C>T and 3730G>A. The effective dose for people with 1173CC (6.2 mg/
d) is much higher than those with 1173TT (4.8 mg/d) (Yuan et al. 2005).

Clopidogrel is an antiplatelet agent. Clopidogrel is used in patients with acute coronary syndrome (ACS) and percutaneous coronary stent implantation for if reduces the risk of myocardial infarction (MI) and stroke in these patients (Plosker et al. 2007). Clinical research indicated that some patients were Clopidogrel poor metabolizers as they had no enzymatic activity to activate Clopidogrel via the CYP2C19 pathway, therefore the drug had no effect. Approximately 14 percent of Chinese people are CYP2C19 poor metabolizers. CYP2C19*2, *3, *4, *5, *7, *8 are the mutant version of CYP2C19 that account for the lower activity of the enzyme, CYP2C19*17 is the only mutant with increased function (Frère et al. 2009; Pettersen et al. 2011; Tantry et al. 2011).

Aspirin resistance is usual in CVD patients, which raises the risk of acute myocardial ischemia (ACS) and cardiovascular ischemia. Mutations on GPb III a PLA2, PEAR1 and PTGS1 genes are related to Aspirin resistance (Li et al. 2014; Varenhorst et al. 2015; Li et al. 2016; Liu et al. 2016; Peng et al. 2016). The metabolism of nitroglycerin relies on ALDH2 (Aldehyde Dehydrogenase II), which helps to transfer nitroglycerin to Nitric Oxide. An SNP (G1510A) on its coding region reduces the activity of the enzyme significantly, and the SNP carriers are mainly distributed in East Asia including China they are not suitable for taking nitroglycerin in the case of angina pectoris (Zhang et al. 2013). Moreover, SNPs on the gene of SLCO1B1 (rs4149056) and APOE (rs429358 and rs7412) influence the metabolism of statin.

With increasing attention paid to the individualized medication in clinic, various techniques have been used in clinical diagnosis for SNPs. DNA sequencing, Taqman PCR and Restriction Fragment Length Polymorphism (RFLP) used to be golden standard in traditional SNP typing (Zhang et al. 2016). Allele-specific PCR, molecular beacon and minisequencing (Sequenom) make SNP typing more accessible and popular. However, these methodologies have distinct sensitivity, specificity and multiplexity, as well as cost based on their respective principles. Taqman PCR and molecular beacon are of satisfactory specificity, but limited multiplexity.

The complicated procedure of RFLP makes it tedious and time-consuming though it’s cost effective and can be manipulated in routine laboratory. SNP typing can also be accomplished by special instrument, such as Sequenom, which is based on the technology of primer extension and mass spectrometry. Sequenom can provide satisfactory accuracy and multiplexity, but its application is limited by expensive instrument and consumables.

PCR-LDR (ligase detection reaction) is an ideal solution for media throughput SNP typing (Li et al. 2009). Ligase-based technologies rely on the capacity of DNA ligase to discriminate a single nucleotide difference. A pair of DNA probes is ligated if they are complementary at the ligation site (SNP locus) to the PCR amplimers. The LDR reaction can be multiplexed by designing probes with different length for distinct loci. The fluorescence-labeled products can be easily separated and identified on a DNA sequencer. PCR-LDR is convenient to test tens of SNPs in a single tube with relatively low cost, that’s why it was widely used in detecting the polymorphism of several loci related to one or two diseases or phenotypes in hundreds of subjects (Cai et al. 2019; Wu et al. 2019; Zhang et al. 2019). Multiplex PCR-LDR can be an appropriate option in testing a panel of SNPs related to clinical medications for CVD.

METHODOLOGY

Sample Collection and DNA Preparation

237 blood samples were collected from the patients came to Songjiang Central Hospital for treatment because of cardiovascular diseases including hypertension, Coronary heart disease, chronic heart failure, Hyperlipidemia or miocardial infarction, etc. The information of the patients is listed in Table 1. The patients were drawn 2ml of venous blood from their arms, and were informed that their blood would be used for research besides normal inspection. The blood sample was collected by using ethylene diamine tetra acetic acid (EDTA) tubes and then stored in a -80°C freezer for further investigation. This study was approved by the Ethics Committee of Shanghai Songjiang Central Hospital and every patient included signed written
consent. 200 μL blood was used to extract DNA using an Axygen DNA Extraction Kit according to the manufacturer’s instruction. The quality of the DNA was checked by 0.8 percent agarose electrophoresis and the concentration was estimated by the value of OD260/280.

PCR

19 SNPs related to the metabolism of anti-CVD medicines are listed in Table 2. PCR primers for each SNP locus are listed in Table 3. Nineteen pairs of primers were divided into two

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<th>Alleles</th>
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</tr>
<tr>
<td></td>
<td></td>
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<td>G&gt;A</td>
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<td>statin</td>
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<td>AGCTCCCTGAGGCCTGAG</td>
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<td>rs671</td>
<td>TGGTGGCTAAGATGTCCCG</td>
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<td>AGGTGGAGGTTATCCGTATGC</td>
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</table>
groups. Primers in the same group were mixed together for multiplex PCR. Group 1 contained primers for 10 SNPs (rs4149056, rs12248560, rs4986893, rs10306114, rs4343, rs5186, rs4244285, rs1057910, rs776746, rs2108622), and group 2 for 9 (rs429358, rs671, rs5918, rs7412, rs9923231, rs5065, rs1801253, rs1065852, rs12041331). 15 μl PCR mixture contained 1.5mM MgCl₂, 0.2mM each dNTP, 2 μl of primer mix, 2 μl DNA template, 1U of HotStar Taq polymerase (Takara Co. LTD). PCR cycling conditions were: 10 min of denaturation at 95 °C, 40 cycles of 30 s at 95 °C, 90s at 56 °C, 1 min at 74 °C, and a final 10 min extension step at 72 °C, 10 min at 98 °C to denature the Taq polymerase.

LDR

LDR probes are listed in Table 4. For each SNP site, three probes were provided: one was the modify probe and the others were discriminating probes for the two different alleles. Modify probe was composed of three parts: complementary sequence to the target region, stuffing sequence with variable length and universal sequence for ligation to a Fam labeled universal probe templated by a universal template. Discriminating probes differed in the length and the last nucleotide complementary to the different alleles. The probes for each SNP locus were grouped according to the multiplex PCR grouping mentioned above. The oligonucleotides for LDR were mixed together at the ratio as: discriminating probe (Allele 1): discriminating probe (Allele 2): modify probe: universal probe: universal template=1: 1: 2: n: n (n referred to the number of SNPs tested in the same reaction, ex: for group1, n=10).

LDR was carried out in 5 μl reaction mixture consisting of 20 mM Tris–HCl (pH 8.5), 5 mM MgCl₂, 100 mM KCl, 10 mM DTT, 1 mM NAD⁺, 1 U of Taq DNA ligase (New England Biolabs, USA) with 2μl probe mixture and 2 μl of PCR product. The reaction program was performed as follows: an initial heating at 94 °C for 2 min, followed by 30 cycles of 15 s at 94 °C and 25s at 50°C.

The LDR products were loaded on ABI 3730XL sequencing instrument (Applied Biosystems Incorporation, USA) and electrophoresed with 3 KV for 30 minutes and the data analysis was conducted using GeneMapper software, version 3.3 (Applied Biosystems Incorporation, USA).

RESULTS

Method Assessment

In order to do the genotyping for 19 SNPs simultaneously, probes for LDR detection were designed with different lengths. Probes for the same locus differed in 2nt, and those for different locus differed in at least 3 nt. The results showed that 19 SNP loci can be clarified clearly in a single lane at one time (Fig. 1). 3 SNP loci (rs1057910, rs776746 and rs4244285) were checked by Sanger sequencing in 8 samples, and the results confirmed those from the PCR-LDR detection (Fig. 2).

The Genotypes of the 19 SNP in Clinical Samples Detected by PCR-LDR

237 patients included in this research all accepted antithrombotic therapy. The genotypic distributions, allelic frequencies of 19 SNPs in all subjects were listed and compared with the data from public databases, the genotype distribution of 17 SNPs except for rs4343 and rs2108622 met the Hardy-Weinberg principle (p<0.05) (Table 5). The results showed that the genotypic distribution in the patients was similar to that of the large population. However, only 22 percent people had no variations affecting drug metabolism, 78 percent people carried at least one variation: 99 people were affected by one variation, representing 42 percent; another 25 percent people had 2 SNPs, and 3 people carried as many as four variations (Table 6).

DISCUSSION

The researchers applied PCR-LDR to do the genotyping for 19 SNPs, grouping them into two panels. Each panel can be detected by multiplex PCR and LDR in one tube. Both specific (discriminating probe) and universal (modify probe) LDR probes were applied in the multiples LDR detection, and a universal ligation template was used to help the ligation of the two kind of probes.
### Table 4: LDR probes

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Probes with different length were designed to clarify different allele of a single SNP, as well as different locus. The stuffing sequence could be included in either modify probe or discriminating probe to widen the range of detection. Only one probe needs to be fluorescent labeled as it can be integrated in all of the LDR products. Comparing with labeling discriminating probe for every SNP, this method is much more economic. The results of this study showed that the accuracy of PCR-LDR detection is comparable to that of Sanger sequencing, while with higher multiplexity and lower cost. PCR-LDR method is an efficient clinical detection tool with median multiplex and low cost. Cai et al. (2019) detected eight SNPs on Complement C3 gene in 1017 subjects by using PCR-LDR method, and concluded that C3 gene polymorphisms were associated with the lipid levels, but not Coronary artery disease susceptibility in Chinese population. Multiplex LDR was also used in genotyping the polymorphisms on the genes of interferon gamma (IFNG) and IFNG receptor 1 (IFNGR1), six SNPs were detected in 565 subjects (Wu et al. 2019).

Clinical treatment has entered in the precision medicine era, after the successful application in oncology and cystic fibrosis diagnosis and treatment, precision medicine is playing an increasingly important role in the treatment of cardiovascular diseases (Dainis et al. 2018). Take warfarin as an example, Pirmohamed et al. (2013) reported that genetic testing in warfarin dosing decisions significantly increased the amount of time patients spent in a therapeutic indicator range. A research in Asian patients showed that genotype-guided warfarin dosing group required fewer dose titrations during the first 2 weeks (Syn et al. 2018). The researchers tested 237 people for their genotype of 19 SNPs affecting the metabolism of anti-CVD medicines. Most of the patients needed this kind of detection to guide their medicine selection, as 78 percent of them carried at least one related allele of these SNPs. Gene testing before medication can be helpful

<table>
<thead>
<tr>
<th>SNP ID</th>
<th>Sequence</th>
<th>Length</th>
<th>Product length</th>
</tr>
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<tr>
<td>rs9923231_modify</td>
<td>GGTGCGTGCTGATCGTAAATCGAAGGCGGAGAAGGCTGGGCGG</td>
<td>59</td>
<td></td>
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<tr>
<td>rs9923231_C</td>
<td>CAACACCTGGTACTCGCATCA</td>
<td>37</td>
<td>166</td>
</tr>
<tr>
<td>rs9923231_T</td>
<td>CAACACCTGGTACTCGCATCA</td>
<td>39</td>
<td>168</td>
</tr>
<tr>
<td>rs5065_modify</td>
<td>GCAAAACCTGGTACTCGCATCA</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>rs5065_A</td>
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<td>41</td>
<td>170</td>
</tr>
<tr>
<td>rs5065_G</td>
<td>CAACACCTGGTACTCGCATCA</td>
<td>43</td>
<td>172</td>
</tr>
<tr>
<td>rs1801253_modify</td>
<td>CAACACCTGGTACTCGCATCA</td>
<td>59</td>
<td></td>
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<tr>
<td>rs1801253_C</td>
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<td>45</td>
<td>174</td>
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<tr>
<td>rs1801253_T</td>
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<td>47</td>
<td>176</td>
</tr>
<tr>
<td>rs1065852_modify</td>
<td>CAACACCTGGTACTCGCATCA</td>
<td>59</td>
<td></td>
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<tr>
<td>rs1065852_C</td>
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<td>178</td>
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<td>rs12041331_A</td>
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<td>CAACACCTGGTACTCGCATCA</td>
<td>55</td>
<td>184</td>
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</table>

Italic letters refer to the sequence complementary to the universal ligation template; underlined letters refer to the stuffing sequence; and the letters in bold refer to the SNP to be detected.
Fig. 1. The graph of LDR products of two panels of SNP separated on 3730 sequencer. Upper: panel 1 containing 10 loci; lower: panel 2 containing 9 loci
Table 5: The genotype distribution of 19 SNPs in 237 samples

<table>
<thead>
<tr>
<th>SNP</th>
<th>Genotype/Allele</th>
<th>Number (%)</th>
<th>$\chi^2$</th>
<th>p value</th>
<th>Percentage in Chinese people</th>
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<td></td>
</tr>
<tr>
<td></td>
<td>GC</td>
<td>110 (46.4)</td>
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<td></td>
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<td>99.95</td>
</tr>
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<td></td>
<td>0.4</td>
</tr>
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<td>0.004273</td>
<td>0.948</td>
<td>97.6</td>
</tr>
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<tr>
<td></td>
<td>CC</td>
<td>1 (0.4)</td>
<td></td>
<td></td>
<td>7.8</td>
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</table>

*: Ensemble waston
**: CASAgilent
to guide these CVD patients and their doctors to choose medicines to avoid side effect and reduce economic burden caused by potential wrong drugs or dosage.

**CONCLUSION**

PCR-LDR method is an ideal clinical solution for CVD drug-related SNP detection for its convenient manipulation, high accuracy and time and cost effectiveness, as well as satisfactory multiplicity. The 19 selected SNPs are able to represent most of the CVD medicine usage associated genetic polymorphisms in Chinese people, and the detection for these SNPs can be a helpful guidance for the doctors and patients to achieve better outcome and less side effects of the medicines.

**RECOMMENDATIONS**

The tested SNP panel can be expanded to more genes if more medicines were included, and the multiplex PCR-LDR can be accomplished in one tube by optimizing the PCR primers and reaction conditions.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


![Fig. 2. Sanger sequencing for rs1057910, rs776746 and rs4244285 from 8 samples](image)

<table>
<thead>
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<th>Number of SNPs carried by each patient</th>
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<th>2</th>
<th>3</th>
<th>4</th>
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<td>41.7</td>
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</table>

Table 6: The allelic distribution of the 19 SNPs in 237 samples


