RESEARCH ARTICLE

Evaluation of a High Throughput Method for the Detection of Mutations Associated with Thrombosis and Hereditary Hemochromatosis in Brazilian Blood Donors

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Abstract

Background

The aim of this study was to evaluate the OpenArray platform for genetic testing of blood donors and to assess the genotype frequencies of nucleotide-polymorphisms (SNPs) associated with venous thrombosis (G1691A and G20210A), hyperhomocysteinemia (C677T, A1298C), and hereditary hemochromatosis (C282Y, H63D and S65C) in blood donors from Sao Paulo, Brazil.

Methods

We examined 400 blood donor samples collected from October to November 2011. The SNPs were detected using OpenArray technology. The blood samples were also examined using a real-time PCR–FRET system to compare the results and determine the accuracy of the OpenArray method.

Results

We observed 100% agreement in all assays tested, except HFE C282Y, which showed 99.75% agreement. The HFE C282Y assay was further confirmed through direct sequencing, and the results showed that OpenArray analysis was accurate. The calculated frequencies of each SNP were FV G1691A 98.8% (G/G), 1.2% (G/A); FII G2021A 99.5% (G/G), 0.5% (G/A); MTHFR C677T 45.5% (C/C), 44.8% (C/T), 9.8% (T/T); MTHFR A1298C 60.3% (A/A), 33.6% (A/C), 6.1% (C/C); HFE C282Y 96%(G/G), 4%(G/A), HFE H63D 78.1%(C/C), 20.3% (C/G), 1.6% (G/G); and HFE S65C 98.1% (A/A), 1.9% (A/T).
Conclusion

Taken together, these results describe the frequencies of SNPs associated with diseases and are important to enhance our current knowledge of the genetic profiles of Brazilian blood donors, although a larger study is needed for a more accurate determination of the frequency of the alleles. Furthermore, the OpenArray platform showed a high concordance rate with standard FRET RT-PCR.

Introduction

Blood donors are typically screened for the presence of infectious diseases to prevent transmission through blood transfusion. However, blood banks have recently begun testing for other factors, such as cholesterol and glucose levels, to evaluate the health of the donor and provide an incentive for blood donation [1, 2]. Blood typing has also become a routine test administered in blood banks [3, 4]. The presence of single nucleotide-polymorphisms (SNPs) associated with common genetic diseases could represent another factor for testing in blood donors, particularly if the cost of this test does not substantially increase the costs of tests routinely performed.

Genotyping tests for high throughput routines have been widely used in clinical laboratories. The OpenArray platform (Life Technologies, Carlsbad, CA) provides high density and requires a very low volume of sample based on nanoliters. It is a flexible system that uses TaqMan technology, capable of accommodating 3072 reactions per array [5].

Among the genetic factors related to diseases studied, Factor V Leiden (FV) (rs 6025) [6, 7] is the leading cause of genetic thrombophilia [8] and is observed in 5% of the Caucasian population [9]. The relative risk for venous thrombosis is 3–10 times higher for heterozygotes and 50–100 times higher for homozygotes carriers when compared to wild type subjects [8, 10]. The second most frequent genetic prothrombotic factor in humans is a mutation in prothrombin or coagulation factor II (rs1799963) [6, 7, 11]. Its prevalence in the Caucasian population is approximately 1 to 4%, and the frequency of this mutation among patients with venous thrombosis is 5 to 7% [11]. In addition, previous studies have indicated that the recurrence of venous thrombosis is higher for heterozygous individuals with mutations in FV Leiden and FII G20210A [12].

An increased level of homocysteine in plasma (hyperhomocysteinemia) also leads to prothrombotic events and is related to the presence of C677T (rs1801133) and A1298C (rs1801131) mutations [13–15]. Previous studies have reported a association between hemorrhagic (677TT and 677TT/1298AA genotypes) and ischemic stroke (1298CC and 677TT/1298CC genotypes) in cases of homozygous mutations, alone or in combination [16].

Another frequent disease also associated with SNPs is hereditary hemochromatosis (HH), an inherited disorder of iron metabolism resulting from mutations in the HFE gene. Clinically, HH is characterized as a multisystemic disease resulting in dysregulated intestinal iron absorption and progressive iron deposition in the liver, heart, skin, endocrine glands, and joints [17]. Thus, patients with HH might benefit from frequent blood donation [18, 19]. The most common genetic variants associated with HH are C282Y (rs1800562), H63D (rs1799945) and S65C (rs1800730) [20, 21].

In Brazil, the incidence of FV Leiden, FII G20210A and MTHFR C677T described in general population ranges from 0.7–4.8% for FV Leiden, 0.7–3.6% for FII 20210A and 35%–44% for MTHFR C677T [22–29]. Few studies have evaluated the frequency of the HFE C282Y, H63D and S65C mutation in Brazilian healthy individuals. The allelic frequencies described for HFE C282Y, H63D, S65C were 2.1%-2.3%, 13.6% and 0.6% respectively [30–32].
The aim of this study was to evaluate the OpenArray platform for genetic testing of blood donors. We also described the prevalence of these mutations among a Brazilian blood donor population.

**Materials and Methods**

**Ethics Statements**

A total of 400 blood samples were collected from October 24 to November 8, 2011, from blood donors at Fundação Pró-Sangue Hemocentro de São Paulo. There were 229 (52.3%) males and 171 (47.7%) females. The mean age of participants was 34.98 (SD±10.15) years-old. Participants were not classified into ethnic groups or skin color, as previous reports have shown genetic similarities among Brazilian population [33, 34]. Written informed consent was obtained from all participants. The study was approved by the Ethical Committee of the University São Paulo Medical School (asset n° 275/11), the ethical review board of Fundação Pró-Sangue Hemocentro de São Paulo.

**Controls**

DNA samples with different alleles for all SNPs examined in this study were previously determined using a Real Time PCR—FRET system reaction and used as controls to validate the OpenArray method. The numbers of controls used for each SNP are described in **Table 1**.

**DNA isolation**

DNA was isolated from 200 μl of whole blood using the QIAamp DNA Mini kit (Qiagen, Hilden Germany), according to manufacturer’s instructions. The quantity and quality of the DNA was determined through spectrophotometry using a NanoDrop 2000 (Thermo Scientific, Wilmington, DE). All samples were diluted to a final concentration of 35 ng/μl.

**Genotyping**

Genotyping was performed using TaqMan assays (Life Technologies). The rs numbers, mutation, gene and assay numbers for selected SNPs are described in **Table 2**. The assays included primers and probes labeled with VIC and FAM fluorophores, and each probe was designed for one particular allele type. The primers and probes were disposed using a solid platform (chip) that allows the performance of multiple samples and targets in the same reaction. The DNA and master mix were incorporated into the array using the OpenArray AccuFill System (Life Technologies), and the primers and probes were subsequently

| Gene     | SNP Number | Number of Controls for Genotyping | Number of controls with discordant Genotyping | Number of samples with Genotyping through OpenArray | Number of samples with discordant Genotyping through LC |
|----------|------------|----------------------------------|---------------------------------------------|--------------------------------------------------|--------------------------------------------------------|
| Factor V Leiden | G1691A      | 19                               | 0                                           | 392                                              | 0                                                      |
| Factor II | G20210A     | 18                               | 0                                           | 390                                              | 0                                                      |
| MTHFR    | C677T       | 24                               | 0                                           | 388                                              | 0                                                      |
| MTHFR    | A1298C      | 22                               | 0                                           | 392                                              | 0                                                      |
| HFE      | C282Y       | 22                               | 1                                           | 392                                              | 1                                                      |
| HFE      | H63 D       | 32                               | 0                                           | 385                                              | 0                                                      |
| HFE      | S65C        | 16                               | 0                                           | 388                                              | 0                                                      |

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solubilized, followed by genotyping according to the manufacturer’s instructions. A non-template control (NTC), comprising DNase-free water, was introduced in each assay. The multiplex TaqMan assay reactions were performed using the GeneAmp PCR System 9700 (Life Technologies) with the following PCR cycle: an initial step at 93°C for 10 minutes, followed by 50 cycles of 45 seconds at 95°C, 13 seconds at 94°C and 2 minutes and 14 seconds at 53°C, with a final step at 25°C for 2 minutes and holding at 4°C.

The end point fluorescence was read using the BioTrove OpenArray SNP genotyping platform (Life Technologies). The genotyping analysis was performed using TaqMan Genotyper software version 1.3 (Life Technologies—available at http://www.lifetechnologies.com/br/en/home/global/forms/taqman-genotyper-softwaredownload-reg.html) with auto calling. The results were considered valid when the quality value call rate was 90% or higher.

The samples were classified as homozygous or heterozygous for alleles 1 or 2. The reproducibility of the OpenArray platform method was evaluated using 9 triplicate samples in three different runs. The accuracy was determined through a comparison of the genotyped results using Real-Time PCR—FRET methodology.

The real-time PCR-FRET (Fluorescence resonance energy transfer) was performed using a Light Cycler (LC) 2.0 (Roche Diagnostics, Meylan, France) instrument. The primers and probes for FV Leiden, FII and for HFE SNP detection have been previously described [35, 36]. The following commercial reagents were used for MTHFR C677T and A1298C: Light Mix C677T (Cat. No. 40-0095-16, TibMol Biol, Berlin, Germany) and Light Mix A1298C (Cat. No. 40-0269-16, TibMol Biol). The results of both methodologies were compared.

The inconsistent results were confirmed after sequencing the specific PCR product using the ABI Prism BigDye terminator kit v3.1 (Life Technologies) and the previously described primers [35–37] according to the manufacturer’s instructions. The reactions were run on an ABI Prism 3730 Genetic Analyzer (Life Technologies) and the results were analyzed using Sequencher—DNA Sequencing Analysis Software 4.1.4 (Life Technologies).

**Results**

Among the blood donor samples tested, eight samples were excluded for call rates below 90%, as determined using Taqman Genotyper v1.3 software. Seven samples failed in at least one SNP, but because the call rates were higher than 90%, these samples were included in the analysis. None of the controls were excluded from the analysis.

Table 1 summarizes the comparison between the OpenArray system and the LC instrument. All blood donor and control samples had 100% agreement for all SNPs tested, except for HFE C282Y in two samples, which presented inconsistent results. One sample was classified as wild type using the OpenArray system and heterozygous using the LC, which showed an abnormal pattern of melting curve (the Tm observed was 51°C and 55°C, while the standard Tm for

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**Table 2. SNPs identification and Assay Part Numbers for OpenArray testing.**

| Gene          | SNP   | Rs number | Life Technologies Assay Part Number |
|---------------|-------|-----------|-------------------------------------|
| Factor V Leiden | G1691A | rs6025    | C__11975250_10                      |
| Factor II     | G20210A | rs1799663 | C__8726802_20                       |
| MTHFR         | C677T | rs1801133 | C__1202883_20                       |
| MTHFR         | A1298C | rs1801131 | C__850486_20                        |
| HFE           | C282Y | rs1800562 | C__1085595_10                       |
| HFE           | H63 D | rs1799945 | C__1085600_10                       |
| HFE           | S65C  | rs1800730 | C__1085599_20                       |

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heterozygous C282Y is 55°C and 64°C). This sample was further assessed using Sanger Sequencing, revealing a heterozygous silent mutation in another position (G843A instead of G845A). This finding was consistent with the result of the OpenArray system, as sequencing revealed that the sample was wild type for C282Y. The other sample was classified as heterozygous (G/A) using the OpenArray system and homozygous mutant (A/A) using the LC. Sequencing confirmed the OpenArray result.

To evaluate the reproducibility of the assay, nine samples were tested in triplicate, and no discrepancies were detected.

The genotypic and allelic frequencies of each SNP tested in the blood donor population were calculated using TaqMan Genotyper Software, and the results are shown in Table 3.

Table 3. Genotypic and allelic frequencies observed in the studied population using the OpenArray method.

| Gene/SNP | WT genotype | HET genotype | MUT genotype | Allele 1 | Allele 2 |
|----------|--------------|--------------|--------------|----------|----------|
| FV G1691A | 98.8% (G/G) | 1.2% (G/A) | 0% (A/A) | 99.4% (G) | 0.6% (A) |
| FII G20210A | 99.5% (G/G) | 0.5% (G/A) | 0% (A/A) | 99.8% (G) | 0.2% (A) |
| MTHF C677T | 45.5% (C/C) | 44.8% (C/T) | 9.8% (T/T) | 67.9% (C) | 32.1% (T) |
| MTHF A1298C | 60.3% (A/A) | 33.6% (A/C) | 6.1% (C/C) | 22.9% (C) | 77.1% (A) |
| HFE C282Y | 96.0% (G/G) | 4.0% (G/A) | 0% (A/A) | 98.0% (G) | 2.0% (A) |
| HFE H63D | 78.1% (C/C) | 20.3% (C/G) | 1.6% (G/G) | 86.9% (C) | 13.1% (G) |
| HFE S65C | 98.1% (A/A) | 1.9% (A/T) | 0% (T/T) | 99.0% (A) | 1.0% (T) |

WT—Wild Type; HET—Heterozygous; MUT—Homozygous for the mutation.

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heterozygous C282Y is 55°C and 64°C). This sample was further assessed using Sanger Sequencing, revealing a heterozygous silent mutation in another position (G843A instead of G845A). This finding was consistent with the result of the OpenArray system, as sequencing revealed that the sample was wild type for C282Y. The other sample was classified as heterozygous (G/A) using the OpenArray system and homozygous mutant (A/A) using the LC. Sequencing confirmed the OpenArray result.

To evaluate the reproducibility of the assay, nine samples were tested in triplicate, and no discrepancies were detected.

The genotypic and allelic frequencies of each SNP tested in the blood donor population were calculated using TaqMan Genotyper Software, and the results are shown in Table 3. The most frequent mutations found among the studied SNPs were C677T and A1298C in the MTHFR gene. Heterozygous genotypes were present in 44.8% (174/388) and 33.6% (132/392) of the blood donor samples tested for C677T and A1298C, respectively, while homozygous mutant genotypes were present in 9.8% (38/388) and 6.1% (24/392) of the samples, respectively. However, less frequent mutations were observed at Factor V (G1691A) and Factor II (G20210A), and these mutations were only present in heterozygous forms (1.2%- 5/392 for G1691A and 0.5%- 2/390 for G20210A).

**Discussion**

The OpenArray genotyping method was used to simultaneously detect seven different mutations in genes associated with thrombophilia and hemochromatosis. For evaluation purposes, the results were compared with FRET based real time PCR and a high concordance level was observed between methods, with only a single discrepancy in 2,727 determinations. Further studies may confirm the accuracy of OpenArray method.

In the present study, we found that the frequencies of the heterozygous genotype for FV Leiden (G1691A) and FII (G20210A) were low, and the homozygous FV 1691AA and FII 20210AA genotype were completely absent among the studied group. Similar results were reported previously in Brazilian healthy subjects [22–24]. These data suggest that the prevalence of FV Leiden and FII G20210A, associated with thrombophilic events, is lower in Brazilian blood donors than that reported in previous studies concerning blood donors of other countries [38–40]. For HFE mutations (C282Y, H63D and S65C), associated with an iron overload in homozygous or compound genotypes characteristic of HH, we observed a higher frequency (20.3% heterozygous and 1.6% homozygous) of H63D, consistent with previous studies reported internationally [21] and in a Brazilian population of blood donors from São Paulo [41]. We did not observe the presence of a homozygous genotype for either C282Y or S65C among
the studied population, and the heterozygous compound genotype C282Y/H63D was observed in only one sample. The MTHFR mutations showed a prevalence of 44.8% (C/T) and 9.8% (T/T) for C677T and 33.6% (A/C) and 6.1% (C/C) for A1298C, consistent with the results obtained from previous studies in Brazilian children and other control groups [22–24, 26, 42]. A comparison of the results obtained in the present study with those obtained in a recent prevalence study in middle-southern Italian blood donors revealed that the cohort used in this study presented a higher number of wild-type subjects for both C677T and A1298C [43]. We also observed that none of the individuals tested carried a 677TT/1298CC genotype. Because some SNPs have not been found in mutant homozygous state and show a very low frequency in heterozygous state, such as FV G1691A (1.2%), FII G20210A (0.5%), HFE C282Y (4%) and HFE S65C (1.9%), the screening of a larger number of samples is required for a more accurate determination of the frequency of the alleles in blood donors from São Paulo, Brazil.

Penetrance is defined as the proportion of individuals of a particular genotype who express the corresponding phenotype [44]. Although we found that some subjects carry the mutant allele, they may not express the disorder due to the reduced (or incomplete) penetrance showed by some autosomal dominant and recessive inherited mutations, such as FV G1691A and HFE C282Y respectively. Genotyping studies of apparently healthy individuals may be an approach to understand the penetrance of pathological variants [45].

Genetic testing may also be used for the identification of the FV Leiden mutation among women. Hormone therapies that primarily contain estrogens enhance the risk of thrombosis among this population [46, 47]. Although, current guidelines consider genetic testing to identify carriers of high-risk thrombophilia are only worthwhile when the subject has a family history of venous thromboembolism [48], the identification of this deficiency could provide more information to weigh the risks and benefits of hormonal contraceptive therapy in young women and hormone replacement in menopausal women. Indeed, further studies may contribute to certainly define whether offering genetic tests to blood donors may be beneficial.

In conclusion, the OpenArray methodology showed good concordance of results when compared with FRET based real time PCR. Although a larger study is required to have a more accurate frequency of the alleles, the frequency of SNPs related with thrombophilias and hemochromatosis in Brazilian blood donors are very similar with results previously reported by other groups.

**Author Contributions**

Conceived and designed the experiments: VDTN JVSB CAN NG ECS. Performed the experiments: VDTN JVSB. Analyzed the data: VDTN JVSB CAN NG ECS. Contributed reagents/materials/analysis tools: VDTN JVSB NG ECS. Wrote the paper: VDTN JVSB CAN NG ECS.

Obtained permission to perform real time PCR reactions at Light Cycler 2.0: NG.

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