Allele-Specific Detection of SLC22A2 rs316019 Variants Associated with Metformin Disposition through the Kidney

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Keywords

Allele-specific polymerase chain reaction · Diabetes mellitus · Metformin · OCT2 · rs316019 · SLC22A2

Abstract

Background: Metformin is prescribed as a first-line drug to treat type 2 diabetes. It is excreted directly and primarily through the SLC22A2 gene-encoded OCT2 transporter in the kidney. rs316019 (c.808G>T, p.270A>S) is the most common variant of SLC22A2, which affects its capacity to clear metformin from the body. Metformin increases the plasma lactate level in a concentration-dependent manner by inhibiting mitochondrial respiration and may lead to a condition known as metformin-associated lactic acidosis (MALA). MALA is a potentially life-threatening complication that can occur within the clinical doses of metformin. Therefore, dose adjustments based on the SLC22A2 rs316019 variants may be beneficial to maximize the efficacy and minimize the toxicity of metformin. **Objective:** This study was carried out to develop a simple and fast method to define genotype at the rs316019 locus. This method was applied to estimate the rs316019 allele frequencies in the Bangladeshi population. **Methods:** We designed allele-specific primers to determine

genotype at the rs316019 locus using allele-specific polymerase chain reaction (AS-PCR). AS-PCR data were confirmed by targeted sequencing of randomly selected samples. **Results:** The DNA sequence chromatograms showed the exact genotypes predicted through the AS-PCR method. A proportion of 79.62, 18.01, and 2.37% of Bangladeshi individuals have GG, GT, and TT genotypes, respectively. **Conclusion:** We report here a simple and fast method to define genotypes at the rs316019 locus in diabetic patients who are under metformin regimen. Allele frequencies at the rs316019 locus in the Bangladeshi population are close to those reported in other populations.

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Introduction

Diabetes mellitus, commonly referred to as diabetes, is a chronic metabolic disease characterized by hyperglycemia over a prolonged period [1]. The incidence of diabetes is growing in epidemic proportions worldwide. Globally the number of diabetic individuals is speculated to rise from 415 million in 2015 to 642 million in 2040 [2]. Diabetes is a complex condition which affects the entire

body. Prolonged hyperglycemia in diabetic patients may result in macro- and microvascular complications leading to a greater risk for heart disease and stroke as well as damage to the nervous system, retina, kidneys, and other organs [3]. The major goal of diabetes treatment, therefore, is to continuously maintain the blood glucose level within the physiological range. Currently this is approached through dietary control, injection of exogenous insulin, and/or a range of oral medications, such as insulin secretagogues and sensitizers [4]. Among the oral medications, metformin is recommended as the first-line treatment in most clinical guidelines on the management of type 2 diabetes, which accounts for nearly 90% of all cases of the disease [5, 6]. It is one of the two oral glucose-lowering agents in the list of essential medications of the World Health Organization [6].

Metformin reduces hepatic glucose output and increases glucose uptake by the peripheral cells and tissues [7]. Besides, metformin does not affect insulin secretion, unlike the other antihyperglycemic agents [8]. However, concern remains regarding the possible side effect of lactic acidosis, also known as metformin-associated lactic acidosis (MALA) [9]. Lactic acidosis is a condition characterized by serum lactate >5 mmol/L and blood pH <7.35 [10]. Though MALA is a relatively rare condition, it is associated with a high mortality rate (30–50%), and cases continue to be reported [9, 11].

Metformin does not undergo any modification in the liver and is excreted directly in urine through the kidneys [12]. Studies suggest that MALA can occur in patients with even mild renal dysfunction [9, 11]. An approximately 2- to 4-fold higher concentration of metformin is observed in the plasma of type 2 diabetic patients with moderate to severe kidney impairment compared to healthy subjects [9, 13]. Metformin can increase plasma lactate levels in a concentration-dependent manner [9, 14, 15].

Metformin is excreted primarily through the SLC22A2 gene-encoded OCT2 transporter in the kidney [16, 17]. SLC22A2 (OCT2) is a member of the SLC22 family of the SLC superfamily. OCT2 transports diverse molecules (including metformin), which remain positively charged under physiological conditions [16–18]. rs316019 (c.808G>T, p.270A>S) is the most common missense variant of SLC22A2 and has a significant impact on the disposition of metformin [19–24]. While the mechanistic aspect of MALA is still not well defined, recent studies suggested that rs316019 (p.270A>S) polymorphism in SLC22A2 (OCT2) substantially contributes to this complication [21, 23]. A marked influence of homozygous

variant TT genotype was observed on metformin clearance from the body [14, 19, 20]. This particular polymorphism of SLC22A2 should, therefore, be taken into consideration when determining the optimum dosage of metformin.

The International Diabetes Federation has projected that the prevalence of diabetes in Bangladesh will be 13% by 2030, placing Bangladesh as the 8th largest diabetic populous country in the world [25]. Nearly half of the diagnosed diabetic patients in Bangladesh are treated with metformin alone or in combination with other medicines [26]. There are no Bangladeshi population-specific data on the prevalence of MALA and SLC22A2 rs316019 variants. In this study, we developed a simple and fast allele-specific polymerase chain reaction (AS-PCR)-based method to determine genotype at the rs316019 locus in individuals. We also applied this method to determine the genetic architecture of the Bangladeshi population at the rs316019 locus.

Materials and Methods

Samples Used in the Study

Buccal swab samples from 125 random Bangladeshi individuals were collected. Briefly, cheek cells from the rinsed and clean mouths of the study subjects were collected using sterile cotton swabs. The cotton swabs were firmly rubbed and rotated along the inside of the cheek for 10–15 s to ensure good contact with the cheek. The swabs were air-dried for a while and carefully placed in collection tubes. The tubes were appropriately labeled and stored at 4 °C until DNA extraction.

DNA Extraction, Primer Design, and AS-PCR

DNA was extracted from the buccal cells using the PureLink™ Genomic DNA Mini Kit (K1820-01; Thermo Fisher Scientific) following the manufacturer's protocol. rs316019 allele-specific primers were designed applying the principle described by Wang-kumhang et al. [27]. Primers for glucose-6-phosphate isomerase (GPI) were designed using the Primer-BLAST tool [28]. The specificity of all the primers was checked using the Primer-BLAST tool. Sequences of all primers and other relevant information are given in Table 1. GPI and rs316019 allele-specific sequences were amplified in a duplex PCR in a thermal cycler (Gene Atlas G; As-tec Co. Ltd.). An amount of 10–50 ng of genomic DNA template was used for amplification in a final reaction volume of 25 µL with 2.5 µL of 10× PCR buffer (EP0702; Thermo Fisher Scientific), 1.0 µL of 10 mM dNTP mix (110-002; GeneON), 0.5 µL of 10 µM rs316019 allele-specific forward primer, 0.5 µL of 10 µM common reverse primer for rs316019, 0.5 µL of 10 µM GPI forward primer, 0.5 µL of 10 µM GPI reverse primer, 0.2 µL of Taq DNA polymerase (EP0702), and PCR grade water. All primers were purchased from Macrogen Inc. (South Korea). In the negative control PCR, an equal volume of PCR grade water was added instead of genomic DNA. The reaction cycle condition was as follows: an initial denaturation step at 94 °C for 5 min, then 33 cycles each

Table 1. Primers used in the study

Combination of primers in PCR	Primer ID	Sequence, 5' → 3'	Size, bp
For the G allele of rs316019	SLC22A2_rs316019_G_For*	GGTGGTTGCAGTTCACAGTCG	439
	SLC22A2_Rev	ATGCTCTGCTTTCTCCACCC	
	GPI_For	GGTACACAGGCAAGACCATC	255
	GPI_Rev	ACCTCCTGAAGAGTATGGCTT	
For the T allele of rs316019	SLC22A2_rs316019_T_For*	GGTGGTTGCAGTTCACAGTCT	439
	SLC22A2_Rev	ATGCTCTGCTTTCTCCACCC	
	GPI_For	GGTACACAGGCAAGACCATC	255
	GPI_Rev	ACCTCCTGAAGAGTATGGCTT	
For sequencing	SLC22A2_rs316019_Seq_For	TGTTTGCTTATGTTGGCAGGC	798
	SLC22A2_Rev	ATGCTCTGCTTTCTCCACCC	

PCR, polymerase chain reaction. * Allele-specific forward primers.

with denaturation at 94 °C for 30 s, annealing at 58.5 °C for 1 min, and elongation at 72 °C for 25 s followed by a hold at 4 °C. The PCR amplified sequences were resolved in 1.5% agarose gel (0710; Amresco®) using 0.5× Tris-acetate-EDTA (TAE) buffer along with DNA size markers (300003; GeneON). Amplicons were observed in a gel documentation system (WGD-30; Witeg) following incubation with ethidium bromide (0492; Amresco®) in TAE buffer and photographed with the WiseCapture II™ software. The genotype at the rs316019 locus was determined from the banding pattern in the gel photographs.

Sequencing of SLC22A2 rs316019 Variants

To confirm the genotype data obtained through AS-PCR, randomly selected DNA samples were amplified by PCR in a thermal cycler (Gene Atlas G) using human SLC22A2 gene-specific primers that encompass the rs316019 locus. An amount of 10–50 ng of genomic DNA template was used for amplification in a final reaction volume of 25 µL with 2.5 µL of 10× PCR buffer (EP0702), 1.0 µL of 10 mM dNTP mix (110-002), 0.5 µL of 10 µM forward primer, 0.5 µL of 10 µM reverse primer, 0.2 µL of Taq DNA polymerase (EP0702), and PCR grade water. The reaction cycle condition was as follows: an initial denaturation step at 94 °C for 5 min, then 33 cycles each with denaturation at 94 °C for 30 s, annealing at 58.5 °C for 1 min, and elongation at 72 °C for 45 s followed by a final extension at 72 °C for 5 min once. The PCR amplified sequences were resolved in 1.0% agarose gel (0710) and observed in a gel documentation system (WGD-30). PCR products were purified using the FavorPrep™ GEL/PCR Purification Kit (FAGCK 001; Favorgen Biotech Corp.) following the manufacturer's protocol. The concentration and purity of the purified PCR products were checked in a NanoDrop™ 2000 spectrophotometer. Sequences of the purified PCR products were obtained through commercial service from Macrogen Inc. (South Korea). Sequences were analyzed using SnapGene Viewer (GSL Biotech LLC).

Data Analysis

The SLC22A2 rs316019 allele frequencies in the Bangladeshi population and genotypes of individuals were calculated and presented using the Microsoft Excel and the GraphPad Prism® software.

Results

Duplex PCR was performed to determine genotype at the SLC22A2 rs316019 locus. Gene-specific primers were used to amplify GPI gene sequence as an internal control in PCR. Figure 1 shows the AS-PCR of the SLC22A2 rs316019 variants.

The allele-specific genotypic data were confirmed by sequencing the region of rs316019 locus of randomly selected samples. Figure 2 shows the sequence chromatograms along with corresponding rs316019 allele-specific duplex PCR. The DNA sequence chromatograms showed the exact genotype predicted through the AS-PCR method.

Of the 125 participants in this study, 54.81 and 45.19% were female and male, respectively. In this representative Bangladeshi population, the G and T alleles at the rs316019 locus had frequencies of 0.86 and 0.14, respectively (Fig. 3a). Out of the 125 participants, 95 had homozygous G (GG) genotype, which comprised 76% of the studied population (Fig. 3b). The TT genotype, which can lead to hyperlactacidemia in patients undergoing metformin therapy [14], comprised only 3.2% of the studied population. The 1000 Genomes Browser harbors the whole genome sequence information of 86 Bangladeshi

Fig. 1. AS-PCR of SLC22A2 rs316019 variants. Amplified products were separated in 1.5% agarose gel in 0.5× TAE. GPI was used as an internal control. Amplicon sizes of GPI and the rs316019 allele-specific products were 255 and 439 bp, respectively. AS-PCR, allele-specific polymerase chain reaction; GPI, glucose-6-phosphate isomerase; TAE, Tris-acetate-EDTA.

Fig. 2. Targeted sequencing to assess the AS-PCR data. **a** Partial gene sequence of the human SLC22A2 gene (NCBI reference sequence: NC_000006.12). Sequences shown in the chromatograms below are highlighted in yellow. **b-d** DNA sequence chromatograms of representative samples alongside the corresponding

rs316019 AS-PCR data. A different pair of primers (Table 1) was used in singleplex PCR to amplify the region encompassing the rs316019 site for the sequencing purpose. AS-PCR, allele-specific polymerase chain reaction.

individuals [29]. G and T allele frequencies in the combined population of 211 individuals (125 and 86 individuals from our study and the 1000 Genomes Browser, respectively) are 0.89 and 0.11, respectively (Fig. 3a). In these combined population data, 79.62 and 2.37% of the individuals have GG and TT genotypes, respectively (Fig. 3b). In the combined population, 53.16 and 46.84% are female and male, respectively. The distribution of rs316019 alleles follows Hardy-Weinberg equilibrium in the Bangladeshi population.

Discussion

The SLC22A2 gene encoded OCT2 is a polyspecific transporter which transports diverse structurally unrelated cations down their electrochemical gradients across the cell membrane [16, 17]. Alongside the endogenous metabolites, it transports exogenous compounds, including many drugs which have at least one positively charged moiety at physiological pH [16–18]. From the perspective that 40% or more of the prescribed drugs are positively

Fig. 3. Genetic architecture of the Bangladeshi population at the SLC22A2 rs316019 locus. **a** Frequencies of G and T alleles at the rs316019 locus. **b** Percentages of different genotypes at the rs316019 locus.

charged, it is apparent that the function of this transporter in the kidney has important pharmacological implications [30]. rs316019 (c.808G>T, p.270A>S) is the most common variant of SLC22A2, which affects its capacity to bind metformin, reduces clearance from circulation, and increases half-life in the body [14, 18–21, 23]. Metformin can increase plasma lactate levels in a concentration-dependent manner by inhibiting mitochondrial respiration, ultimately leading to MALA [9, 14, 15]. MALA is a potentially life-threatening complication which can occur within the clinical doses of metformin [11, 15]. Pre-existing clinical conditions, such as renal impairment, sepsis, anoxia, etc. may make an individual under metformin regimen more prone to MALA [9, 31]. Therefore, dose adjustments based on the SLC22A2 rs316019 variant may be beneficial to maximize the efficacy and to minimize the toxicity of metformin [32].

We here report a simple and fast AS-PCR-based method to define the genotype of individuals at the SLC22A2 rs316019 locus (Fig. 1). The allele-specific genotypic data showed perfect matching to their corresponding sequence chromatograms (Fig. 2). This simple method can be performed in a basic laboratory setting within 3 h starting from DNA extraction to allele detection.

The most critical point to ensure in AS-PCR is the avoidance of false-positive amplification as the single nucleotide polymorphism alleles predominantly differ by single base only. The rs316019 allele-specific primers were designed following the principle described by Wangkumhang et al. [27]. In the rs316019 allele-specific prim-

ers, one deliberate mismatch at the penultimate base to the rs316019 locus (second to last base at the 3' end of the primer) was made to introduce a destabilizing effect to eliminate the false-positive amplification. This special feature adds more to the accuracy of alleles-specific primers. The specificity of the primers to the human SLC22A2 gene was checked using the NCBI Primer-BLAST tool as well. To ensure good amplification of the alleles, the annealing temperature of the primers was optimized in thermal gradient PCR.

There is always a possibility of technical error in PCR. Therefore, GPI gene-specific primers were used as an internal control to eliminate false-negative results as well as erroneous interpretation to be homozygous at the rs316019 locus.

Hybridization- or restriction enzyme-based methods are often used to interrogate particular single nucleotide polymorphisms [33, 34]. One intractable challenge of hybridization-based single nucleotide polymorphism detection is the reduction of cross-hybridization of allele-specific probes, since these differ by a single base [33]. On the other hand, the restriction enzyme-mediated single nucleotide polymorphism detection method is labor intensive, time consuming, relatively more expensive, prone to erroneous result due to incomplete digestion, and difficult to adapt for high-throughput genotyping [33, 35]. Compared to these, AS-PCR may be considered as a simple and cost-effective method.

By 2040 the number of diabetic individuals in Bangladesh is predicted to rise as high as 13.6 million [36]. Al-

though metformin is prescribed as a first-line antihyperglycemic drug alone or in combination with other agents to diabetic individuals in Bangladesh [26], there are no Bangladeshi population-specific data on the prevalence of SLC22A2 rs316019 variants. In this study, we applied the AS-PCR method to determine the genetic architecture of the Bangladeshi population at the rs316019 locus. The observed frequency of rs316019 alleles in the Bangladeshi population is similar to those reported in other populations, particularly South Asians (0.87 and 0.13, respectively) [37].

The AS-PCR-based method described in this study is relatively simple but accurate. This method may be used to detect genotype at the SLC22A2 rs316019 locus in diabetic patients who are under metformin regimen. Dosage adjustments based on the SLC22A2 rs316019 variant may be beneficial to maximize the efficacy and to minimize the toxicity of metformin among diabetic individuals.

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Statement of Ethics

Buccal swab samples were collected from individuals upon receiving their consent. This study was approved by the Ethics Review Committee of the Faculty of Biological Sciences, University of Dhaka.

Disclosure Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Idea and experiment design: A.A. Sajib. Experiment: T. Islam, M.S. Rahman, and A.A. Sajib. Evaluation of results and manuscript writing: N. Paul, S. Akhteruzzaman, and A.A. Sajib.

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