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Multiplex allele-specific PCR to determine genotypes at statin metabolizing SNP loci- rs1135840 and rs776746

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Abstract

Single nucleotide polymorphisms (SNPs) play crucial roles in determining the extent of susceptibility to various diseases as well as actions of proteins involved in drug metabolism and transport. Statins are the first-line drugs for lowering cholesterol level and one of the commonly prescribed classes of drugs to patients with cardiovascular diseases (CVD). CVDs are the most prevalent cause of co-morbidity and mortality among patients with diabetes. Multiple studies have reported diabetogenic effect of statins. rs1135840 and rs776746 are two SNPs that affect statin metabolism and consequently increase their concentration in the blood. We have developed a simple, reliable and cost-effective allele specific PCR (ASPCR) based method to determine the genotypes at rs1135840 and rs776746 loci and applied to calculate the allele and genotypic frequencies in Bangladeshi population. Both rs1135840 and rs776746 variant alleles are present at high frequencies (0.43 and 0.36, respectively) in Bangladeshi population. Although the percentage of homozygous rs776746 variant genotype is relatively low (7.0%) in Bangladeshi population, homozygous rs1135840 variant frequency is quite high (22.0%). On the other hand, 21.94% and 2.55% of the Bangladeshi individuals are heterozygous and homozygous, respectively, for both variant alleles. The AS-PCR method described here may be used to optimize the dose of statin guided by an individual's genotype and, therefore, increase the efficacy of statin treatment.

Key words: rs1135840; CYP2D6; rs776746; CYP3A5; Allele Specific PCR; Diabetes; Statins.

INTRODUCTION

3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoAR) inhibitors, more commonly known as statins, are commonly prescribed as drugs to individuals suffering from dyslipidemia [1]. Statins are taken to reduce the risks of myocardial infarction, stroke and death due to CVDs [1]. Though their high efficacy and safety profiles as drugs to treat dyslipidemia are well established, there have been reports on dose-dependent adverse effects associated with statin administration [2]. Multiple studies have reported association between prolonged statin therapy and development of type 2 diabetes mellitus (T2DM) [3,4]. Statins also worsen hyperglycemia in those with pre-existing diabetes or glucose intolerance [5,6]. Though the benefits of statin intake overweigh the associated risks [7], responses to statins vary among individuals with some being more susceptible to the adverse outcomes [8]. Therefore, individual specific dose adjustments based on genetic profiles may reduce the risk of developing diabetes and associated complications.

Studies have revealed certain genetic variants that greatly influence the efficacy of statin therapy [9,10]. For instance, members of the P450 family of enzymes, particularly the CYP enzyme system, are most significantly involved in the phase I metabolism of different categories of statins [11-13]. These enzymes have genetic variants, some of which exert effects on their rates and extent of metabolism [14]. Distribution of these variant alleles display significant interethnic variability [13,15,16].

CYP2D6 is dominantly involved in the metabolism of about 20% of all commonly used clinical drugs, including statins [14,17,18]. One particular variant- rs1135840 in CYP2D6 (c.4180G>C or p.Ser486Thr), plays roles in the development of diabetes, hypertension, prostate cancer, etc. Theoretically, poor metabolizers have higher levels of statin in blood and subsequently face greater risk of adverse effects [19,20]. CYP3A5 is another drug-metabolizing enzyme found in the liver and intestinal tissues. The variant allele rs776746 (CYP3A5*3) is one of the common polymorphisms that reduces metabolism of several drugs [21]. The variant allele of rs776746 leads to alternative splicing and protein truncation, causing low or undetectable CYP3A5 activity [22-25]. Homozygous CYP3A5*3 (mutant) individuals are termed non-expressors of CYP3A5 [21]. Studies investigating interindividual pharmacokinetic variability revealed that the mean plasma concentration of simvastatin was very high in CYP3A5*3/*3 3 individuals [26]. Several other studies correlated rs776746 variant allele to poor metabolism and subsequent buildup of the immunosuppressive drug tacrolimus beyond the safe dosage level during organ transplantation [27-29].

Individuals who are more prone to developing diabetes because of genetic predisposition, may become even more susceptible due to secondary effects from drugs like statin. Here we report a simple, reliable and cost-effective multiplex method to determine genotypes at the rs1135840 and rs776746 loci. Since significant interpopulation differences exist in the variant allele frequencies of drug-metabolizing genes [30], we applied this method to study the distribution of these variants in Bangladeshi population to generate a population specific representation.

MATERIALS AND METHODS

Sample collection and DNA extraction: Buccal swab samples were collected from random Bangladeshi individuals upon approval from the ethics review committee of the Faculty of Biological Sciences, University of Dhaka. Buccal cells from the rinsed and clean mouths of the study subjects were collected using sterile cotton swabs after rubbing against the inner cheek wall around 10 times. The swabs were air-dried for a while and carefully placed in sterile separate collection tubes and stored at 4°C. DNA was extracted from the collected buccal samples using the PureLinkTM Genomic DNA Mini Kit (K1820-01, Thermo Fisher Scientific Corp.) following the manufacturer's protocol.

Allele-specific multiplex PCR: Allele specific primers were designed for the rs1135840 and rs776746 loci following the principle described by Wangkumhang et al. [31]. For each of the two loci, two separate allele-specific forward primers and one common reverse primers were designed (Table 1). Specificities of the designed primers were checked in silico using the Primer-BLAST tool [32]. As an internal amplification control in PCR, a 255 bp region of glucose phosphate isomerase (GPI) gene sequence was amplified using human specific primers [33]. Temperature-gradient PCR was performed in a thermal cycler (Gene Atlas G; Astec Co. Ltd.) to obtain the common optimum annealing condition for the primer pairs in multiplex PCR.

Table 1: Primers used in the study.

Gene	Primer ID	Primer	Sequence, 5'→3'	Amplicon size, bp
CYP2D6	rs1135840_W	**Forward**	GTCTTTGCTTTCCTGGTGGC	282
	rs1135840_M	**Forward**	GTCTTTGCTTTCCTGGTGGG	
	rs1135840_Common	**Reverse**	GGATTATGGGCAAGGGTAAC	
	rs1135840_ Sequencing	**Forward**	TTCCTGCCTTTCTCAGCAGG	519
CYP3A5	rs776746_W	**Forward**	TGGTCCAAACAGGGAAGAGATTT	222
	rs776746_M	**Forward**	TGGTCCAAACAGGGAAGAGATTC	
	rs776746_Common	**Reverse**	GTCCTTGTGAGCACTTGATGA	
	rs776746_Sequencing	**Forward**	GGATGCTTACCCTTCGATTTGTG	546

10–50 ng of genomic DNA template was used for amplification in a final reaction volume of 50 µL with 5 µL of 10x reaction buffer (S102, GeneON), 3.0 µL of 10 mM dNTP mix (110-002, GeneON), 1.0 µL of each primer (10 µM), 0.3 µL of Maximo Taq DNA Polymerase (S102, GeneON), 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 4 min, then 34 cycles each with denaturation at 94°C for 30 sec, annealing at 58°C for 1 min, and elongation at 72°C for 20 sec followed by a hold at 4°C. The PCR amplified sequences were resolved in 1.5% agarose gel (0710, Amresco®) using 0.5x Tris-acetate-EDTA (TAE) buffer along with DNA size markers (300003, GeneON). Amplicons were observed and photographed in a gel documentation system (INFINITY imaging system, Vilber) following incubation with ethidium bromide (0492, Amresco®) in TAE buffer. The genotypes at the rs1135840 and rs776746 loci were determined from the banding pattern in the gel photographs.

DNA sequencing: A different primer in combination with the common reverse primer (Table 1) for each locus was used to amplify the corresponding SNP encompassing region. 10–50 ng of genomic DNA template was used for amplification in a final reaction volume of 25 µL with 2.5 µL of 10x reaction buffer (S102, GeneON), 0.75 µL of 10 mM dNTP mix (110-002, GeneON), 0.5 µL of each primer (10 µM), 0.25 µL of Maximo Taq DNA Polymerase (S102, GeneON) and PCR grade water. The reaction cycle condition was as follows: an initial denaturation step at 94°C for 4 min, then 34 cycles each with denaturation at 94°C for 30 sec, annealing at 60.5°C for 1 min, and elongation at 72°C for 30 sec followed by a single elongation step at 72°C for 5 min and hold at 4°C. The PCR amplified sequences were resolved in 1.0% agarose gel (0710, Amresco®) using 0.5x Tris-acetate-EDTA (TAE) buffer along with DNA size markers (300003, GeneON). Amplicons were observed in a gel documentation system (INFINITY imaging system, Vilber) following incubation with ethidium bromide (0492, Amresco®) in TAE buffer. PCR products were purified using the FavorPrepTM 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 NanoDropTM 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).

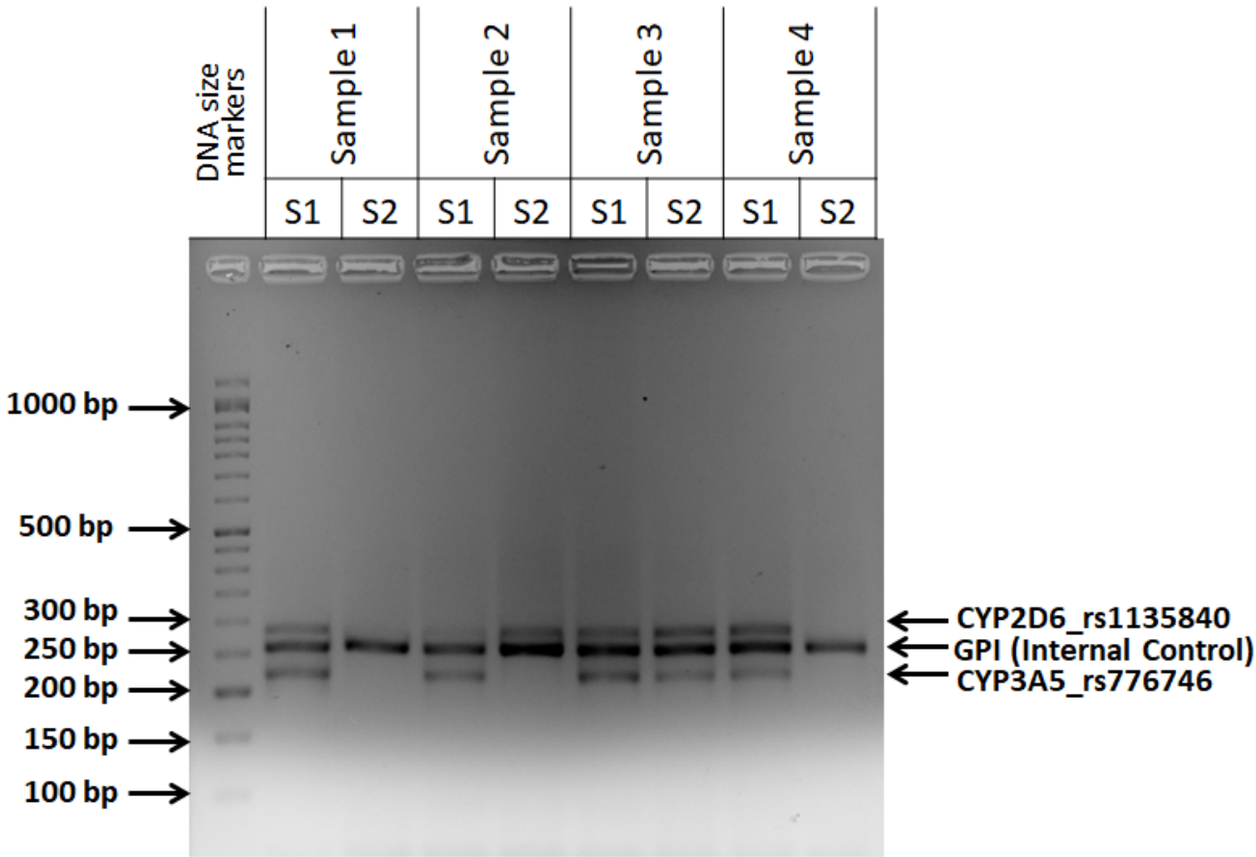
Data analysis: The allele frequencies of rs1135840 and rs776746 in the Bangladeshi population and genotypes at these loci were calculated and presented using the Microsoft Excel and the GraphPad Prism® software.

RESULTS AND DISCUSSION

In this study a multiplex allele specific PCR (AS-PCR) method was developed and applied to determine the genotypes at the rs1135840 and rs776746 loci (Figure 1). The GPI amplicon was used as an internal control in PCR to avoid misleading genotypic interpretation [33]. Allele specific PCR data was confirmed by sequencing the DNA regions encompassing the SNPs (Figure 2). The DNA sequence chromatograms matched to the genotypes predicted using the AS-PCR method.

There are different methods for determining the genotype of an individual for a specific SNP locus. Restriction fragment length polymorphism (RFLP) method is one of the most widely used methods. The main disadvantage of using RFLP on a daily basis is that it is time-consuming, difficult to scale up and often troublesome. A few other methods that are also used for genotyping SNP of interest include next generation sequencing (NGS), denaturing HPLC (DHPLC), TaqManTM SNP assay, SNaPshot sequencing, and PCR with fluorescently labeled probe, etc. These methods require highly expensive instrumentation and may not be easily affordable. On the other hand, AS-PCR based genotyping is inexpensive, easy to perform and, does not call for high-end laboratory equipment. This method requires only a thermal cycler and horizontal gel electrophoresis system, which are available in almost every molecular biology laboratory and diagnostic centers around the globe.

However, there is always a possibility of technical error in PCR. To avoid PCR bias, especially to get rid of technical error, two special sets of primers were used. GPI gene-specific primers were used as an internal control to eliminate false-negative results. To avoid false positive results in AS-PCR, primers were designed with an intentional insertion of mis-match at the penultimate position based on the theory described by Wangkumhang et al. [31]. We could combine this method to a recently published protocol for detection of SLC22A2 rs316019 variants associated with metformin disposition through the Kidney (Figure 4) [33].



SNP ID	Set 1 (S1)	Set 2 (S2)	Product size, bp
CYP2D6_rs1135840	W*(C)	M# (G)	282
CYP3A5_rs776746	W* (C)	M# (T)	222
GPI	-	-	255

* Wild type allele # Variant allele

Figure 1: Multiplex AS-PCR of CYP2D6 rs1135840 and CYP3A5 rs776746 alleles. Amplified products were separated in 1.5% agarose gel in 0.5x TAE. GPI was used as an internal amplification control.

When we combined our experimental data to the genotypes of 86 Bangladeshi individuals deposited in the 1000 genomes database [34], the variant allele frequencies among 201 Bangladeshi individuals at rs1135840 and rs776746 loci were 0.43 and 0.36, respectively (Figure 3A). This is quite similar to the global frequency data from the 1000 genome database (Figure 5). It is estimated that 40.1% individuals carry the mutant allele at rs1135840 globally, which is marginally lower than the frequency in Bangladesh found in this experiment. On the other hand, variation at rs776746 is found at a frequency of 0.379, slightly higher than our population under study. 35.32% among these 201 Bangladeshi individuals are homozygous (G/G) for the wild type allele, 42.79% are heterozygous (G/C) and 21.89% were homozygous (C/C) for the variant allele at the rs1135840 locus (Figure 3B). At the rs776746 locus, 45.66% were homozygous (G/G) for the wild type allele, 7.31% homozygous (A/A) for the variant allele and 47.03% heterozygous (G/A) (Figure 3B).

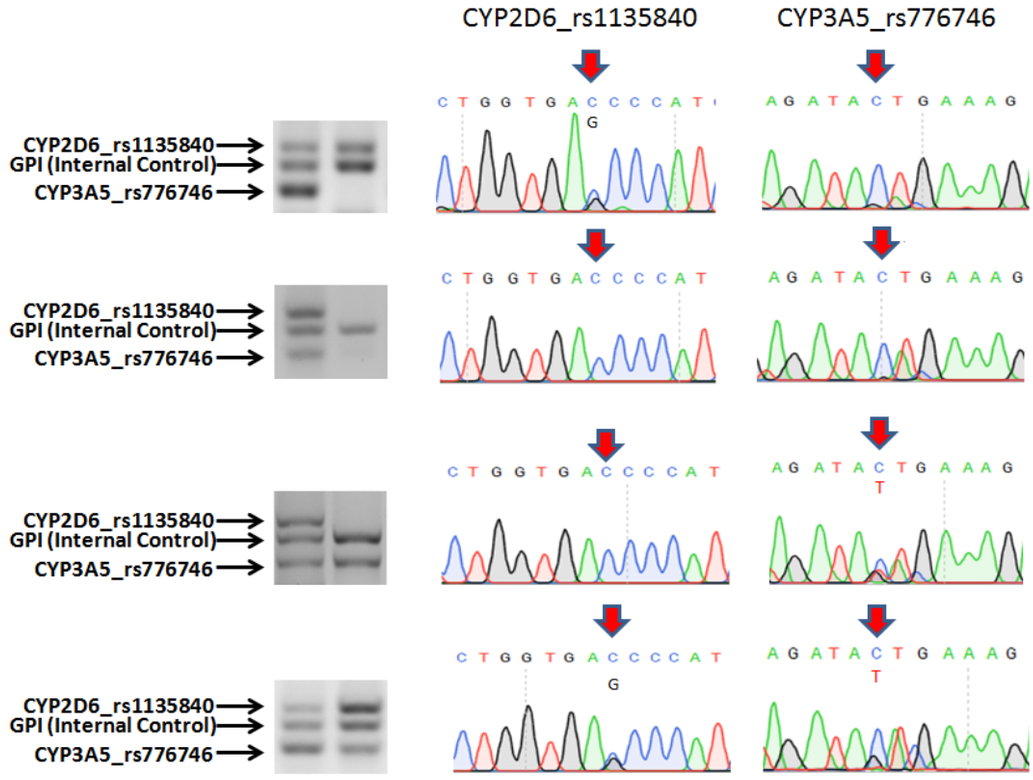


Figure 2: Targeted sequencing to assess the multiplex AS-PCR data. A-D. DNA sequence chromatograms of representative samples alongside the corresponding CYP2D6 rs1135840 and CYP3A5 rs776746 AS-PCR data. A different pair of primers (Table 1) was used in singleplex PCR to amplify the region encompassing the CYP2D6 rs1135840 and CYP3A5 rs776746 sites for the sequencing purpose.

Figure 3C shows the proportion of Bangladeshi individuals with different combinations of alleles at the rs1135840 and rs776746 loci. 2.55% of Bangladeshi individuals possessed homozygous variant genotype (CC_AA) at both loci. 37.76% of the individuals had at least one variant allele (GC_GA, GC_AA, CC_GA or CC_AA) at both loci. 11.73% of the individuals were homozygous for the variant alleles (CC_GG or GG_AA) at any one of the loci. Using LDpair tool at LDlink suite [35] to assess whether these variants are inherited non-randomly, we found a D' value of 0.0159 and r2 value of 0.0001 for the Bangladeshi population, which suggest that the Bangladeshi individuals having homozygous and heterozygous mutants at both the loci inherited them completely by chance. This is quite expected as rs1135840 and rs776746 are located in chromosome 22 and 7, respectively.

CVDs have caused much global apprehension, especially in recent years, due to its alarmingly high rate of incidence worldwide. CVDs are the number 1 cause of death globally with an estimated 17.9 million death in 2016 alone (WHO Fact sheet on CVD, available at- https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds)). Diabetes mellitus (DM) is another highly concerning public health issues in today's world. In the past three decades, prevalence of this chronic metabolic disease has risen dramatically in countries of all income levels. The number of people suffering from diabetes is predicted to soar up to 642 million by 2040 [36]. Each year almost 7 million people develop diabetes and the number of deaths attributed annually is around 3.2 million [2].

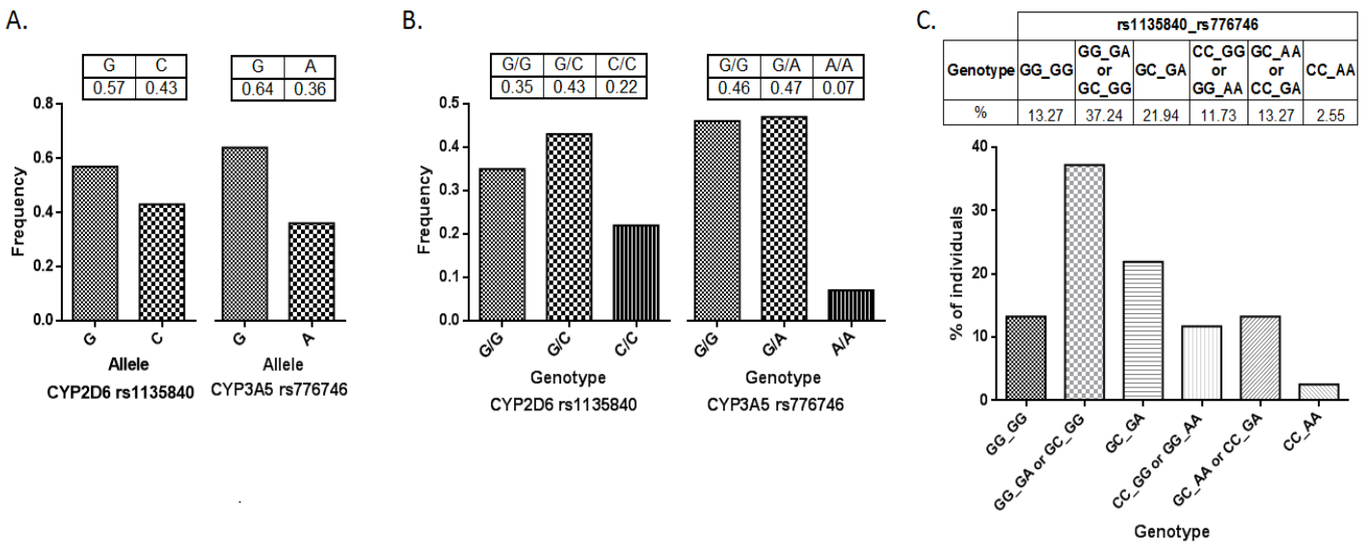
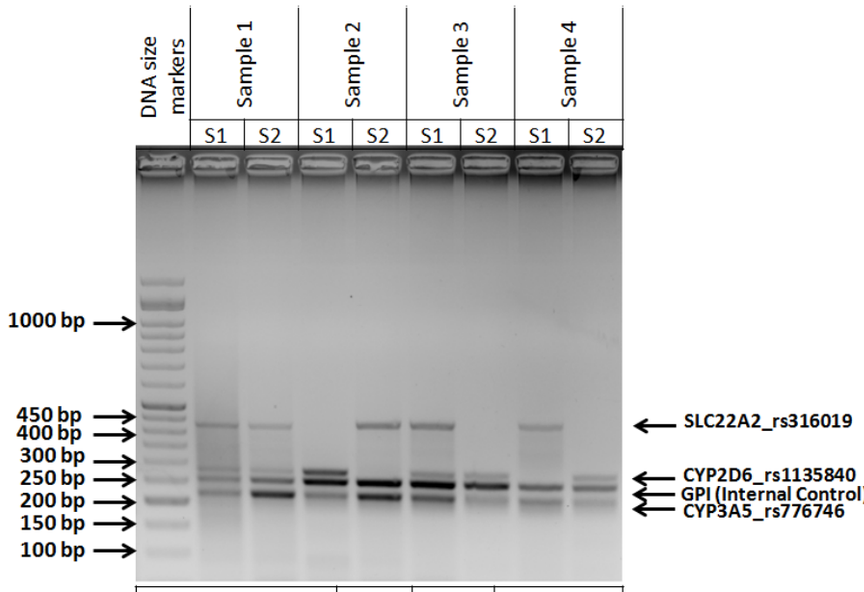


Figure 3: Allele and genotype distribution at CYP2D6 rs1135840 and CYP3A5 rs776746 loci in Bangladeshi population.



SNP ID	Set 1 (S1)	Set 2 (S2)	Product size, bp
CYP2D6_rs1135840	W*(C)	M# (G)	282
CYP3A5_rs776746	W* (C)	M# (T)	222
SLC22A2_rs316019	W* (G)	M# (T)	439
GPI	-	-	255

* Wild type allele # Variant allele

Figure 4: Multiplex AS-PCR of CYP2D6 rs1135840, CYP3A5 rs776746 and SLC22A2 rs316019 alleles. Amplified products were separated in 1.5% agarose gel in 0.5x TAE. GPI was used as an internal amplification control.

A.

Global	African	East Asian	Admixed American	European	South Asian	Bangladeshi
0.401	0.324	0.296	0.52	0.454	0.47	0.43

B.

Global	African	East Asian	Admixed American	European	South Asian	Bangladeshi
0.379	0.82	0.287	0.2	0.057	0.33	0.36

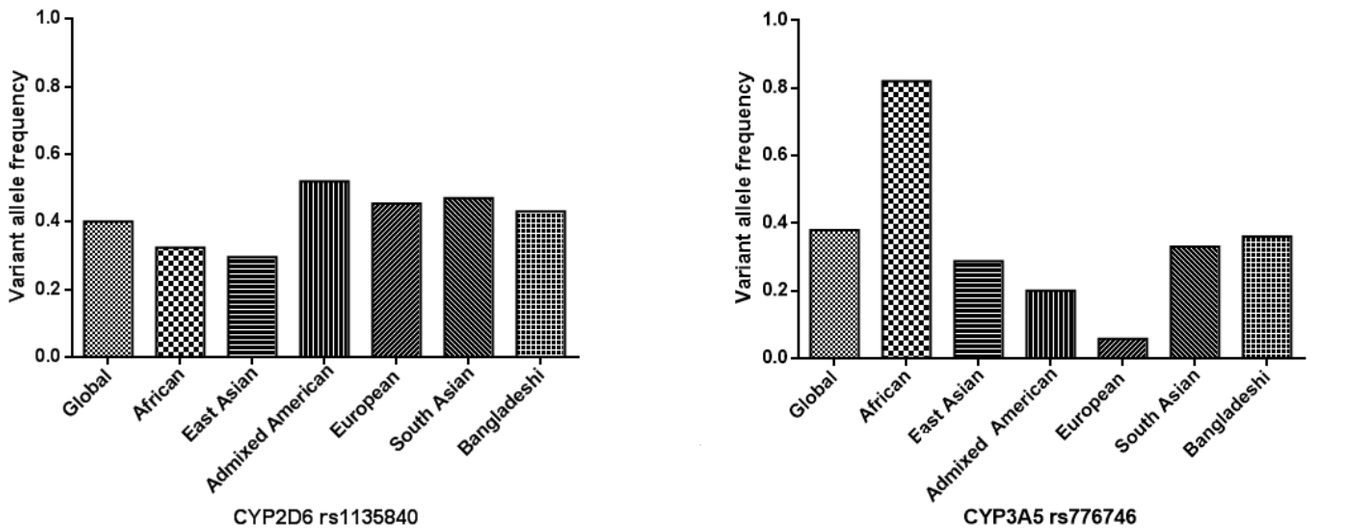


Figure 5: Allelic distribution at CYP2D6 rs1135840 and CYP3A5 rs776746 loci in world populations.

The scenario in developing countries like Bangladesh is quite perturbing. According to the International Diabetes Federation (IDF), there were 6,926,300 cases of diabetes in Bangladesh in 2017, which is equivalent to 6.9% of the adults in Bangladesh. The number of diabetic patients in Bangladesh is expected to rise over 13 million by 2045. Some well-known factors partly held responsible for this soaring trend include increased life expectancy of the population, higher presence of certain risk factors, high use of tobacco, physical inactivity, poor food habit, etc [37,38]. However, there are definitely other contributing issues that need to be addressed. Studies have revealed certain genetic variants that may greatly increase bioavailability of statins. Although statins are proven to be safe and effective for its relevant treatment purpose, these have diabetogenic potential [4]. Some of these may directly or indirectly influence and play a part in the rising prevalence of T2DM due to 'non-tailored' doses of statins prescribed for CVDs. The AS-PCR method described here may be used to optimize the dose of statin guided by an individual's genotype and, therefore, increase efficacy of statin treatment.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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