RESEARCH ARTICLE

Genotyping of Lipoprotein(a) Gene Variants in Coronary Artery Disease in Indian Population

Anu RI [1], Charanjeet Kaur[2], Prabhash Bhavsar[3], Jagdish Prasad[4]

Abstract

India is undergoing an epidemiological transition and an alarming rise in the prevalence of coronary artery disease (CAD). The etiopathology of the disease is evolving to a more complex setting with the discovery of novel risk factors. In the quest for non-traditional risk factors, we found that Lipoprotein(a) gene and its product are unique and pertinent to the Indian population. To unravel the genetic blueprint of the variations within the *LPA* gene in Indians, we studied novel exonic and intronic SNPs in 60 patients with angiographically proven CAD and matched them with healthy volunteers. From the study, we observed that in our patients, *LPA* aspirin genotype rs3798220 did not cause variation in Lp(a) levels in either of the two groups which is a novel finding as it is at odds with most published studies from other populations, on this variant. The polymorphic allele of SNP rs1321196 increased serum Lp(a) levels but the presence of the variant was however not limited to the patient population. The SNP rs9364564 did not influence Lp(a) levels in either of the two groups of the study. The study is unique as it bears witness to the novelty of the genotype background in Indian patients with CAD.

Keywords: Allele-specific PCR, Cardiovascular genetics, Coronary artery disease, Lipoprotein(a), *LPA* aspirin genotype, *LPA* gene polymorphism, Molecular cardiology, rs1321196, rs3798220, rs9364564.

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Introduction

The prevalence of coronary artery disease (CAD) is reaching epidemic proportions in India with a fourfold rise in the past 40 years.[1] The estimated prevalence of cardiovascular disease in India is 54.5 million, as of 2016.[2,3] Traditional risk factors for CAD do not provide an adequate explanation for the epidemic proportions of the disease. The non-traditional risk factors have proved to bridge this gap to an extent, together with the fact that CAD being highly heritable, bringing to light the complex polygenic backdrop of the setting of the disease.

A multi-centered case-control study, PROCARDIS (Precocious Coronary Artery Disease) investigated 3,145 cases and 3,352 control subjects to evaluate 2,100 candidate genes using evaluation of single-nucleotide polymorphisms (SNPs). The study found three genomic loci associated with CAD *viz*. 6q26-27, 9p 21, and 1p13. Of these, the locus 6q26-27 encompassing the *LPA* gene that encodes for Lipoprotein(a) had the strongest association with CAD.[4,5]

Numerous trials have shown us that patients with Lp(a) levels above 30 mg/dL had markedly increased risk of CAD and susceptibility to occlusive complications after various interventions.[6] A high level of Lipoprotein(a) is an important determinant of CAD and is projected to be one of the strongest biomarkers of premature heart disease.[7] It is noteworthy that Lp(a) has been proved to be an independent risk factor of CAD by the Framingham Heart Study and Premature CAD attributable to high Lp(a) was double that of hypertension or diabetes mellitus as concluded by the Copenhagen City Heart Study.

Structurally, Lp(a) is a plasma lipoprotein consisting of a cholesterol-rich LDL-like particle, having apolipoprotein(a) attached to apo B via a disulfide bond.[8] The basic LDL component has a lipid core formed by cholesteryl esters and triglycerides, which is surrounded by a layer of unesterified cholesterol, phospholipids, and an apolipoprotein B-100 (apo B-100) molecule. The apo "a" particle surrounds this complex and is attached by a disulfide bond to the apo B-100 component as shown in Figure 1. The apo(a) moiety is encoded by the *LPA* gene located on chromosome 6q 26-27, consists of multiple repeated segments or domains called Kringles. The Kringles are tri-looped structural units containing about 77–79 amino acids. They are connected by linker regions of 26–36 amino acids.[9] Apo "a" has Kringles numbered IV and V, and a protease domain that is catalytically inactive.[10] Kringle IV types I and III to 10 exist only as single copies, whereas Kringle IV type II is present in multiple copies in an individual. This special variation in the number of copies from 3 to more than 40 copies, is responsible for the apo "a" size and molecular weight heterogeneity in a person[11] with a range between 300 and 800 kDa.[10,12] The isoform size of the apo "a", which thus depends on the number of repeats of the KIV type II repeats, has high inter-individual variation. Each Kringle has

[1]Department of Clinical Biochemistry, MVR Cancer Center and Research Institute, Calicut, Kerala, India

[2]Department of Biochemistry, Vardhman Mahavir Medical College and Safdarjung Hospital, New Delhi, India

[3]Department of Biochemistry, Government Medical College and Shri Haridev Joshi General Hospital, Dungarpur, Rajasthan, India

[4]Department of Cardiothoracic and Vascular Surgery, Vardhman Mahavir Medical College and Safdarjung Hospital, New Delhi, India

Corresponding Author: Anu RI, Department of Clinical Biochemistry, MVR Cancer Center and Research Institute, Calicut, Kerala, India, Phone: +91 9971272384, e-mail: anuevangelineiris@gmail.com

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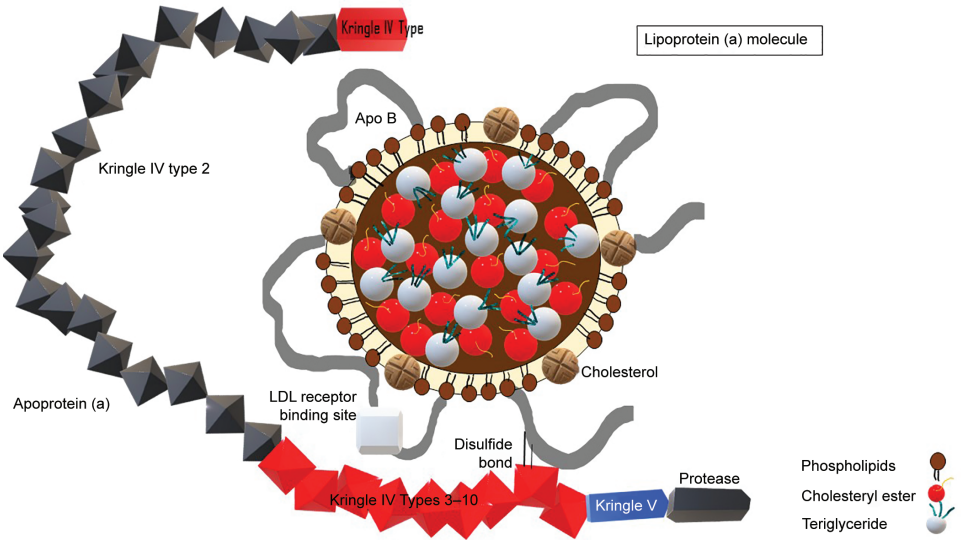

Fig. 1: Graphical structure of lipoprotein (a) molecule

three disulfide bonds and a peculiar folding pattern that serves as binding motifs[13] probably for lysine or lysine analogs.[14] Serum Lp(a) levels display high inter-individual variations and may range from <1 to >1,000 mg/dL, with a possibility of being absent totally from the blood.[15] Lp(a) concentrations and their relationship with the cardiovascular disease vary across races or ethnicities.[16] *LPA* gene polymorphism is responsible for this wide variation in the serum Lp(a) levels with a heritability of 73–90%.[17,18]

Despite a few studies proving the association between risk of development of CAD and increased Lipoprotein(a) levels, there is a paucity of literature revealing the existence or association of the *LPA* gene polymorphism and risk of development of atherosclerotic cardiovascular diseases (ASCVD) in Indian population. Hence, this study aims in bringing to light the SNPs in the *LPA* gene which may prove to be significant in the Indian population. This will further help us in further research in the arena of treatment and prevention of patients with CAD.

Materials and Methods

The study was conducted on 60 patients with angiographically proven CAD, admitted for coronary artery bypass graft (CABG) and the age group varied from 45 to 70 years. Fifty apparently healthy individuals were selected as a control group. Patients suffering from hepatic, renal, or thyroid dysfunction and patients taking drugs such as niacin were excluded from the study. After obtaining informed consent, venous blood was collected in two vacutainer collection tubes. The blood from the EDTA tube was used to extract genomic DNA and that from the "no additive" tube was used for testing Lipoprotein(a) using Immunoturbidimetry, and routine parameters. Ethical approval was obtained from Institution Ethics Committee before the commencement of the study. Statistical analysis was performed using SPSS 22.0 software. Data are presented as mean (standard deviation) unless otherwise specified. The difference in the mean baseline values of various parameters was analyzed by unpaired *t*-test or Mann–Whitney test depending upon whether the values were parametric or non-parametric. Nominal variables were analyzed using χ^2 and Fisher's exact tests. Correlation between two parameters was made using Pearson's correlation. A *p* value of <0.05 was considered significant. The risk of development of CAD was evaluated by odds ratio calculation.

Molecular Genetics Experiment

Extraction of DNA was performed using QIAamp Blood Mini Kit from Qiagen. The extracted DNA was subjected to spectrophotometric analysis for quality and yield estimation. DNA was then stored at −80°C until further analysis.

Three variants (SNPs) rs1321196, rs9364564, and rs3798220 in the Lipoprotein(a) (*LPA*) gene were genotyped in this study. Target regions were amplified from gDNA using the PCR technique. Primers were designed using the Primer-BLAST tool from NCBI.

Genotyping of exonic missense variant rs3798220 (Location chr 6: 160540105, GRCh38.p12)

The technique of allele-specific PCR (AS-PCR) was used for this variant. Primers were designed with the following sequence:

Forward ancestral: CAAGAACAGCCTAGACACTACT

Forward polymorphic: CAAGAACAGCCTAGACACTACC

Common reverse: ACTGATTCTGGGTGGCCGAG

Genotyping of intronic variants rs1321196 (Location chr 6: 160660810, GRCh38.p12) and rs9364564 (Location chr 6: 160578008, GRCh38.p12)

For rs1321196, forward and reverse primer sequences were: GGCAGTATTGGTCCCAGGTT and ACCTTGGAGGCCATTGTTTGA, respectively.

For rs9364564, forward and reverse primer sequences were: CCCTTGCGCATTTCCCTAGA and CTTTAGGAAGGGCACGGTGT, respectively.

The amplicon was subjected to the RFLP technique to determine the presence or absence of the target variant. Restriction endonuclease Hae III was used for both variants. The products were then separated by agarose gel electrophoresis and visualized using a gel documentation system. Table 1 depicts details of primers, restriction enzymes, amplicon size, and size of digestion products described in the experiments.

The amplicon obtained was directly visualized in a gel documentation system after agarose gel electrophoresis.

Table 1: Genotyping experiment details of variants with primer sequences, restriction enzyme, amplicon, and digestion product sizes (bp)

S. no.	*rs ID*	*Primer sequence*	*Restriction enzyme*	*Size of amplicon (bp)*	*Size of digestion products (bp)*
1	rs3798220	Forward ancestral/wild-CAAGAACAGCCTAGACACTACT Forward polymorphic/mutant-CAAGAACAGCCTAGACACTACC Common reverse-ACTGATTCTGGGTGGCCGAG	NA	211	NA
2	rs1321196	Forward-GGCAGTATTGGTCCCAGGTT Reverse-ACCTTGGAGGCCATTGTTTGA	Hae III	359	GG: 216-133-10 AA: 349-10 GA: 349-216-133-10
3	rs9364564	Forward-CCCTTGCGCATTTCCCTAGA Reverse-CTTTAGGAAGGGCACGGTGT	Hae III	661	GG: 332-224-105 AA: 437-224 GA: 437-332-224-105

Results

Genotyping of *LPA* Variants

rs3798220 (LPA Aspirin Genotype)

The mean values of serum Lp(a) in the homozygous ancestral (TT) group were 50.13 ± 3.42 mg/dL, in the heterozygous (CT) group 50.45 ± 4.17 mg/dL, and in the homozygous polymorphic (CC) 55 mg/dL. There were no statistically significant differences observed between the mean Lp(a) levels with the presence of ancestral or polymorphic allele (*p* value = 0.953, 0.862, and 0.872). The MAF of this variant, as observed in our study is *C* = 0.4090 (NCBI: *C* = 0.05549/246246 gnomAD-Exomes). There was no statistically significant difference between the occurrence of the variant allele among the case and control groups (*p* value = 0.590).

rs1321196

It is observed that with the introduction of the polymorphic allele A, there is an increase in the mean level of serum Lipoprotein(a), the difference being statistically significant (*p* value = 0.023 and 0.002). The MAF of this variant, as observed in our study is *G* = 0.7455 (NCBI: *G* = 0.38968/125568 TopMed Study). However, there was no statistically significant difference between the occurrence of the variant allele among the case and control groups (*p* value = 0.350).

rs9364564

There were no statistically significant differences observed between the mean Lp(a) levels with the presence of ancestral or polymorphic allele (*p* value = 0.726, 0.846, and 0.776). The MAF of this variant, as observed in our study is *G* = 0.4636 (NCBI: *G* = 0.82349/125568 TopMed Study). However, we could not establish a statistically significant difference between the occurrence of the variant allele among the case and control groups (*p* value = 0.219).

Genomic variant analysis revealed significant differences from findings published elsewhere in the world.

Serum Lipoprotein(a) level was analyzed in 60 patients with CAD and 50 controls (Fig. 2). Our results show a statistically significant difference between case and control analyte levels as hypothesized. Receiver operating characteristic curve analysis using the present study revealed the cutoff value of Lp(a) is 45 mg/dL, below which the serum level may be considered as normal for our population. Accordingly, the odds ratio was calculated using the Pearson Chi-square test, which revealed that high levels of serum Lipoprotein(a) is a risk factor for CAD (OR = 3.836; 95% CI = 1.735–8.481; *p* value = 0.001). Figure 3 shows the bar diagram for distribution of Lp(a) using this cutoff, within the study groups. The baseline characteristics of the cases and control are enumerated in Table 2 and the biochemical parameters are listed in Table 3. Traditional coronary risk factors were significantly different between the cases and controls. The lipid parameters were significantly different with levels being significantly lower in cases compared with control owing to the use of cholesterol-lowering medications in this group compared with controls.

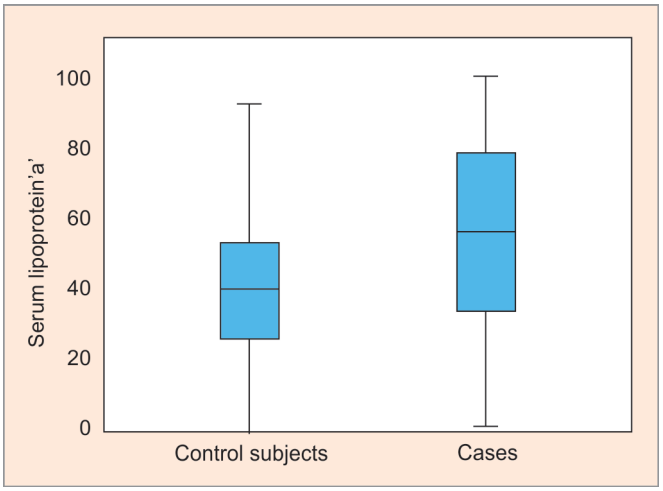

Fig. 2: Comparison of serum lipoprotein (a) between two groups depicted using Box and Whisker plot

Discussion and Clinical Significance

The mean age of patients suffering from CAD was observed to be 56 years with the majority of patients within the age group of 50–59 years. The mean BMI was 23.55 in the case group, comparable to 23.25 in the control group, both fitting within the normal range of BMI according to the WHO criteria. These findings were disquieting and were comparable to the mean BMI observed by Burman et al.

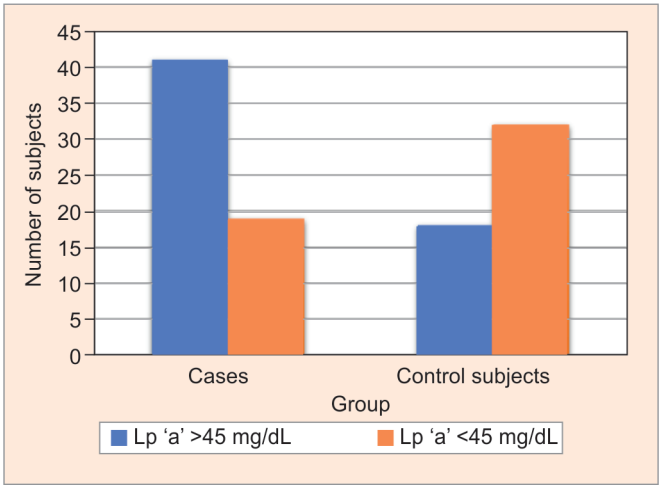

Fig. 3: Distribution of lipoprotein (a) levels within groups along with cutoff decided by ROC analysis

Table 2: Comparison of baseline characteristics between groups

Parameter	*CASE Mean or percentage of subjects*	*CONTROL Mean or percentage of subjects*	*p value*
Age (Years)	56.13	52.94	0.07
Gender ratio (M:F)	54:6	43:7	0.518
BMI (kg/m^2)	23.55	23.25	0.695
Hypertension	21.7	–	–
History of MI in the past	20	–	–
History of smoking	16.7	–	–
History of tobacco use	3.3	–	–
History of alcohol intake	10	–	–

in a North Indian study comprised of patients with CAD.[19] Hence, we may highlight the finding that, despite having a normal BMI, these patients have CAD with triple vessel disease. Blood for fasting lipid profile was drawn from patients admitted for elective CABG surgery, and hence there was no history of recent myocardial infarction. Serum total cholesterol levels were significantly less with a mean value of 124 mg/dL in patients compared with 181 mg/dL in controls, as patients were on high dose statin therapy (40–80 mg HS). Serum LDL-C levels were low in patients, mean value of 68 mg/dL compared with 113 mg/dL in controls. Serum HDL-C was significantly lower in patients, mean value of 34 mg/dL when compared with the control group (44 mg/dL). These findings are taken together to project the risk posed to the body when it is faced with an amalgamation of unacceptably low levels of protective lipoprotein HDL and lower than optimal levels of LDL (<100 mg/dL as per NCEP ATPIII guidelines), which may affect the normal functioning of the immune and nervous system from a biochemical viewpoint.[20,21] Serum alkaline phosphatase was found to be significantly higher in the patient group (p value < 0.003) dedicating to the view that it can serve as a potential biomarker for the disease. Serum Lipoprotein(a) levels were raised in both patients (mean value 57.49 ± 3.67 mg/dL) and healthy controls

Table 3: Comparison of biochemical parameters between the groups

Parameter	*Case mean*	*Control mean*	*p value*
Serum triglycerides (mg/dL)	133	144	0.389
Serum total cholesterol (mg/dL)	124	181	0.000*
HDL-C (mg/dL)	34	44	0.000*
LDL-C (mg/dL)	68	113	0.000*
Total bilirubin (mg/dL)	0.6	0.6	0.475
AST (U/L)	29	26	0.096
ALT (U/L)	30	26	0.335
ALP (U/L)	90	75	0.003
Blood urea (mg/dL)	28	25	0.584
Serum creatinine (mg/dL)	0.7	0.8	0.327

*p values < 0.001

(mean value 41.68 ± 3.34 mg/dL) with significantly higher mean values in patients (p value < 0.002) probably leading to CAD. These results were comparable to the findings of studies by Vandana et al., Burman et al., and Ashfaq et al.[13,19,22] An important finding is that we have observed a significantly higher mean Lp(a) level (41.68 ± 3.34 mg/dL) in the control group than expected, with a p value <0.002. It could be due to the strikingly different genotype and baseline hyperinflammatory state that the Indian population harbors which raise their mean Lp(a) levels, yet without causing disease. Another reason to substantiate this finding could be that en-mass measurement of serum Lp(a) in mg/dL is an inferior predictor of the atherogenic potential of the molecule when compared with measurement of the Lp(a) particle size. As the number of particles increase, there is a stronger propensity for the development of atherosclerosis. This is a direct contributory effect of the difference in the isoform size of apo(a) each individual carries. This fact must be probed into by further large-scale population studies. An isoform insensitive assay may therefore lead to under or over-estimation of the serum values. Despite the above-mentioned reasons, genetic polymorphisms or epigenetic mechanisms play a sturdy role in the quantity and quality of the molecule generated. A cutoff value of 45 mg/dL for Lp(a) was obtained from the ROC curve analysis pointing toward the notion that normal reference range cutoff values must be based on the ethnicity of the population and that we need to re-evaluate our population's baseline values to formulate therapeutic guidelines.

Analysis of SNPs: SNP rs1321196 in Intron 2 caused an increase in serum Lp(a) values in both cases and control subjects. This allele has been observed to increase serum levels of Lp(a) probably through a mechanism that involves the binding of transcription factors (YY1, NR4A2) and increased gene transcription. A study by Deo et al. involving 4,464 patients observed a significant influence of the polymorphic allele over serum Lp(a) levels.[23] However, we noted that the presence of the variant was not limited to patients from our population, as opposed to literature. rs9364564, another less studied intronic variant did not raise serum Lp(a) values in our population. In the Third National Health and Nutrition Examination Survey (NHANES III), this polymorphism revealed significant influence in raising serum Lp(a) levels in two subpopulations: non-Hispanic whites and non-Hispanic blacks but did not show a similar influence on Mexican Americans[16] akin to our observation. This may be because SNPs of the *LPA* gene are not solely responsible for the development of disease. Other factors like variation in the

number of copies of the Kringle IV type II domain influences the major portion of the quantity of Lp(a) in serum.

The exonic variant rs3798220, commonly known as the "*LPA* aspirin genotype" surprisingly did not cause variation in serum Lp(a) levels in our population. It is a T to C polymorphism in the apparently inactive protease domain of apo(a), causing isoleucine to be substituted by methionine. This polymorphism is one of the vastly studied missense variants that raise Lp(a) levels, from within the *LPA* gene, across the world.[4,24,25] Most studies have observed that the presence of the polymorphic allele C increases the risk of CAD twofold. This variant is of clinical interest because carriers of this allele have been shown to benefit from low-dose aspirin therapy as this drug is thought to affect gene transcription.[26] Hence, this variant carries pharmacogenetic importance to CAD patients worldwide. Unlike as in Western literature, unexpectedly, we found that the presence of the polymorphic allele did not lead to a significant increase in serum Lp(a) levels; mean Lp(a) levels for genotypes TT (homozygous ancestral), CT (heterozygous), and CC (homozygous polymorphic) being 50.13 $\pm$ 3.42, 50.45 $\pm$ 4.17, and 55 mg/dL, respectively. There was no statistically significant difference between the cases and control group when tallied for the presence of the variant (p value < 0.590). This fact has been corroborated in a pilot study by Khalifa et al. using samples of patients with CAD from India where they found that this variant did not increase serum Lp(a) levels and was not associated with CNVs.[27]

This is an important finding influencing pharmacogenomics and genetic epidemiology of India as we may conclude that Indians may not be affected by this variant even in its presence as the polymorphic allele was distributed evenly between cases and the control subjects. This opens further questions with regards to the exact molecular or epigenetic mechanisms underlying the variation, as proposed transcription factor binding and oxidation of methionine residue in the protein leading to altered binding of apo(a) to extracellular matrix proteins are not sufficient to understand the role of the variant. The observed minor allele frequencies of all variants were significantly higher when compared with the population database of other countries. However, large population-based genome-wide association studies (GWAS) must be conducted to strengthen our findings. The exact role of SNP markers can be elucidated rightly only by large sample size. This study primarily targeted an analytical search for the genetic markers of hyperlipoprotein(a)emia within the *LPA* gene and their association with the occurrence of CAD. Our study poses few limitations such as restricted sample size, the inclusion of only three variants from the *LPA* gene, and en-mass measurement of serum Lp(a) instead of particle size assessment. However, these factors were outside the scope of the present study.

Conclusion

Preventive measures and risk stratification of individuals with a predisposition to the development of CAD must be strengthened as the mean age of patients is decreasing over time and non-traditional markers are surfacing strongly against a backdrop of traditional risk factors in India. Forty-eight percent of male and 100% of female patients fall under the category of premature CAD in our study. We found that serum Lp(a) levels were not affected by any other variable in the study. Hence, it has been validated as an independent risk factor for the development of CAD. We propose a cutoff value of 45 mg/dL for Lp(a) for primary prevention strategies in Indians, after corroboration from larger cohorts in India. High serum Lp(a) levels, though proved to be a causative factor for the development of CAD, a uniform isoform insensitive assay needs to be introduced for measurement of the analyte across the country and preferably measured as Lp(a) particle number. It is striking to note that the *LPA* aspirin genotype is not prevalent in our population, which warrants GWAS for CAD specific for India. Furthermore, treatment protocols influenced by the presence of genetic markers may be undertaken only after the creation of a population-specific database as the Indian genotype is heterogeneous and unique.

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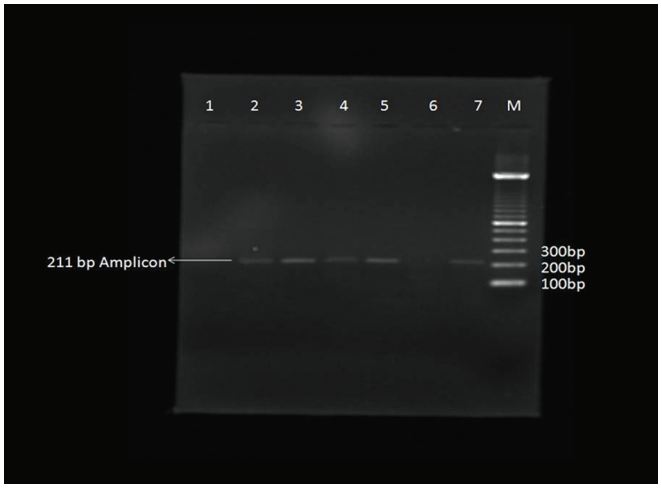
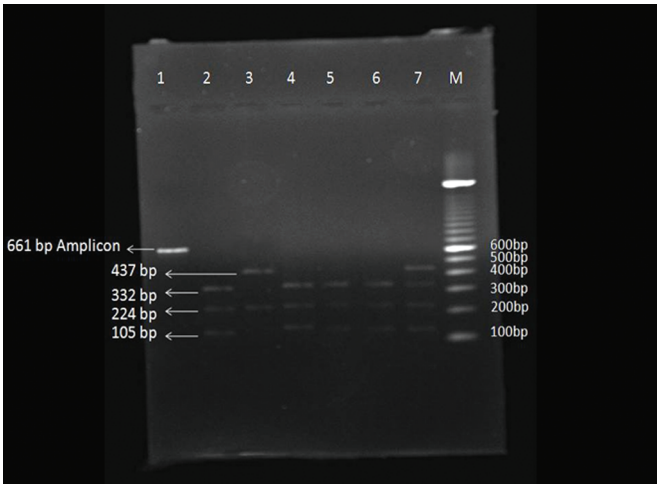
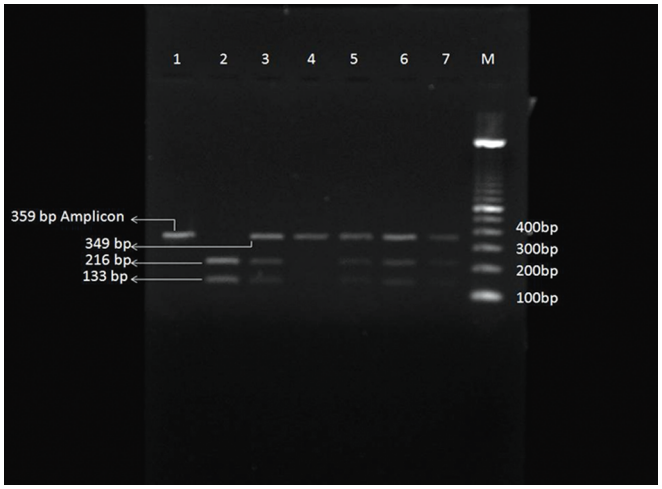


Supplementary Fig. 1: Agarose gel electrophoresis for rs3798220; M: molecular weight marker, Lane 2 and 3, 4 and 5: heterozygous (TC), Lane 6 and 7: homozygous ancestral (TT)



Supplementary Fig. 2: Agarose gel electrophoresis for rs9364564; M: molecular weight marker, Lane 2, 4, 5, 6: homozygous ancestral (GG), Lane 3: homozygous polymorphic (AA), Lane 7: heterozygous (GA)



Supplementary Fig. 3: Agarose gel electrophoresis for rs1321196; M: molecular weight marker, Lane 2: homozygous ancestral (GG), Lanes 3, 5, 6, 7: heterozygous (GA), Lane 4: homozygous polymorphic (AA)

Supplementary Table 1: SNP variant rs3798220: the size of amplicon and details of AS-PCR products

Size of amplicon (bp)	*Products visualized*
211	TT: Product amplified only in the tube with forward ancestral primer and reverse primer
	CC: Product amplified only in the tube with forward polymorphic primer and reverse primer
	TC: Product amplified in both tubes

Supplementary Table 2: SNP variant rs9364564: the size of amplicon and Hae III digested products

rs9364564	
Size of amplicon (bp)	*Size of digested products (bp)*
661	GG: 332-224-105
	AA: 437-224
	GA: 437-332-224-105

Supplementary Table 3: SNP variant rs1321196: the size of amplicon and Hae III digested products

rs1321196	
Size of amplicon (bp)	*Size of digested products (bp)*
359	GG: 216-133-10
	AA: 349-10
	GA: 349-216-133-10