Apolipoprotein A1 Gene Polymorphism (G–75A and C+83T) in Patients With Myocardial Infarction

A Pilot Study in a North Indian Population

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Key Words: Myocardial infarction; Apolipoprotein A1; High-density lipoprotein; Apolipoprotein B; Coronary artery disease

Abstract

The apolipoprotein A1 gene polymorphism (G–75A and C+83T) was studied in 100 subjects (50 patients diagnosed with myocardial infarction and 50 healthy subjects). Serum apolipoprotein (apo) A1 and apo B levels were estimated immunoturbidometrically. Extracted DNA from blood was amplified by polymerase chain reaction, digested with MspI restriction enzyme, run on 8% polyacrylamide gel, and restriction fragment length polymorphism was studied by using a gel documentation system. Serum (mean ± SD) apo A1 levels were significantly higher in control subjects than the study group (100.80 ± 7.06 mg/dL [1.0 ± 0.07 g/L] and 72.56 ± 9.86 mg/dL [0.73 ± 0.1 g/L], respectively; P < .0001), whereas apo B levels were significantly lower (72.12 ± 11.32 mg/dL [0.7 ± 0.1 g/L] and 97.45 ± 9.04 mg/dL [1.0 ± 0.09 g/L], respectively; P < .0001). The G allele frequency at the –75-base-pair (bp) site was higher in the study group (79%) compared with the control group (58%). The T allele frequency at the +83-bp site was higher in the study group (56%) than in the control group (32%). G at –75 bp upstream from the start of transcription and T at +83 bp in the first intron may be susceptibility alleles for myocardial infarction.
Materials and Methods

The study was carried out in the Department of Biochemistry in collaboration with the Department of Medicine, Lady Hardinge Medical College and Smt. Sucheta Kriplani Hospital, New Delhi, India, during the period May 2008 to April 2009. The study was approved by the Lady Hardinge Medical College Scientific Review Board and Ethics Committee.

A total of 100 subjects were included in the study with informed consent. They were selected from the wards and outpatient department of the medicine department, Lady Hardinge Medical College and Smt. Sucheta Kriplani Hospital. Subjects were divided into a study group and a control group.

The study group consisted of 50 patients with documented MI of either sex and older than 40 years. They were selected randomly from patients admitted to the hospital and who fulfilled the inclusion criteria. The selection criteria for MI cases included the typical rise and/or fall in the levels of biochemical markers of myocardial necrosis with at least 1 of the following: ischemic symptoms, development of pathologic Q waves in the electrocardiogram, electrocardiographic changes indicative of ischemia (ST segment elevation or depression), and imaging evidence of new loss of viable myocardium or new regional wall motion abnormality.

The control group consisted of 50 age- and sex-matched control subjects with no history or clinical evidence suggestive of CAD.

All cases and controls were subjected to detailed history with special reference to cardiovascular disease risk factors followed by clinical examination. Venous blood was collected from the antecubital vein with informed consent and under sterile conditions after overnight fasting. From the study subjects, the sample was collected within 3 days of the episode of MI. For the study, 4 mL of blood was collected in plain vials for routine biochemical investigations and extended lipid profiles. Serum samples were stored in aliquots at −20°C and were not thawed until the batch was analyzed for serum apo A1 and apo B levels. For gene polymorphism studies, blood was collected with anticoagulant (1 part of 5% EDTA for each 9 parts of blood) and mixed by gentle shaking. This sample was centrifuged at 2,500g for 10 minutes. Plasma was separated, and the remaining cell aggregate was stored at −20°C until analyzed by polymerase chain reaction (PCR) and restriction fragment length polymorphism after DNA extraction.

Routine biochemical investigations were carried out in an autoanalyzer (SYNCHRON CX9, Beckman Coulter, Brea, CA) using standard reagents and kits. Determination of apo A1 and apo B levels in serum was performed by immunoturbidimetric assay on the SYNCHRON CX9 using a kit from Randox Laboratories (Crumlin, England). It was based on the reaction of a sample containing human apo A1/apo B and specific antisera to form an insoluble complex, which was measured turbidimetrically at 340 nm. By constructing a standard curve from the absorbance of standards, the concentration of apo A1 and apo B in the serum sample was determined.

Study of APOA1 Gene Polymorphism

DNA extraction was done by using the Fermentas Genomic DNA Purification Kit (No. K0512, Fermentas, Burlington, Canada). Double-stranded DNA was isolated from human blood by lysis of leukocytes with subsequent selective DNA precipitation with detergent. This DNA was concentrated and desalted by ethanol precipitation. The typical DNA yield was in the range of 2 to 10 μg from 200 μL of fresh blood. The purity of DNA was ascertained by calculating the A260/A280 ratio. It was found to be in the acceptable purity range of 1.5 to 1.8, ruling out RNA contamination.

The genomic DNA was amplified by PCR using primers flanking the polymorphic region of the APOA1 gene. The primer pair used was as follows: forward: 5′-AGG GAC AGA GCT GAT CCT TGA ACT TAA-3′, and reverse: 5′-TTA GGG GAC ACC TAG CCC TCA GGA AGA GCA-3′.

Preparation of the reaction mixture was carried out taking all necessary precautions to prevent contamination. The constituents of the cocktail mixture for 10 reactions were as follows: nuclease-free water, 153 μL; 10× buffer, 25 μL; magnesium chloride, 25 μL; deoxynucleoside triphosphate, 5 μL; primer (forward), 10 μL; primer (reverse), 10 μL; and Taq DNA polymerase, 2 μL.

The DNA cocktail mix thus prepared was “vortexed” briefly. It was divided into aliquots in 0.2-mL PCR tubes.
To 23 μL of this PCR mix in each tube, 2 μL of DNA was added to make 25 μL of reaction mixture. Tubes were vortexed and then centrifuged. After this step, they were put in a preprogrammed MJ thermal cycler (programmable thermal controller, Bio-Rad, Hercules, CA) for DNA amplification. The program for the APOA1 PCR assay was as follows: initial denaturation at 95°C for 5 minutes, cycle denaturation at 94°C for 1 minute, cycle annealing at 62°C for 1 minute, cycle extension at 72°C for 1 minute, and final extension at 72°C for 10 minutes. There were 35 cycles, and it was held at 4°C till the PCR product was taken out.

Following PCR, the presence of a 433-bp product was ascertained by using 2% agarose gel where it was visualized as a discrete band using a gel documentation system (Alpha DigiDoc, Alpha Innotech, San Leandro, CA). The 433-bp fragment obtained was digested with the restriction enzyme MspI to ascertain the APOA1 gene polymorphism. For this step, 9 μL of PCR product was digested with 10 units of MspI restriction enzyme overnight at 37°C in the presence of 1 μL of 10× buffer provided with the restriction enzyme. The digested PCR product was run on 8% polyacrylamide gel. For the samples, 11 μL of digested PCR product and 4 μL 6× loading dye and for the marker, 2 μL of 50-bp/100-bp ladder, 8 μL of nuclease-free water, and 2 μL of loading dye were used. It was run in 1× Tris/borate/EDTA buffer in a vertical electrophoresis apparatus at 200 V for 1.5 hours. The gel was stained with ethidium bromide and then viewed in a gel documentation system (Alpha DigiDoc). Restriction fragment length polymorphism was determined by interpretation of the restriction fragment length on the gel picture and comparison with the DNA bp marker, which was run simultaneously. There are 3 restriction sites for MspI in the APOA1 gene at −75, +37, and +83 bp, and, therefore, 4 fragments are produced that are 45, 66, 113, and 209 bp. When there is G to A transition at −75 bp, this restriction site is lost, and it produces a fragment of 179 bp instead of 113 and 66 bp. Similarly when a C to T transition takes place, the +83 bp restriction site is lost and a fragment of 254 bp is produced instead of 209 and 45 bp. The gel picture is interpreted using this information. This was done for each subject, and interpretation (with respect to −75 bp and +83 bp site polymorphism) was documented as an allelic profile of each subject.

Statistical Analysis

Statistical analysis was done by using the GraphPad Prism (version 5.02) software program (La Jolla, CA [http://www.graphpad.com]). The mean and standard deviation for all parameters were calculated. The Student t test was used to analyze clinical and laboratory data, and the χ² test was used wherever required. A P value of .05 or less was considered statistically significant; a P value of .001 or less, highly significant; and a P value of .0001 or less, highly significant.

The frequency of genotypes (GG, GA, AA) at −75 bp in the promoter region of the APOA1 gene and the frequency of genotypes (+ +, + –, and − –) at +83 bp in the first intron of the APOA1 gene were assessed by using the χ² test. Allele frequency was calculated by using the equation p² + q² = 1 where p and q are the frequencies of each allele at the particular locus. Hardy Weinberg equation equilibrium was followed as shown by p² + q² + 2pq = 1. The unpaired Student t test and analysis of variance were used to analyze the significance of difference in values of HDL, apo A1, and apo B in different genotypes at −75 bp and +83 bp, respectively.

Results

The study and control groups were matched for age and sex, which was further verified by statistical evaluation. Table I shows the serum levels of HDL, apo A1, and apo B and the apo B/apo A1 ratio in the 2 groups. The HDL level was significantly lower in cases compared with controls (24.78 ± 4.28 vs 37.23 ± 7.73; P < .0001). The serum apo A1 level was significantly reduced in the study group (72.56 ± 9.86 mg/dL [0.73 ± 0.1 g/L]) compared with the control group (100.80 ± 7.06 mg/dL [1.0 ± 0.07 g/L]; P < .0001). The serum apo B level in the study group was significantly higher (97.45 ± 9.04 mg/dL [1.0 ± 0.09 g/L]) than in the control group (72.12 ± 11.32 mg/dL [0.7 ± 0.1 g/L]; P < .0001). The serum apo B/apo A1 ratio in the study group was higher (1.37 ± 0.25) than in the control group (0.73 ± 0.13; P < .0001).

Table II

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Study Group (n = 50)</th>
<th>Control Group (n = 50)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SD age (y)</td>
<td>47.18 ± 4.83</td>
<td>47.36 ± 4.81</td>
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</tr>
<tr>
<td>Sex distribution</td>
<td></td>
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<td>.687</td>
</tr>
<tr>
<td>Males</td>
<td>23 (46)</td>
<td>20 (40)</td>
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</tr>
<tr>
<td>Females</td>
<td>27 (54)</td>
<td>30 (60)</td>
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<td>Family history</td>
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<tr>
<td>Hypertension</td>
<td>31 (62)</td>
<td>11 (22)</td>
<td>&lt;.001</td>
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<tr>
<td>Diabetes mellitus</td>
<td>27 (54)</td>
<td>11 (22)</td>
<td>&lt;.001</td>
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<td>Coronary artery disease</td>
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<td>3 (6)</td>
<td>.002</td>
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<td>Personal history</td>
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</tr>
<tr>
<td>Diabetes mellitus</td>
<td>5 (10)</td>
<td>3 (6)</td>
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<td>Smoking</td>
<td>15 (30)</td>
<td>6 (12)</td>
<td>.048</td>
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<tr>
<td>Alcohol intake</td>
<td>9 (18)</td>
<td>7 (14)</td>
<td>.585</td>
</tr>
</tbody>
</table>

* Data are given as number (percentage) unless otherwise indicated.
Table 3 shows the distribution of genotypes and alleles of the APOA1 (G–75A) polymorphism in the study and control groups. The GG genotype was found in 29 (58%) of the subjects in the study group compared with 8 (16%) of subjects in the control group. The GA genotype was found in 21 (42%) of the subjects in the study group compared with 42 (84%) of the subjects in the control group. No AA genotype was found in either group. The difference was highly significant ($\chi^2 = 18.919; P < .0001$). The frequency of the G allele was higher (79%) in the study group than in the control group (58%). On the other hand, A allele frequency was higher in the control group (42%) compared with the study group (21%).

Table 4 shows the distribution of genotypes and alleles of the APOA1 (C+83T) polymorphism in the study and control groups. The ++ genotype (homozygous CC at +83) was found in 12 (24%) of the subjects in the study group and 18 (36%) of the subjects in the control group. The +– genotype (heterozygous CT at +83) was found in 20 (40%) of the subjects in the study group and 32 (64%) of the subjects in the control group. The genotype –– (homozygous TT at +83) was found in 18 (36%) of the study group but in none of the control group. The frequency of the + allele (C) was 44% in the study group and 68% in the control group. The frequency of the – allele (T) was higher (56%) in the study group than in the control group (32%). The frequency values of the – (T) allele (56%) and the G (79%) allele were higher in the study group than the control group, where the values were 32% and 21%, respectively (Tables 3 and 4).

Table 5 shows the comparison of HDL, apo A1, and apo B levels in different genotypes at –75 bp and +83 bp. The HDL and apo A1 levels were significantly lower ($P < .0001$ for both) in the GG genotype compared with the GA genotype, and the apo B level was significantly higher in the GG genotype. No AA genotype was found in either group. The difference was highly significant ($\chi^2 = 18.919; P < .0001$). The frequency of the G allele was higher (79%) in the study group than in the control group (58%). On the other hand, A allele frequency was higher in the control group (42%) compared with the study group (21%).
(P < .05) compared with the GA genotype. A similar analysis at +83 bp suggested that the TT (––) genotype showed the lowest and heterozygosity at this site showed the highest level of protective HDL and apo A1 in all subjects. Table 6 depicts the levels of HDL, apo A1, and apo B with respect to combined genotype at –75 bp and +83 bp. The highest levels of HDL and apo A1 were found in the GA/+– genotype and the lowest levels in the GG/–– genotype.

Lanes 1 and 2 have 5 fragments of 45, 66, 113, 209, and 254 bp, which means it is heterozygous at +83 bp because of the presence of a 254-bp fragment, suggesting that it is the GG/+– genotype. Lane 3 has 45, 66, 113, 179, and 209 bp fragments, which suggests it to be heterozygous at a –75 bp site that is GA/++. Lane 4 has 45, 66, 113, 179, 209, and 254 bp fragments, suggesting it to be heterozygous at both sites and thereby GA/+–. Lane 5 has 45, 66, 113, 209, and 254 bp fragments, which means it is GG/+–. Lanes 6, 7, and 8 have 45, 66, 113, 179, and 209 bp fragments, suggesting it to be GA/++.

Lanes 9 and 10 have 4 fragments of 45, 66, 113, and 209 bp, suggesting it to be GG/++.

Discussion

We measured the serum levels of HDL, apo A1, and apo B and the apo B/apo A1 ratio in 2 age- and sex-matched groups. The study group included 50 patients who had documented MI, and the control group included 50 healthy subjects.

The HDL level was significantly lower in cases compared with controls. Our findings that the serum apo A1 level was significantly lower in the study group than in the control group and that the serum apo B level in the study group was significantly higher than in the control group are consistent with the findings of various other studies.17-20 We found a highly significant difference (P < .0001) in serum apo B/apo A1 ratios in the study group vs the control group. Our study findings are in accord with the findings of the AMORIS study,17 the MONICA/KORA Augsburg Cohort Study,21 and the Thrombo Study.22 The apo B/apo A1 ratio reflects the status of the major atherogenic and antiatherogenic pathways of lipid metabolism.

Polymorphism of the APOA1 gene was studied at 2 sites: –75 bp in the promoter region and +83 bp in the first intron of APOA1 gene. The GG genotype was found more frequently in patients with MI compared with healthy control subjects, in whom the GA genotype was more frequent. This difference was highly significant ($\chi^2 = 18.919; P < .0001$). The small sample could be the reason that we found no AA genotype in either group. The frequency of the G allele in the study group was highly significantly greater than in the control group, showing that the G allele may be a susceptibility

<table>
<thead>
<tr>
<th>Genotype</th>
<th>HDL, mg/dL (mmol/L)</th>
<th>Apo A1, mg/dL (g/L)</th>
<th>Apo B, mg/dL [g/L]</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA/++</td>
<td>31.76 ± 5.69 (0.82 ± 0.15)</td>
<td>94.53 ± 10.73 (0.82 ± 0.15)</td>
<td>78.47 ± 13.47 (0.8 ± 0.15)</td>
</tr>
<tr>
<td>GA/+</td>
<td>37.03 ± 8.95 (0.96 ± 0.23)</td>
<td>97.55 ± 10.25 (1.0 ± 0.1)</td>
<td>79.31 ± 16.85 (0.8 ± 0.2)</td>
</tr>
<tr>
<td>GA/––</td>
<td>24.80 ± 3.62 (0.64 ± 0.09)</td>
<td>72.79 ± 5.79 (0.7 ± 0.1)</td>
<td>99.04 ± 7.39 (1.0 ± 0.1)</td>
</tr>
<tr>
<td>GG/+</td>
<td>62.28 ± 5.14 (0.86 ± 0.11)</td>
<td>81.58 ± 17.02 (0.8 ± 0.2)</td>
<td>85.65 ± 17.13 (0.9 ± 0.2)</td>
</tr>
<tr>
<td>GG/–</td>
<td>24.38 ± 3.43 (0.63 ± 0.09)</td>
<td>72.29 ± 2.86 (0.7 ± 0.09)</td>
<td>95.64 ± 3.41 (1.0 ± 0.03)</td>
</tr>
<tr>
<td>GG/––</td>
<td>22.72 ± 3.63 (0.59 ± 0.09)</td>
<td>62.16 ± 6.39 (0.6 ± 0.1)</td>
<td>95.02 ± 13.10 (1.0 ± 0.1)</td>
</tr>
</tbody>
</table>

Apo, apolipoprotein; HDL, high-density lipoprotein.

* Data are given as mean ± SD.
allele for CAD in the Indian population. On the other hand, frequency of the A allele was greater in the control group than in the study group. Our study findings are consistent with the findings of studies by Saha et al., Pischon et al., Yangchun et al., and Kamboh et al. These groups also evaluated the effect of the A allele on HDL and apo A1 levels and found a positive association for those and, thereby, a decreased cardiac risk. In contrast, Reguero et al. showed there was higher frequency of the A allele in subjects younger than 50 years with a diagnosed MI (0.30) than in a control group (0.24), which was not in agreement with our findings. Also in an Australian population, it was found that the presence of the A allele increased the severity of CAD, which was not consistent with our findings.

A proposed explanation for the effect of the A allele on apo A1 levels suggests that the presence of the A allele at –75 bp from transcription start site of the gene increases the transcriptional efficiency of the promoter. The mechanism is probably due to decreased stability of a DNA-protein complex that inhibits transcription. Deletion analysis of the APOA1 promoter has suggested that the 199 to –40 bp region is needed for repression of transcription by the binding factor, with a molecular weight of 90 kDa and that is present in all cell types other than liver and intestine. The nucleotide at the –75 position of the APOA1 promoter lies in a GC-rich sequence (5’-GCC[A/G]GGG-3’). The transcription of these promoters has been shown to be negatively regulated by this GC box. A GC binding factor has been cloned and shown to repress the transcription of promoters carrying these sequence elements. This 90-kDa factor could mediate the repression of transcription observed in the G allele. The G to A transition decreases its binding affinity to –75 bp position and alleviates the repression of APOA1 gene transcription.

The C+83T polymorphism is associated with CAD in some but not all studies. In our study, the CC (++) and CT (–+) genotypes were found more often in the control group, whereas the TT (––) was found exclusively in patients with an MI, although CC and CT were also present. The difference was highly significant (\( \chi^2 = 21.969; P < .0001 \)). The frequency of the + allele (C) was lower in the study group than in the control group, whereas the frequency of the – allele (T) was higher in the study group than in the control group. These findings are in agreement with the findings of studies by Shioji et al and Wang et al.

Reguero et al., in a study of Spanish subjects younger than 50 years with diagnosed MI, found no significant difference in the frequency of the – allele in study and control groups (0.04 vs 0.07), a finding not in agreement with our findings. The discrepancy could be due to the different ethnicity of the study population. In contrast, a study by Wang et al suggested a protective effect of the – allele on CAD risk by raising the levels of HDL and apo A1. The mechanism mediating the association between the C to T substitution and an increased HDL cholesterol level is yet to be resolved. However, it is proposed that the methylation pattern of the 5’ region of the APOA1 gene reflects the extent of its expression. The 5’ region of the APOA1 gene has been found to be hypomethylated in tissues that express the gene (ie, liver) but heavily methylated in nonexpressing tissues. The MspI site at +83 bp (containing a CpG dinucleotide) is known to be methylated in nonexpressing cells but undermethylated in cells expressing apo A1. It is possible that T substitution at this site may lead to further demethylation, resulting in more cells expressing the gene. Alternatively, the T substitution could influence the translation of APOA1 messenger RNA (mRNA) because the substitution occurs in the 5’ end leader region for APOA1 mRNA, and this could be important for the initiation of mRNA translation.

Discrepancies in the findings of these studies and our study could be attributed to differences in the genetic susceptibility between different ethnic groups. Also, the APOA1 gene locus lies in a cluster with CIII and AIV loci, which could be in linkage disequilibrium with the APOA1 alleles in some populations but not in others.

The significantly lower HDL and apo A1 levels in the GG genotype compared with the GA genotype and the significantly higher apo B level in the GG compared with the GA genotype suggest that the A allele is associated with increased HDL and apolipoprotein levels. A similar analysis at +83 bp revealed the TT (––) genotype to have the lowest and heterozygosity at this site to have the highest level of protective HDL and apo A1 in all subjects. The highest levels of HDL and apo A1 were found in the GA/+– genotype and the lowest levels in the GG/–– genotype.

We conclude that APOA1 polymorphism (G–75A and C+83T) may be associated with MI by affecting the levels of HDL and apo A1 as suggested by our study. G at –75 bp and (T) at the +83 bp site may be susceptibility alleles for CAD in the Indian population. However, more studies with larger samples are needed to confirm genotypic risk associated with the G and – alleles.

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References