ABSTRACT

Background: Adiponectin is an abundant adipose tissue–derived protein with anti-atherogenic, anti-inflammatory and antidiabetic properties. Plasma adiponectin levels are decreased in obesity, type 2 diabetes, and coronary artery disease and low adiponectin levels also predict insulin resistance (IR).

Methods: Case-control study in which 642 male and female subjects were participated from the North Indian population. Lipid, insulin, leptin and adiponectin level were estimated using standard protocols by commercially available test kits. Single nucleotide polymorphisms +45T>G and +276G>T of the AMP1 (adiponectin) gene was genotyped by polymerase chain reaction restriction fragment length polymorphism method.

Results: Levels of adiponectin, insulin, homeostasis model assessment-IR index (HOMA-IR index), systolic blood pressure and fat mass showed significant differences between male and female subjects. Serum adiponectin level showed highly significant association with both the +45 and the +276 genotypes. The common haplotype triglyceride (TG) showed a significantly lower adiponectin value than other haplotypes (P = 0.0001). A clear trend of decreasing adiponectin levels per copy of the common haplotype was observed. Nonobese insulin sensitive subjects showed a higher adiponectin value (P = 0.0006) than nonobese insulin resistant subjects. The values of blood pressure, adiponectin, insulin, HOMA-IR, total-cholesterol, and low-density lipoprotein-cholesterol significantly associated with TG haplotype.

Conclusions: We observed the very strong association of the adiponectin 45-276 genotypes and haplotypes with adiponectin levels in healthy north Indian population and TG haplotypes also associated with metabolic parameters of the IR syndrome.

Keywords: Adiponectin, AMP1, haplotype, insulin resistance, polymorphism

INTRODUCTION

Adipose tissue is an active endocrine organ that secretes adiponectin,[1] which is a potent insulin sensitizer in muscle and liver, regulating energy homeostasis and glucose tolerance.[2] There is large evidence that the adipose tissue is an active endocrine organ, not only the
major tissue for energy storage and secreting a different type of proteins that influence the metabolism of the body and affect energy and glucose homeostasis.\[3\]

Adiponectin is the product of the AMP1 (Adiponectin) gene, which spans approximately 15.8 kb and three exons. It is sited on chromosome 3q27, which has been linked to a susceptibility locus for metabolic syndrome, type 2 diabetes and cardiovascular disease.\[4\]

In a previous study, adiponectin concentrations were positively correlated with insulin sensitivity and decreased significantly with deteriorating glucose tolerance in Pima Indians and Caucasians,\[5\] while adiponectin concentrations increased after weight reduction.\[6\] In addition, administration of intravenous recombinant adiponectin to rodent models of insulin resistance (IR) restored normal insulin sensitivity.\[7\]

In animal model of obesity and diabetes, administration of adiponectin or its globular domain produces weight loss and improves insulin sensitivity and glucose tolerance. The adiponectin acts on skeletal muscle to increase fatty acid oxidation and on liver to increase sensitivity to the anti-glucogenesis effects of insulin.\[7,8\] With such profound effects on metabolism, genetic variability in adiponectin may be a determinant of IR. Results of two linkage studies are consistent with this hypothesis: A genome scan for IR loci in U.S. Caucasian families detected a signal at 3q27 the locus of the AMP1 gene.\[9\] The early-onset type 2 diabetes has been linked to the same position in French families.\[10\] In addition, association study from Japan points out these linkage results directly to the AMP1 gene.\[4\] The gene is very polymorphic; associations with adiponectin level and/or the metabolic syndrome have been reported for genetic variants in many populations.

Two single nucleotide polymorphisms in the AMP1 gene, a silent T to G substitution in exon 2, 45T > G (rs2241766) and another G to T substitution in intron 2, 276 G > T (rs266729) have been associated with adiponectin levels, obesity, type 2 diabetes and coronary artery disease patients, although there are inconsistencies among these results.\[11-19\]

Therefore, in the present study, we investigated the association of AMP1 45-276 genotypes and haplotypes with serum adiponectin levels and their association with IR syndrome, in large ethnically homogeneous and healthy northern Indian population.

**METHODS**

**Study subjects**

All individuals were of north Indian origin, and the population was homogeneous with regard to ethnic background. A total of 642 subjects were enrolled from the out patients department of King George’s Medical University, Lucknow and volunteers from the general population of Lucknow (Uttar Pradesh, India). Of these, 88.20% were Hindu north Indian (Hindi spoken residing in Lucknow) while 21.8% represented other religions or languages. The possibility of population admixture was slight, because, in this part of the country, inter-religion marriage is very rare because of the traditional socioeconomic differences among different religions (for details).\[20\] 309 obese (body mass index [BMI] > 30 kg/m\(^2\)) and 333 nonobese (BMI ≤ 30 kg/m\(^2\)) individual were selected on the basis of the BMI. In all subjects, body height, body weight, waist circumferences and hip circumferences were measured for the calculation of BMI and waist to hip ratio. Subjects with established diabetes mellitus, coronary artery disease, congestive heart failure, and pregnant women were excluded. Subjects were classified as type 2 diabetics if they were on hypoglycemic medications or when their fasting glucose concentrations exceeded 126 mg/dL. Tobacco smokers (12.6%) included subjects with present or past smoking or any other tobacco use. About alcohol usage, 88.6% were nonalcoholic. In our study participants, 56.1% of participants were pure vegetarians (no consumption of meat, egg or fish). Informed consent was obtained from each participant, and the study was carried out in accordance with the local ethics committee. At baseline, all study participants were subjected to a thorough screening program that included assessment of a detailed personal and family history, physical examination, determination of anthropometric indices and measurement of various biochemical parameters.

**Estimation of body fat composition**

The body composition was measured by bioelectrical impedance analyzer (Tanita–TBF–310, Tokyo, Japan). The impedance measurements were obtained on the morning of the examination after the individuals had voided while fasting. The results obtained were BMI, the body fat mass, fat-FM, and percent of body fat (fat %).

**Laboratory measurements**

Venous blood was collected after an overnight fast, and plasma, and serum samples were either used immediately for analysis or were stored frozen at −80°C. Commercial enzymatic test kits were used for determining high-density lipoprotein-cholesterol (HDL-C), triglyceride (TG) concentrations and total-cholesterol (T-cholesterol), low-density lipoprotein-cholesterol (LDL-C) was calculated by the formula of Friedewald (LDL-C = T-cholesterol − HDL-C − TG/5 mg/dL). Insulin level was determined by enzyme-linked radio immnosorbent assay (Linco Research, St Charles, USA). The degree of insulin sensitivity/resistance
was calculated according to the homeostasis model assessment (HOMA) which is a good index for assessing insulin sensitivity/resistance. IR was calculated as follows: IR = FI × g/22.5; where FI = fasting insulin (μu/mL) and g = fasting glucose (mmol/L). [21]

Adiponectin was assayed with enzyme-linked immunosorbent assay method. The fasting glucose concentration was measured by glucose oxidase-peroxidase (GOD-POD) method, [22] systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured twice on the right arm, after a 15-min rest, using a mercury sphygmomanometer. [23] All protocols were approved by the Institutional Review Board or Ethical Committee (IRB number - XXIV ECM/P6) at King George’s Medical University, Lucknow, Uttar Pradesh, India and all the subjects gave informed consent.

Genotyping
The genomic DNA was extracted from peripheral blood leucocytes pellet using the standard salting-out method. [24] Single nucleotide polymorphism (SNP) +45T>G in the AMP1 gene was genotyped by the amplification of genomic DNA using the following primers: Forward, 5'- TGT GTC TGT CCG GTG TGT CT-3' and reverse, 5'- TGT GAT GAA AGA GCC CAG AA-3'. The amplification conditions were used as follows: 94°C for 10-min, followed by 35 cycles of 30 s at 94°C, 30 s at 57°C, and 30 s at 72°C, and ending with a single 10-min extension step at 72°C. The polymerase chain reaction fragment was 305-bp in length and was digested with the enzyme Ava I at 37°C. +276G>T in the AMP1 gene was genotyped by the amplification of genomic DNA using another pair of primers: Forward, 5'- CTA CAC TGA TAT AAA CTA TAT GGA G-3' and reverse, 5'- CCC CAA ATC ACT TCA GGT TG-3'. The amplification conditions were as follows: 94°C for 10-min, followed by 35 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C, and ending with a single 10-min extension step at 72°C. The resulting fragment was 107 bp in length. The polymorphism was typed using the enzyme Hinf I at 37°C. In the genotyping experiments for both SNPs, the digestion products separated on 15% polyacrylamide gels. Bands were visualized by ethidium bromide staining.

Statistical analysis
Genotype and allele distribution was compared between obese and nonobese subjects using Chi-square test. The independent segregation of alleles was tested for the Hardy–Weinberg equilibrium, comparing the observed genotype frequencies with those expected (Chi-square test). Haplotype analysis was done by SNP analyzer version 1.2 by expectation–maximization algorithm. [25] The association of adiponectin concentrations with the genotypes and the haplotypes was estimated using a general linear regression model. Two-tailed tests were performed with the significance level of 0.05. The results for continuous variables are given as the mean ± standard deviation. The differences among three groups were assessed by one-way analysis of variance for continuous variables. Further, we analyzed the change in adiponectin concentrations per copy of one haplotype. A trend per copy of the haplotype with the mean adiponectin concentrations was ascertained in three groups: Subjects with two, one or zero copies of the rare haplotype. All analyses were adjusted for gender, age and BMI.

The association between haplotypes and serum adiponectin concentrations was also evaluated in four subgroups: (1) Nonobese insulin-sensitive (n = 255), (2) nonobese insulin-resistant (n = 78), (3) obese insulin-sensitive (n = 166), (4) obese insulin resistant subjects (n = 145). In a secondary analysis, the parameters SBP and DBP, fasting sugar, adiponectin, FI concentrations, HOMA-IR, T-Cholesterol, HDL-C and LDL-C and TGs were tested using a general linear regression model to view their association with the APM1 + 45 and + 276 loci.

RESULTS

Association of the +45T>G and +276G>T polymorphisms with adiponectin levels
Demographic characteristics of study subjects are summarized in Table 1. Levels of adiponectin, insulin, and HOMA-IR index showed significant differences between male and female subjects. SBP and FM also showed a significant difference between both the group while other factors like BMI, fasting blood sugar and lipid profile data did not show any significant sex-specific differences. In Table 2a, frequencies of APM1 genotypes are summarized. The G allele of the APM1 +45 showed a frequency (MAF) of 14.72% while MAF of APM1 +276 was 10.98%. Serum adiponectin level showed highly significant association with both the +45 and the +276 genotypes (P < 0.01 and < 0.01 respectively). After comparing the mean (95% confidence intervals) serum adiponectin concentrations per genotype [Table 2a], TT genotype (Wild) of +45 APM1 showed significantly lower adiponectin value than variant genotype GG. Similarly, GG genotype of +276 APM1 showed significantly lower adiponectin value than variant genotype TT. A clear trend of decreasing adiponectin levels per copy of the common allele was also observed for +45 SNP P < 0.01, and +276 SNP, P < 0.01.

When APM1 haplotypes were constructed, the TG haplotype was the most common, with a frequency of 75.39%. The other significant haplotypes in this sample were the GG (14.02%), TT (9.35%) and the GT (1.24%). The common haplotype TG showed significantly lower adiponectin value than other haplotypes (P < 0.01). The other two haplotypes (GG and TT) with one major and
Table 1: Demographic characteristic of the study subjects by sex

<table>
<thead>
<tr>
<th></th>
<th>Male subjects (347)</th>
<th>Female subjects (295)</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td><strong>Demographic profile</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>28.70±5.52</td>
<td>29.67±5.53</td>
<td>NS</td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>123.20±11.68</td>
<td>125.70±15.19</td>
<td>0.014</td>
</tr>
<tr>
<td>DBP (mm Hg)</td>
<td>82.34±7.68</td>
<td>84.64±8.05</td>
<td>NS</td>
</tr>
<tr>
<td>Fat (%)</td>
<td>36.54±7.58</td>
<td>36.27±6.97</td>
<td>NS</td>
</tr>
<tr>
<td>FM (kg)</td>
<td>31.63±8.18</td>
<td>35.21±9.37</td>
<td>0.007</td>
</tr>
<tr>
<td>Free FM (kg)</td>
<td>48.83±9.78</td>
<td>48.64±11.02</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Hormone profile</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting sugar (mg/dL)</td>
<td>108.98±15.94</td>
<td>109.95±18.62</td>
<td>NS</td>
</tr>
<tr>
<td>Insulin (µU/mL)</td>
<td>11.45±6.01</td>
<td>13.83±9.73</td>
<td>0.0001</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>3.13±1.83</td>
<td>3.86±2.87</td>
<td>0.0001</td>
</tr>
<tr>
<td>Adiponectin (µg/mL)</td>
<td>7.01±6.11</td>
<td>7.90±2.63</td>
<td>0.002</td>
</tr>
<tr>
<td><strong>Lipid profile</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T cholesterol (mg/dL)</td>
<td>186.03±44.85</td>
<td>187.40±35.72</td>
<td>NS</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>44.58±10.91</td>
<td>44.70±7.13</td>
<td>NS</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>117.40±30.13</td>
<td>119.32±31.79</td>
<td>NS</td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>125.02±37.11</td>
<td>123.94±32.32</td>
<td>NS</td>
</tr>
<tr>
<td>VLDL-C (mg/dL)</td>
<td>23.48±5.25</td>
<td>23.86±11.18</td>
<td>NS</td>
</tr>
</tbody>
</table>

All values are expressed in mean±SD. NS=Non-significant, BMI=Body mass index, WHR=Waist to hip ratio; SBP=Systolic blood pressure; DBP=Diastolic blood pressure; Fat (%)=Percentage body fat; T-Cholesterol=Total cholesterol; HDL-C=High-density lipoprotein cholesterol; LDL-C=Low-density lipoprotein cholesterol; VLDL-C=Very low-density lipoprotein cholesterol; HOMA-IR=Homeostasis model assessment-insulin resistance, FM=Fat mass, SD=Standard deviation.

We divided study subjects in four subgroups: (1) Nonobese insulin-sensitive, (2) nonobese insulin-resistant, (3) obese insulin-sensitive, (4) obese insulin resistant subjects [Table 3]. Nonobese insulin sensitive subjects showed higher adiponectin value \( P < 0.01 \) than nonobese insulin resistant subjects. However, obese insulin sensitive subjects also showed higher adiponectin value \( P < 0.01 \) in comparison with obese insulin resistant subjects. It shows that higher adiponectin levels in both obese and nonobese insulin sensitive subjects. After comparing adiponectin levels in subjects carrying TG haplotype, it emerge that carriers of this haplotype had lower adiponectin levels both in insulin sensitive and insulin resistant subjects.

The association of the TG haplotype with decreasing adiponectin concentrations was most pronounced in the nonobese insulin-sensitive subjects \( P < 0.01 \) assuming a trend per copy. The association was nonsignificant in the obese insulin-resistant subjects, and in the obese insulin-sensitive subjects \( P < 0.01 \) assuming a trend per copy). In a secondary analysis, a series of anthropometric and biochemical parameters (T-cholesterol, HDL-C and LDL-C, very low density lipoprotein-cholesterol (VLDL-C), TGs, SBP and DBP, HOMA-IR, fasting glucose and F1 concentrations) were analyzed with respect to their association with the TG haplotype [Table 4]. The values of SBP and DBP, adiponectin, insulin, HOMA-IR, T-cholesterol, and LDL-C significantly associated with TG haplotype [Table 4].
DISCUSSION

The adipose tissue secretes a variety of molecules having endocrine, paracrine, and autocrine actions and has a role in the etiology of the metabolic abnormalities. Among several proteins secreted by adipocytes, adiponectin is prominent because of its insulin-enhancing actions in the liver and muscle, as well as its anti-inflammatory effects. The present study suggests that two common SNPs, AMP1 G>T substitution in intron 2 (+276G>T) in a silent T>G substitution in exon 2 (+45T>G) and a prominent because of its insulin‑enhancing actions in the liver and muscle, as well as its anti‑inflammatory effects. The adipose tissue secretes a variety of molecules having endocrine, paracrine, and autocrine actions and has a role in the etiology of the metabolic abnormalities. Among several proteins secreted by adipocytes, adiponectin is prominent because of its insulin‑enhancing actions in the liver and muscle, as well as its anti‑inflammatory effects. The present study suggests that two common SNPs, AMP1 G>T substitution in intron 2 (+276G>T) in a silent T>G substitution in exon 2 (+45T>G) and a T‑cholesterol (mg/dL) 45‑276 haplotype TG haplotype group

Table 3: Association of the AMP1 45‑276 haplotype and adiponectin concentrations in insulin sensitive categorized subgroups

Table 4: Association of clinical and metabolic characteristics of the study population with adiponectin AMP1 45‑276 haplotype TG

AMP1 +45T>G gene polymorphism associated with reduced circulating adiponectin levels in nonalcoholic fatty liver disease patients. Ong et al. found that genetic variants in the adiponectin gene influenced plasma adiponectin levels in the Chinese population. Chung et al. observed that subjects with the SNP 45TT genotype showed increased adiponectin levels and AMP1 genetic variants can affect insulin resistance indexes. Youpeng et al. observed significant association of polymorphism +276G>T with serum adiponectin level. On the other hand, Demirci et al. reported that serum adiponectin levels do not associate with +45T>G gene polymorphism. Sanghera et al. observed significant association of AMP1 variant with body weight, waist circumference, and hip circumference. On the other hand, genetic polymorphisms in FTO[33,34] and other...
genes associated with obesity may also contribute to the above association. Systematic testing of these genes is a challenge for any study that follows.

The chromosomal region 3q27, contains APM1, the gene encoding adiponectin. In genome wide linkage scans several susceptibility loci for type 2 diabetes were identified.[85] Recently, AMP1 gene polymorphism may play an important role in the development of type 2 diabetes in South Indian population.[86] Stronger associations were observed with the insulin resistance syndrome when the two SNPs were considered together as haplotypes. Menzaghi et al. reported association of the haplotype TG with higher plasma insulin and lower adiponectin levels in healthy individuals.[79] In the present study, it appears that adiponectin levels are influenced by multiple factors including TG haplotype and obesity phenotype. These finding are also in agreement with a previous study.[88]

The result showed that, hypoadiponectinemia is a principal, genetically determined defect that contributes into the etiology of obesity and insulin resistance. Adiponectin is significantly associated with obesity, insulin resistance and other obesity-related phenotypes our previous study.[99]

Animal models studies have reported that adiponectin is a strong insulin enhancer, regulating energy homeostasis and glucose tolerance.[87,88] Mice fed a high-fat diet reported weight loss when constantly treated with a proteolytic fragment of adiponectin.[89] In addition, Insulin resistance decreased in mice after administration of adiponectin, which are genetically predisposed to obesity and diabetes by fatty acid oxidation and by augmenting insulin’s ability to reduces hepatic glucose production.[87,88] Huang et al. reported that the serum adiponectin level was significantly correlated with HOMA-IR and insulin resistance.[40]

In the present study insulin and HOMA index showed a significant association with TG haplotype. We found that plasma adiponectin concentrations are associated with insulin sensitivity. Our finding showed that adiponectin concentrations could modulate insulin action by its positive relationship between adiponectin and insulin sensitivity. A dose-dependent effect of adiponectin deficiency on insulin sensitivity observed in heterozygous,[41] homozygous AMP1 null,[42] and develop marked diet-induced insulin resistance mice.[43]

In previous study, plasma adiponectin level was positively associated with insulin sensitivity,[5] while low concentrations of adiponectin associated with decreased insulin sensitivity in diabetic obese mothers.[44]

In further analysis to confirm the association among the TG haplotype with insulin sensitivity, we analyzed the impact of the TG haplotype in four subgroups, according to BMI as well as the insulin sensitivity. The association between the serum adiponectin levels and TG haplotype was most prominent in the subgroup of nonobese insulin-sensitive individuals (P < 0.01). This result is supported by a significant relation of the TG haplotype with obesity and insulin resistance. In this context, Melistas et al. reported that insulin resistance associated with the APM1 +45T>G, +276G>T genotype’s in healthy subjects.[45] In addition, this could also function as physiological factors related to the metabolic syndrome, such as blood levels of F1, [46] which themselves incline to aggregate in families.[47]

Other than association of TG haplotype with adiponectin levels, the haplotype is also associated with other features of the insulin resistance syndrome, such as SBP and DBP, insulin level, HOMA-IR and T-cholesterol. The association of circulating adiponectin levels with SNPs 45T>G and 276G>T possibly act through decreased adiponectin expression, which may cause increased body weight and insulin resistance. In a previous study, homozygous/heterozygous carriers of the TG haplotype (45TT and 276GG) showed a significant association with the HOMA-IR index and insulin resistance indexes.[50]

In the present study, TG haplotype associated with significantly higher value of SBP and DBP. As TG haplotype showed lower value of adiponectin, this low value of adiponectin may responsible for such association. Chow et al. observed that hypoadiponectinemia predicts the development of hypertension and hypertension associated with the APM1 G276T genotype while de Faria et al. reported that hypoadiponectinemia may be a marker for predisposition to hypertension.[40,49] A significant negative correlation between adiponectin level and SBP and DBP, in the present and previous study, suggesting that adiponectin contributes to the clinical course of essential hypertension.[50] The TG haplotype associated with T-cholesterol, and LDL-C. The insulin-sensitizing action of adiponectin in adipose tissue may be responsible for lipid storage insulin signaling in the adipocyte is important while adipose tissue selective insulin receptor knockout mice are protected from obesity.[51]

CONCLUSIONS

In conclusion, our data show a very strong association of the APM1 45-276 gene polymorphism and TG haplotypes with adiponectin levels in healthy north Indian population and TG haplotypes also showed substantial association with metabolic parameters of the insulin resistance syndrome.

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