One Common Polymorphism of Cholesteryl Ester Transfer Protein Gene in Iranian Subjects With and Without Primary Hypertriglyceridemia

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Abstract: Primary hypertriglyceridemia is considered to be a major risk factor for pancreatitis, atherosclerosis and coronary heart disease. Cholesteryl ester transfer protein gene polymorphisms known to be associated with changes in lipid levels. This study was performed by using polymerase chain reaction and restriction fragment length polymorphisms. Genotype distribution and allelic frequencies of polymorphism were determined and compared in primary hypertriglyceridemic and normotriglyceridemic subjects. The results showed that plasma cholesteryl ester transfer protein activity was significantly higher in primary hypertriglyceridemia than in controls (p = 0.001). In this study all individuals with B,B genotype had lower plasma cholesteryl ester transfer protein activity, higher high-density lipoprotein than B,B and B,B2 genotypes, whereas triglyceride was significantly decreased in this genotype. The genotype and allelic frequencies for this polymorphism differed significantly between primary hypertriglyceridemic patients and controls (p = 0.014 and p = 0.027, respectively). In both groups, CETP Taq 1B polymorphism (presence of B allele) correlated significantly with HDL-C (r = 0.207 and 0.300 in control and patient groups, respectively) and CETP activity (r = -0.193 for controls and r = -0.132 for patients). Taq 1B polymorphism of cholesteryl ester transfer protein gene was associated with changes in lipids profile and plasma cholesteryl ester transfer protein activity in the selected population.

Key words: Lipid levels, Taq 1B polymorphism, cholesteryl ester transfer protein activity, Iranian population

INTRODUCTION

Primary hypertriglyceridemia is the result of various genetic defects leading to disordered triglyceride metabolism. It is considered to be one of the most important risk factors for some disease, specially pancreatitis, atherosclerosis and Coronary Heart Disease (CHD). The plasma Cholesteryl Ester Transfer Protein (CETP), transfers Cholesteryl Ester (CE) from the High-Density Lipoprotein (HDL) to atherogenic apo B-containing lipoproteins, such as the Very Low-Density Lipoprotein (VLDL), Low-Density Lipoprotein (LDL) and chylomicron (Yamashita et al., 2000). Therefore the plasma CETP has a vital role in the metabolism of lipids and lipoproteins so that may alter the susceptibility to CHD (Schaefer, 2002, Twickler et al., 2004).

The gene for human CETP contains 25 kb genomic DNA and is composed of 16 exons. This gene is closely linked to the lecithin: cholesterol acyltransferase gene on chromosome 16q (Agellen et al., 1990) and produces a hydrophobic glycoprotein containing 476 amino acids with six potential N-glycosylation sites (Tall, 1995).

The genetic variation of CETP is a major determinant of inter-individual variation in susceptibility to primary hyperlipidemia and CHD (de Grooth et al., 2004). Several investigation described the human CETP genetic polymorphisms in promoter at position -971 G/A and -629 C/A (Klerx et al., 2003), intron 8 Mspl, intron 9 Eco N1, intron 10 Taq 1A (Kuivenhoven et al., 1997), 1405V (Lottenberg et al., 2003) and R451Q (Kakko et al., 1998) Variations in especial populations. Polymorphisms that cause amino acid changes include A373P, 1405V, R 451Q and D442G (Thompson et al., 2003).

Some of studies have documented the effects of CETP polymorphisms on lipids and lipoproteins profile and CETP activity. A study showed that Taq 1B polymorphism at the CETP gene locus is associated with

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changes in lipoprotein size, CETP activity and HDL cholesterol (HDL-C) levels (Ordovas et al., 2000). According to a literature, -971 G/A polymorphism was significantly related to plasma HDL-C levels and CETP concentrations (Le Goff et al., 2002). This study reported that -971 GG genotype had higher plasma CETP concentrations and lower HDL-C levels than -971 AA genotype.

A Taiwanese study showed that the ECON1’ G allele is associated with a low apo A1 level and the 442Gly with both high Total Cholesterol (TC) and LDL cholesterol (LDL-C) levels. The results of this study indicate a significant difference in frequency distribution for the genotype between the control and CHD groups (Wu et al., 2001). Lu et al. (2003) investigated the relationship of the CETP promoter polymorphisms to CETP concentrations and HDL-C levels in Japanese men. Their investigation indicated that the -2505 C/A variation might explain the changes in the plasma HDL-C levels and CETP concentrations.

Other studies reported that the strength of the relationship between HDL-C levels and polymorphisms in the human CETP gene might be affected by environmental factors (Fumeron et al., 1995).

However, there are no data available regarding Iranian population with CETP gene polymorphisms. Thus, in this study we investigated the association of one common polymorphism in the CETP gene (TaqlB) with plasma lipid levels and CETP activity in Iranian subjects with and without primary hypertriglyceridemia.

**MATERIALS AND METHODS**

All digestion enzymes and Taq DNA polymerase used for Polymerase Chain Reaction (PCR) analysis were obtained from New England Biolabs. All primers, other chemicals and reagents were of molecular grade from Sigma, Merck and Invitrogen.

**Study population:** A total of 316 subjects-214 healthy (111 males; 103 females) and 102 patients with primary hypertriglyceridemia (52 males; 50 females) were selected for present study. All the individuals with primary hypertriglyceridemia (triglyceride > 200 mg dL⁻¹) were those without other disease that could affect lipid levels, such as thyroid disorder, liver disease, renal failure and finally diabetes mellitus. The included patients were not on any lipid-lowering treatment. The average age for control group was 49.4±12.9 and for patients group was 49.8±12.2 years (Mean±SD; range 25-65). Body weight and height were measured and the Body Mass Index (BMI) calculated. All subjects gave their informed consent to participate in this investigation.

**Biochemical measurements:** Blood was drawn after an overnight fasting for the determination of blood TC, triglyceride(TG), HDL-C and VLDL cholesterol (VLDL-C) levels, which were measured by routine biochemical assays. LDL-C was calculated according to the Friedewald formula (Friedewald et al., 1972). For those patients who had a triglyceride level of more than 400 mg dL⁻¹, we were measured LDL-C by special kit. The plasma CETP activity was measured by Roar Biomedical kit, in a perkin Elmer LS55 fluorescence spectrometer. The CETP activity kit includes donor and acceptor particles. Incubation of donor and acceptor with a CETP source results in the CETP activity. CETP-mediated transfer of the fluorescent neutral lipid to the acceptor molecule (VLDL) results in an increase in fluorescence. The assay was read at excitation wavelength of 465 nm and emission wavelength of 535 nm. A standard curve was used, according to the kit’s guidelines, to drive the relationship between mass transfer and fluorescence intensity.

**Genotype detection:** Genomic DNA was extracted from blood leukocytes by the salting out method (Miller et al., 1988) and a fragment of 535 bp from intron 1 of the CETP gene was amplified by PCR using these primers:

Forward 5’-cag tag ccc gag gaa agt ggc-3’
Reverse 5’-ctg aag cca ggc gca cac taa c-3’

The PCR reaction was carried out in a total volume of 50 μL containing 100 ng genomic DNA, 10 mM Tris-HCl pH 8.4, 1.5 mM MgCl₂ with 0.2 mM dNTPs, 40 pM each primer and 0.25 unit Taq DNA polymerase. PCR was performed with initial denaturation at 95°C, for 30 sec. For amplification, 30 cycles were carried out of 30 sec at 95°C, 30 sec at 63°C and 45 sec at 72°C, in a PTC-200 MJ-Research peltier thermocycler. The reaction ended with an additional 5 min of the last extending temperature (72°C). The PCR products were further analyzed by standard restriction fragment length polymorphism (RFLP). An aliquot of 16 μL of PCR products was digested with 4 units of Taq 1B restriction Enzyme at 65°C for 2 h. The digest was analyzed on a 1.5% agarose gel using TBE as the buffer and the gel was stained with ethidium bromide.

**Statistical analysis:** Continuous variable are shown as Mean±Standard Deviation (SD). Analysis of variance (one-way ANOVA) and student’s t-test were used for the comparison of lipid parameters means among the various genotypes. The distributions of genotype and allele frequencies between primary hypertriglyceridemia and control groups were compared using Pearson’s chi-square test. p<0.05 was considered significant. All statistical analysis was performed using SPSS software (Version 11.5).
RESULTS

The characteristics of the control and patient groups are represented in Table 1. Present data show that all parameters, except HDL-C and LDL-C, were significantly higher in primary hypertriglyceridemia compared with control subjects. Age and sex were similar in two groups (p<0.05).

The presence of a restriction site for the enzyme Taq 1B in intron 1 was referred to as B1, and its absence as B2. Digestion of the PCR products of a 535 bp fragment containing a Taq 1B G (B1,B1 genotype) by Taq 1B generated two fragments (361 and 174 bp), whereas one fragment (553 bp) was generated when one A was present at this position (B1,B2 genotype).

The distribution of Taq 1B genotypes/Alleles frequencies among healthy subjects and primary hypertriglyceridemic patients is shown in Table 2. In the control group, 31.3% (67 of 214) were homozygous for B1 and 18.2% (39 of 214) for B2 allele, whereas in the patient group they were 14.7% (15 of 102) and 18.6% (19 of 102), respectively. In control individuals the Taq 1B B1 and B2 alleles were found at frequencies of 0.685 and 0.435, respectively, while in primary hypertriglyceridemic subjects they were 0.481 and 0.519, respectively. There was significant statistical difference in the frequency distribution between the two groups (p = 0.014 for genotypes and p = 0.027 for alleles). The distribution of genotypes observed in both the control and case samples were in Hardy-Weinberg equilibrium.

The serum lipid levels and CETP activity in normal and case subjects according to CETP/Taq 1B genotype are shown in Table 3 and 4, respectively. In both groups, the plasma HDL-C was higher in the B1,B1 genotype than in the B1,B2 and B2,B2 genotypes, whereas the serum TG concentrations and CETP activity were lower in B1,B2 genotype compared with other genotypes (B1,B2 and B2,B2). With regard to other parameters such as LDL-C, VLDL-C and TC/HDL-C ratio, there was not significantly difference between various genotypes in normal individuals. In addition, present data showed that in the patient’s group TC/HDL-C ratio and VLDL-C levels were higher in B1,B2 subjects than in the B1,B1 and B2,B2 subjects, while no statistically significant difference in TC and LDL-C levels with different genotype was observed.

The Pearson correlation coefficients are represented in Table 5 and 6. In both groups, CETP Taq 1B polymorphism (presence of B1 allele) correlated significantly with HDL-C (r = 0.207 and 0.300 in control and patient groups, respectively) and CETP activity (r = -0.193 for controls and r = -0.132 for patients) (Table 5). In both the patients and controls, the CETP activity showed a significant inverse correlation with HDL-C and a significant positive correlation with LDL-C and TC/HDL-C ratio. In addition, the CETP activity indicated a significant inverse correlation with TC and a
significant positive correlation with TG and VLDL-C in primary hypertriglyceridemic and healthy subjects, respectively (Table 6).

The odds ratio for primary hypertriglyceridemia was 1.98 for the Taq1 B B2 B3, but it was not significant.

**DISCUSSION**

The purpose of this investigation was to study the Taq1B polymorphism of CETP gene in Iranian subjects with and without primary hypertriglyceridemia. Lipids and lipoproteins metabolism in humans may be controlled by genes, including the lipoprotein lipase, apo AI, apo E, apo B, apo C, apoAII and lecitthin: cholesterol acyltransferes genes, because of their central position in the lipids metabolism regulation (Girard-Globa, 1997; Aouizerat et al., 1999; Yamada et al., 2002).

The human CETP is one of the most studied polymorphic genes and its clinical relevance and allelic frequency have been extensively investigated in different groups (Kuijvenhoven et al., 1997; Wu et al., 2001; Le Goff et al., 2002), but to date, no data in the Iranian population have been reported.

In this study, the frequency of the human CETP Taq1B genotypes/alleles and their effects on lipid profile and CETP activity in primary hypertriglyceridemic and control subjects were examined. Our results indicated that the CETP Taq1B genotypes/alleles frequencies are slightly different from those reported for other populations (Ordovas et al., 2000; Wu et al., 2001; Le Goff et al., 2002).

The analysis of frequency distribution of Taq1B polymorphism showed a significant difference between primary hypertriglyceridemia and control groups. Wu et al. (2001) investigated the human CETP gene variations frequency in control and CHD patients (2001). They reported a frequency very similar to the European population. 0.44 for Taq1 B2 allele. Their results indicated an increased Taq1 B B2 genotype frequency in the primary hypertriglyceridemic patients, so that the difference with the control group showed a statistically significant difference. Moreover, the data represented here indicate that B2 B2 genotype frequency is lower and B2 B3 genotype frequency is higher than other populations (Le Goff et al., 2002; Hsu et al., 2002). Consistent with previous study, we found that the heterozygous B2 B3 genotype being the most common in the selected population (Boekholdt and Thompson, 2003). In regard to allele frequency our findings show that the B3 allele frequency is significantly higher in primary hypertriglyceridemic than control subjects. Durlach et al. (1999) reported that the B3 allele is associated with higher prevalence of CHD in patients.

Many investigations have documented that genetic variance in lipid and lipoprotein concentrations is associated with the CETP genotypes, in either primary hypertriglyceridemic or normotriglyceridemic individuals (Tato et al., 1995; Hill et al., 1998; Arashiro et al., 2001; Hsu et al., 2002). It has been reported that the B3 B3 genotype for Taq1B polymorphism provides protection against coronary arteriosclerosis (Ordovas et al., 2000). The present study shows that B3 B3 genotype has highest HDL-C concentrations and lowest CETP activity in both groups.

When we tested the Pearson correlation coefficient between Taq1B polymorphism (presence of B3 allele) and other parameters, observed that this polymorphism was significantly correlated with plasma HDL-C levels and also had an inverse correlation with CETP activity. Present findings are in agreement with previous studies, which explained a significant association between common polymorphisms of the human CETP gene and both CETP activity and HDL-C levels (Kuijvenhoven et al., 1997; Dachet et al., 2000) and also confirm the role of B3 allele in protection against CHD. Several reports showed that in normotriglyceridemic subjects, the B3 allele at this polymorphism site has been associated with decreased

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Table 5: Pearson correlation coefficients between Taq1B polymorphism and other parameters

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<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Patient</th>
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<tbody>
<tr>
<td></td>
<td>r</td>
<td>p</td>
</tr>
<tr>
<td>Age</td>
<td>0.032</td>
<td>0.640</td>
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<tr>
<td>Sex</td>
<td>0.046</td>
<td>0.378</td>
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<td>Total cholesterol</td>
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<tr>
<td>Triglyceride</td>
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<tr>
<td>HDL cholesterol</td>
<td>0.207</td>
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<td>LDL cholesterol</td>
<td>0.075</td>
<td>0.278</td>
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<tr>
<td>VLDL cholesterol</td>
<td>-0.108</td>
<td>0.114</td>
</tr>
<tr>
<td>TC/HDL-C ratio</td>
<td>-0.070</td>
<td>0.310</td>
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<tr>
<td>CETP activity</td>
<td>-0.193</td>
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*: Correlation is significant at the 0.01 level (2-tailed); *: Correlation is significant at the 0.05 level (2-tailed)

Table 6: Pearson correlation coefficients between CETP activity and other parameters

<table>
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<tr>
<td></td>
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<td>p</td>
</tr>
<tr>
<td>Age</td>
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<tr>
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<tr>
<td>Total cholesterol</td>
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<tr>
<td>Triglyceride</td>
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<tr>
<td>HDL cholesterol</td>
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<td>0.001*</td>
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<tr>
<td>LDL cholesterol</td>
<td>0.284</td>
<td>0.001*</td>
</tr>
<tr>
<td>VLDL cholesterol</td>
<td>0.140</td>
<td>0.040*</td>
</tr>
<tr>
<td>TC/HDL-C ratio</td>
<td>0.422</td>
<td>0.001*</td>
</tr>
</tbody>
</table>

*: Correlation is significant at the 0.01 level (2-tailed); *: Correlation is significant at the 0.05 level (2-tailed)
CETP levels and increased HDL-C levels (Or dov as et al., 2000; Wu et al., 2001; Le Goff et al., 2002). Although Taq 1B polymorphism is not expected to directly influence CETP transcriptional regulation or RNA splicing but rather to affect CETP gene expression, due to its location in intron 1 (+2796G/A).

In addition, we found that, in all subjects (cases and controls) TG levels were the lowest in individuals with B1B1 genotype, which findings are similar to previous report that described a decreased TG levels in the B1B1 genotype (Kuivenhoven et al., 1998).

When we analyzed the Pearson correlation coefficient between CETP activity and other parameters, we obtained a positive correlation with LDL-C and TC/HDL-C ratio and an inverse correlation with HDL-C, in both groups. One study showed a significant positive correlation between CETP activity with LDL-C and non-HDL/HDL-C in normolipidemic subjects (Tato et al., 1995). It has been also showed that plasma CETP concentration is related to CETP activity and that plasma CETP activity is inversely associated with plasma HDL-C levels (Kakko et al., 1998; Le Goff et al., 2002). The data represented here are consistent with these results.

Present results showed an inverse correlation between HDL-C and TG in the patients group (data not shown). Other studies indicated that low plasma HDL concentration, which is an important risk factor of CHD, is generally observed in hyperlipidemic subjects (Schaefer et al., 1978; Davis et al., 1980; Mann et al., 1991). Since HDL-C concentration is inversely related to plasma TG levels, the low HDL-C levels in hyperlipidemia could result from an increased rate of CE transfer from HDL to TG-rich lipoprotein. Previous study has shown that in primary hypertriglyceridemia, the rate of CE accumulation in VLDL is increased three fold compared to normolipidemia (Mann et al., 1991). Present findings also show that VLDL-C levels significantly increase in primary hypertriglyceridemic individuals.

In summary, the presence of B1 allele is significantly associated with both low plasma CETP activity and high HDL-C levels in entire population (primary hypertriglyceridemia and controls) and also it has a protective role against CHD. Therefore, the association of the CETP Taq1B genotypes with lipid levels and CETP activity suggest that they contribute to the genetic risk of developing atherosclerosis disease.

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