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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_2B_2 genotype had lower plasma cholesteryl ester transfer protein activity, higher high-density lipoprotein than B_1B_1 and B_1B_2 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_2 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 (Agellon *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 (Klerkx *et al*., 2003), intron 8 Msp1, intron 9 Eco N1, intron 10 Taq 1A (Kuivenhoven *et al*., 1997), I405V (Lottenberg *et al*., 2003) and R451Q (Kakko *et al*., 1998) Variations in especial populations. Polymorphisms that cause amino acid changes include A373P, I405V, 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 (Taq1B) 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$^{/}$-cac tag ccc aga gag agg agt gcc-3$^{/}$
Reverse 5$^{/}$-ctg agc cca gcc 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 $MgCl_2$ with 0.2 mM dNTPs, 40 pM each primer and 0.25 unit Taq DNA polymerase. PCR was performed with initial denaturation of 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 B_1 and its absence as B_2. Digestion of the PCR products of a 535 bp fragment containing a Taq 1B G (B_1B_1 genotype) by Taq 1B generated two fragments (361 and 174 bp), whereas one fragment (535 bp) was generated when one A was present at this position (B_2B_2 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 B_2 and 18.2% (39 of 214) for B_1 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 B_2 and B_1 alleles were found at frequencies of 0.565 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 B_2B_2 genotype than in the B_1B_1 and B_1B_2 genotypes, whereas the serum TG concentrations and CETP activity were lower in B_2B_2 genotype compared with other genotypes (B_1B_1 and B_1B_2). 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 B_1B_1 subjects than in the B_1B_2 and B_2B_2 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 B_2 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

Table 1: Characteristics of the study population

Characteristics	Control (n = 214)	Patient (n = 102)	p
Age (years)	49.4±12.9	49.8±12.2	NS
Sex (men/women)	111/103	52/50	NS
Systolic blood pressure (mmHg)	108±11	129.0±15	0.016
Diastolic blood pressure (mmHg)	77.3±11.7	98.0±12.6	0.006
Body mass index (kg m^2)	25.5±5.9	27.4±6.1	0.018
History of hypertrigliceridemia (%)	9.2	37.4	0.001
Total cholesterol (mg dL^{-1})	184.8±31.5	198.4±26.4	0.006
Triglyceride (mg dL^{-1})	131.1±51.1	354.5±118.9	0.001
HDL cholesterol (mg dL^{-1})	53.6±10.9	46.9±7.3	0.001
LDL cholesterol (mg dL^{-1})	104.6±25.9	95.9±25	0.036
VLDL cholesterol (mg dL^{-1})	26.4±11.7	54.8±12.8	0.001
TC/HDL-C ratio	3.45±0.85	4.23±0.93	0.001
CETP activity (pmol/μL h)	107.3±18	170.5±26.5	0.001

Data represent the Mean±SD, NS: Not Significant

Table 2: CETP/Taq1B genotype and allele frequencies in patient and control groups

Parameters	Control n (%) n = 214	Patient n (%) n = 102
Genotype[a]		
B_1B_1	39 (18.2)	19 (18.6)
B_1B_2	108 (50.5)	68 (66.7)
B_2B_2	67 (31.3)	15 (14.7)
Allele[b]		
B_1	0.435	0.519
B_2	0.565	0.481

[a]: p-value for genotype frequency distribution between two groups is 0.014, [b]: p-value for Allele frequency distribution between two groups is 0.027

Table 3: Lipid levels (mg dL^{-1}) and CETP activity (pmol/μL.h) in control group according to CETP/Taq1B genotype[a]

Parameters	B_1B_1	B_1B_2	B_2B_2
N	67.00	108.00	39.00
Total cholesterol	183.20±33.7	183.70±31.1	199.60±28.3
Triglyceride	124.30±49.4	143.40±50.3[b]	108.70±47.5
HDL cholesterol	52.00±9.40	52.40±11.1	57.50±10.8[c]
LDL cholesterol	104.60±29.0	101.90±24.0	112.00±24.9
VLDL cholesterol	26.10±14.3	28.50±10.0	30.10±9.00
TC/HDL-C ratio	3.52±0.75	3.51±0.82	3.47±0.75
CETP activity	109.40±16.7	109.40±18.3	97.80±16.6

[da]: p-value calculated after adjustment for sex, age, BMI, family relation and alcohol use, [b]: $p = 0.037$ vs. B_1B_1 and $p = 0.001$ vs. B_2B_2, [c]: $p = 0.002$ vs. B_1B_1 and $p = 0.001$ vs. B_1B_2, [d]: $p = 0.001$ vs. B_1B_1 and B_1B_2

Table 4: Lipid levels (mg dL^{-1}) and CETP activity (pmol/μL h) in patients group according to CETP/Taq1B genotype[a]

Parameters	B_1B_1	B_1B_2	B_2B_2
N	20.00	59.00	23.00
Total cholesterol	296.00±30.8	280.20±23.8	277.00±11.6
Triglyceride	375.70±96.0	304.80±63.5	271.60±37.1[b]
HDL cholesterol	53.60±7.80	61.40±5.50	66.40±8.70[c]
LDL cholesterol	163.80±16.8	155.90±15.6	156.40±12.6
VLDL cholesterol	78.60±20.4	57.40±14.2[d]	58.40±7.70
TC/HDL-C ratio	05.52±1.10	04.56±0.91	04.17±0.79[e]
CETP activity	172.70±23.2	170.80±25.8	126.60±36.4[f]

[a]: p-value calculated after adjustment for sex, age, BMI, family relation and alcohol use, [b]: $p = 0.001$ vs. B_1B_1, [c]$p = 0.020$ vs. B_1B_1, [d]: $p = 0.039$ vs B_1B_1, [e]: $p = 0.018$ vs. B_1B_2 and $p = 0.001$ vs. B_1B_1, [f]: $p = 0.001$ vs. B_1B_1 and B_1B_2

Table 5: Pearson correlation coefficients between Taq 1B polymorphism and other parameters

	Control		Patient	
Parameters	r	p	r	p
Age	0.032	0.640	-0.077	0.605
Sex	0.061	0.378	0.046	0.754
Total cholesterol	0.072	0.296	0.261	0.073
Triglyceride	-0.055	0.423	0.135	0.359
HDL cholesterol	0.207	0.002[a]	0.300	0.018[b]
LDL cholesterol	0.075	0.278	0.133	0.368
VLDL cholesterol	-0.108	0.114	0.222	0.130
TC/HDL-C ratio	-0.070	0.310	0.093	0.528
CETP activity	-0.193	0.005[a]	-0.132	0.020

[a]: Correlation is significant at the 0.01 level (2-tailed), [b]: Correlation is significant at the 0.05 level (2-tailed)

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

	Control		Patient	
Parameters	r	p	r	p
Age	0.001	0.985	-0.107	0.471
Sex	-0.105	0.167	0.203	0.167
Total cholesterol	-0.192	0.283	-0.364	0.007[a]
Triglyceride	0.157	0.021[b]	0.051	0.731
HDL cholesterol	-0.896	0.001[a]	-0.775	0.001[a]
LDL cholesterol	0.284	0.001[a]	0.256	0.006[a]
VLDL cholesterol	0.140	0.040[b]	-0.179	0.224
TC/HDL-C ratio	0.422	0.001[a]	0.301	0.012

[a]: Correlation is significant at the 0.01 level (2-tailed), [b]: Correlation is significant at the 0.05 level (2-tailed)

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 Taq 1 B B_1B_1, but it was not significant.

DISCUSSION

The purpose of this investigation was to study the Taq 1B 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 lecithin: cholesterol acyltransferase 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 (Kuivenhoven *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 Taq 1B 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 Taq 1B 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 Taq 1B B_2 allele). Their results indicated an increased Taq 1B B_1B_1 genotype frequency in the CHD group. We found a decreased Taq 1B B_2B_2 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 B_1B_1 genotype frequency is lower and B_2B_2 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 B_1B_2 genotype being the most common in the selected population (Boekholdt and Thompson, 2003). In regard to allele frequency our findings show that the B_1 allele frequency is significantly higher in primary hypertriglyceridemic than control subjects. Durlach *et al*. (1999) reported that the B_1 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 B_2B_2 genotype for Taq1B polymorphism provides protection against coronary arteriosclerosis (Ordovas *et al*., 2000). The present study shows that B_2B_2 genotype has highest HDL-C concentrations and lowest CETP activity in the both groups.

When we tested the Pearson correlation coefficient between Taq 1B polymorphism (presence of B_2 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 (Kuivenhoven *et al*., 1997; Dachet *et al*., 2000) and also confirm the role of B_2 allele in protection against CHD. Several reports showed that in normotriglyceridemic subjects, the B_2 allele at this polymorphism site has been associated with decreased

CETP levels and increased HDL-C levels (Ordovas *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 (+279G/A).

In addition, we found that, in all subjects (cases and controls) TG levels were the lowest in individuals with B_2B_2 genotype, which findings are similar to previous report that described a decreased TG levels in the B_2B_2 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 B_2 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 Taq 1B genotypes with lipid levels and CETP activity suggest that they contribute to the genetic risk of developing atherosclerosis disease.

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