Association of the TNF-α-C-857T Polymorphism with Resistance to the Cholesterol-Lowering Effect of HMG-CoA Reductase Inhibitors in Type 2 Diabetic Subjects

Running Title: TNF-α-C-857T polymorphism, LDL-cho & statins

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**Objective:** An association of the C-857T polymorphism of TNF-α gene promoter region with LDL-cholesterol (LDL-C) levels has been reported. This study is designed to evaluate the relationship between TNF-α-C-857T polymorphism and LDL-C levels according to statin treatment in subjects with type 2 diabetes.

**Research Design and Methods:** DNA was obtained from 322 Japanese subjects (160 males and 162 females) with type 2 diabetes and TNF-α-C-857T polymorphisms were determined by direct sequencing. Serum LDL-C was measured by a direct method.

**Results:** Although serum LDL-C levels were significantly higher in the T carriers (C/T+T/T) than those in the non-T carriers (C/C) (3.14 ± 0.86 vs. 2.89 ± 0.75 mmol/l, P<0.05), there was no difference in LDL-C levels between the non-T-carriers and the T carriers in the statin-untreated subjects (2.87 ± 0.73 vs. 2.89 ± 0.76 mmol/l, NS), whereas, in the statin-treated subjects, LDL-C levels were more significantly higher in subjects with the T carriers than the non-T-carriers (3.43 ± 0.89 vs. 2.90 ± 0.78 mmol/l, P = 0.0007). There were no differences in HDL-C and triglyceride levels between the non-T carriers and the T-carriers both in the statin-treated and untreated subjects. Percent decrease in LDL-C levels after administration of statins was significantly smaller in the T-carriers as compared with the non-T-carriers (27.6 vs. 36.4%, P = 0.031).

**Conclusions:** The mutant allele of the C-857T promoter polymorphism of the TNF-α gene may predispose to resistance to the LDL-C-lowering effect of statins, and could be one of markers to predict the efficacy of statins.
Tumor necrosis factor-α (TNF-α) is a potent immunomodulator and proinflammatory cytokine with multiple functions and plays a variety of roles in pathological and physiological conditions. There have been many reports on relationships between TNF-α gene polymorphisms and various diseases including infectious and metabolic disorders (1, 2). As to lipid metabolism, there have been few reports on an association of TNF-α gene polymorphism with serum lipids including cholesterol levels, the most potent risk factor for cardiovascular diseases (3, 4, 5). Shiau et al. have shown that TNF-α-G-238A is associated with LDL-cholesterol (LDL-C) levels in Taiwanese patients with type 2 diabetes (4). We have recently reported that TNF-α-C-857T, a functional TNF-α gene promoter polymorphism with higher transcriptional activity (6), was associated with higher LDL-C levels and carotid plaques in Japanese subjects with type 2 diabetes mellitus (5). In the course of this study, our preliminary analysis indicated that an association of TNF-α-C-857T with higher LDL-C levels was observed only in subjects treated with the 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitors (HMG Co-A reductase inhibitors, statins), but not in those without statin treatment (7), implying the resistance of this polymorphism to the effect of statins. We hereby performed a study to confirm the C-857T promoter polymorphism of the TNF-α gene is associated with resistance to the cholesterol-lowering effect of statins in type 2 diabetic subjects.

RESEARCH DESIGN AND METHODS

Subjects: After obtaining approval from the ethics committee of the Iwate Medical University, and informed consents from all subjects, blood samples were collected from 322 type 2 diabetic subjects (160 males and 162 females). All subjects were Japanese. The present study was performed in accordance with the guidelines expressed in the Declaration of Helsinki.

Identification Of Polymorphisms: Genomic DNAs were obtained from peripheral blood leukocytes by standard phenol-chloroform extraction and ethanol precipitation methods or by the Biomek 3000 Laboratory Automation System (Beckman Coulter Fullerton, CA, USA). The 5’-flanking region of the TNF-α gene, spanning from -188 to -1229, relative to the TNF-α transcription start site, was amplified by polymerase chain reaction (PCR) using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The PCR primers were as follows (6): sense: 5’-GCTTGTGTGTTGTGTGTCTGG-3’; anti-sense: 5’-GGACACACAAGCATCAAGG-3’. PCR conditions were as follows (6): denaturing at 94°C for 1 min, annealing at 55°C for 2 min, extension at 72°C for 3 min, for 40 cycles, final incubation at 72°C for 10 min and cooling to 4°C. The PCR products were purified using NucleoSpin Extract (Macherey-Nagel, Duren, Germany). Sequence analysis was carried out using a BigDye Terminator v3.1 Cycle Sequencing Kit (Perkin-Elmer, Norwalk, CT, USA) with the sequence primer 5’-TGTGGCCATATCTTCTTAAA-3’ to analyze the sequence from -782 to -1209 for polymorphisms at -857, -863, -1031. Finally, the cycle sequencing products were purified again with Dye Terminator Removal Kit (ABgene House, Epsom, Surrey, UK) and analyzed by an Applied Biosystems Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), according to the manufacturer’s instructions.

Laboratory examinations: For all subjects, blood was obtained after fasting for 12 hours or longer, and blood cell counts, fasting plasma glucose (FPG) levels, fasting insulin (immunoreactive insulin: IRI) levels, HbA1c, total cholesterol (TC), triglyceride
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(TG), high density lipoprotein-cholesterol (HDL-C) and low density lipoprotein-cholesterol (LDL-C) were measured at the Central Laboratory in our hospital.

Statistics: The data are expressed as means ± standard deviation (SD). Statistical significance was analyzed by unpaired t-test and χ²-test using StatView-J5.0 (Abacus Concepts, Inc., Berkeley, CA, USA). The level of significance was considered at P < 0.05.

RESULTS

Serum LDL-C levels are higher in diabetic subjects with the T allele of the TNF-α-C-857T promoter gene polymorphism than those with the C allele: The frequencies of C and T alleles of TNF-α-C-857T were 85.1% and 14.9%, respectively, which are not significantly different from ones reported in the Japanese population (5, 6, 8). Hardy-Weinberg’s equilibrium was maintained in this population. We reconfirmed our previous observation (5) for double the number of cases that the T carriers (C/T, T/T) displayed significantly higher serum LDL-C levels than the non-carriers (C/C) (3.14 ± 0.86 vs. 2.89 ± 0.75 mmol/l, P<0.05) (Table 1). Clinical backgrounds, such as gender and age distributions, physiques, blood pressures, HbA1c, and serum lipid levels other than LDL-C showed no difference between these groups (Table 1), as well as the baseline characteristics of medication for dyslipidemia, diabetes, and hypertension (Table 2). Other promoter polymorphisms of the TNF-α gene, including C-863A, and T-1031C, were not associated with the serum LDL-C levels (data not shown).

Higher LDL-C level in the T carriers of the TNF-α-C-857T polymorphism is observed in subjects treated with statins, but not in those without statins: Because the statins affect LDL-C levels, we divided subjects according to the statin treatment, and compared the serum LDL-C levels between the T carriers and the non-T carriers in those with and without statin treatment. As shown in Table 1, the T carriers with statins displayed significantly higher LDL-C levels than the non-T carriers (3.43 ± 0.89 vs. 2.90 ± 0.78 mmol/l, P=0.0007), whereas in subjects without statins the LDL-C levels did not differ irrespective of genotype. Other clinical characteristics did not differ between the T carriers and non-T carriers in both statin-treated and -untreated subjects (Table 1). The distribution of use of statin subclasses including atorvastatin, pitavastatin, pravastatin, fluvastatin, and rosuvastatin was not different between the T carriers and non-T carriers in the statin-treated subjects (data not shown).

The T carriers of the TNF-α-C-857T polymorphism are more resistant to the LDL-C-lowering effect of statin-treatment than the non-T carriers:

Table 1 implies a possibility of a difference in LDL-C-lowering effect of statins between the T-carriers and the non-T-carriers. To see whether the T carriers are resistant to the statin-treatment, we retrospectively analyzed the LDL-L levels before and 3-6 months after statin-administrations among a subset of the diabetic subjects complicated with hypercholesterolemia, whose complete data sets were available for analysis. As shown in Fig. 1, % reduction in LDL-C levels after the statin-administration was significantly smaller in the T-carriers than in the non-T carriers (-27.6% vs -36.4%, P=0.031).

CONCLUSIONS

In this clinical observation, our data implies for the first time that the C-857T polymorphism in the promoter region of TNF-α gene is associated with serum LDL-C levels in statin-treated subjects with type 2 diabetes and the T carrier is resistant to statin
We have confirmed that the T-carriers displayed higher serum LDL-C levels consistent with our previous report (5), after doubling the number of the subjects. The T allele of TNF-α-C-857T generates significantly higher transcriptional promoter activity than the C allele does, possibly leading to elevated TNF-α production (6, 9). The administration of TNF-α in rodents is followed by an increase in serum concentrations of total cholesterol and hepatic cholesterol synthesis (10), probably by stimulating the activity of HMG-CoA reductase (11). However, in our human cases, the increase in HMG-CoA reductase activity is not a mechanism of the increased serum LDL-C in the T-carriers of TNF-α-C-857T, because there was no difference in LDL-C levels between the T-carriers and non-T carriers, who were not treated with statins (Table 1).

A further analysis revealed that the C-857T promoter polymorphism affected the cholesterol lowering effect of statins, but not directly on the cholesterol synthesis. The T carriers displayed significantly higher serum LDL-C levels only in those taking statins, but not in those without (Table 1), indicating that TNF-α productivity possibly affects sensitivity to the LDL-C-lowering effects of statins. Indeed, the T carriers exhibited a significantly smaller LDL-C lowering rate in response to statin treatment than the non-T carriers (Fig. 1). Fibrates, another drug affecting lipid profile, may have not influenced our results, because very few cases were treated with fibrates (Table 2).

Statins are substrates of several drug transporters (12), including the influx transporter solute carrier organic anion transporter family, member 1B1 (SLCO1B1, previously known as OATP1B1/ OATP-C/OATP2/LST-1) (13, 14, 15). It is reasonable to assume that impaired function or expression of the transporters would result in reduced hepatic uptake of statins, and then in reduced cholesterol-lowering efficacy due to lower intracellular statin concentrations of hepatocytes. TNF-α reportedly suppressed protein expression and transport activity of SLCO1B1 (16), which is located on the sinusoidal membrane of hepatocytes (17). This molecule plays a pivotal role as a major transporter of various statins, including atorvastatin, simvastatin, pitavastatin, pravastatin, fluvastatin, and rosuvastatin (13, 14, 15), from the portal blood into hepatocytes. In fact, a retrospective study in caucasian subjects suggested a weaker effect of pravastatin on inhibition of cholesterol synthesis among carriers of the SLCO1B1*17 haplotype, which is associated with impaired OATP1B1 function and/or expression (18). Therefore, T alleles of the TNF-α-C-857T polymorphism with the higher promoter transcriptional activities (6, 9) possibly results in a reduced LDL-C-lowering effect of statins, which may be in line with our present observation. However, serum concentration of TNF-α was not significantly higher in the T carriers of the C-857T than the non-T carriers (5), probably because of dilution of TNF-α in circulation as compared with those in local region. The lower hepatic statin concentrations in the T carriers associated with defective function and/or expression of OATP1B1 remains to be proven. We could not find differences in proportion of the medicines used such as hydrophilic (pravastatin and rosuvastatin) and lipophilic (atorvastatin, simvastatin, pitavastatin, and fluvastatin) statins, or statins with weak or strong cholesterol-lowering effect between the T carriers and non-T carrier, although the number of cases was small (data not shown).

Although it is possible as mentioned above that the TNF-α gene polymorphism is involved in the sensitivity to the LDL-C-lowering effect of statins via TNF-α productivity, other possibilities including linkage disequilibrium of this polymorphism...
with a susceptibility gene to statin’s effect are not ruled out.

In conclusion, these results strongly suggest that the mutant allele of the C-857T promoter polymorphism of the TNF-α gene may predispose to resistance to the LDL-C-lowering effect of statins, and then could be one of markers to predict the efficacy of statins.

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Legend:

**Figure1.** % Reduction of serum LDL-C levels after statin treatment according to the TNF-α-C-857T polymorphism. % Reduction = ([LDL-C levels before statin treatment] – [LDL-C levels 3-6 months after statin treatment]) / [LDL-C levels before statin treatment] x 100

% Reduction in C/C and (C/T, T/T) was -36.4% and -27.6%, respectively (P=0.031).
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Table 1. Comparison of clinical characteristics of diabetic subjects according to the TNF-α-C-857T polymorphism and statin treatment.

|                      | Whole subjects | Statin (-) | Statin (+) |
|----------------------|----------------|------------|------------|
|                      | C/C            | C/T,T/T    | C/C        | C/T,T/T     | C/C        | C/T,T/T     |
| Gender (male/female) | 116 / 115      | 44 / 47    | 77 / 59    | 28 / 20     | 40 / 56    | 15 / 27     |
| Age (year)           | 62.9 ± 10.9    | 62.2 ± 13.6| 62.3 ± 11.7| 62.3 ± 15.8 | 63.4 ± 9.6 | 61.9 ± 11.0 |
| Height (cm)          | 158.6 ± 8.8    | 156.7 ± 15.4| 159.3 ± 9.2| 159.1 ± 8.7 | 157.5 ± 8.4| 156.3 ± 8.7 |
| Body weight (kg)     | 62.5 ± 11.8    | 61.5 ± 12.4| 61.2 ± 11.6| 60.9 ±12.6  | 63.4 ± 13.9| 62.5 ± 12.4 |
| BMI (kg/m²)          | 24.8 ± 4.0     | 24.1 ± 3.5 | 23.8 ± 4.1 | 23.8 ± 3.6  | 25.9 ± 4.2 | 24.8 ± 3.4  |
| SBP (mmHg)           | 134.8 ± 19.9   | 134.1 ± 17.7| 136.2 ± 21.5| 132.3 ± 18.1| 134.9 ± 18.1| 136.8 ± 17.6|
| DBP (mmHg)           | 78.0 ± 13.3    | 74.9 ± 11.6| 78.8 ± 13.9| 73.4 ± 10.6 | 76.1 ± 12.4| 76.5 ± 12.9 |
| HbA1c (%)            | 7.64 ± 1.73    | 7.39 ± 1.65| 7.72 ± 1.91| 7.52 ± 1.70 | 7.59 ± 1.46| 7.30 ± 1.60 |
| TC (mmol/l)          | 5.05 ± 0.92    | 5.21 ± 1.02| 4.95 ± 0.82| 4.83 ± 0.86 | 5.18 ± 1.05| 5.68 ± 1.01*|
| TG (mmol/l)          | 1.51 ± 0.95    | 1.52 ± 0.83| 1.39 ± 0.94| 1.34 ± 0.88 | 1.51 ± 0.84| 1.58 ± 0.68 |
| HDL-C (mmol/l)       | 1.45 ± 0.45    | 1.48 ± 0.45| 1.44 ± 0.46| 1.46 ± 0.49 | 1.46 ± 0.43| 1.53 ± 0.40 |
| LDL-C (mmol/l)       | 2.89 ± 0.75    | 3.14 ± 0.86*| 2.87 ± 0.73| 2.89 ± 0.76 | 2.90 ± 0.78| 3.43 ± 0.89**|

*P < 0.05 (vs. C/C), **P < 0.0001 (vs. C/C)
**Table 2.** Comparison of medications in diabetic subjects between the TNF-α-C-857T polymorphism.

|                      | C/C       | C/T, T/T   | P   |
|----------------------|-----------|------------|-----|
| Statins (- / +)      | 136 / 95  | 49 / 42    | NS  |
| Fibrates (- / +)     | 229 / 3   | 90 / 1     | NS  |
| OAD (- / +)          | 50 / 181  | 22 / 69    | NS  |
| Insulin (- / +)      | 136 / 95  | 49 / 42    | NS  |
| Hypotensive drugs (- / +) | 115 / 72 | 26 / 29    | NS  |

OAD: oral hypoglycemic drugs
NS: not significant

**Figure 1.**

![Graph showing % Reduction of Serum LDL-C](image)

- C/C (N=49)
- C/T, T/T (N=13)

P=0.031