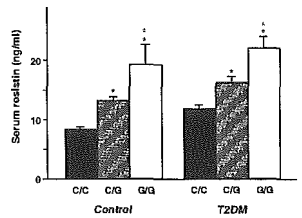

Fig. 1. Serum resistin levels of each genotype in T2DM and control subjects. Serum resistin levels were measured using a human resistin ELISA kit (Linco Research) as described in Materials and methods. Fasting serum samples from 198 T2DM and 157 control subjects were analyzed. The data represent means ± SE for each genotype in either control or T2DM subjects. *Significant difference compared to C/C, **significant difference compared to C/C or C/G. In controls, ANOVA: $F = 15.0$, $P < 0.0001$, Scheffe's test $P < 0.001$ between each pair. In T2DM, ANOVA: $F = 14.3$, $P < 0.0001$; Scheffe's test: $P = 0.0053$ (C/C vs C/G), $P < 0.0001$ (C/C vs G/G), and $P = 0.016$ (C/G vs G/G). When all genotypes were combined, serum resistin levels were significantly higher in T2DM than controls (means ± SE, control vs T2DM; 11.2 ± 0.5 vs 15.1 ± 0.7 ng/ml, Student's t test; $P < 0.0001$). Fasting serum resistin levels were also increased as the number of G alleles increased when T2DM and controls were combined (C/C 10.2 ± 0.4, C/G 15.0 ± 0.7, and G/G 21.1 ± 1.7 ng/ml, ANOVA: $F = 38.3$, $P < 0.0001$; Scheffe's test: $P < 0.0001$ between each pair).

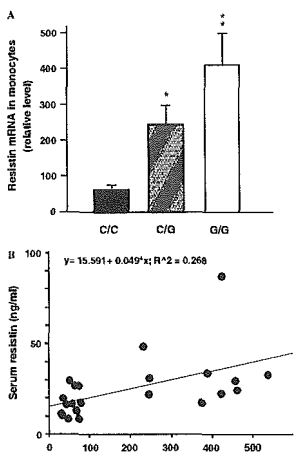

Fig. 2. Resistin mRNA levels in monocytes in healthy volunteers. Resistin mRNA levels in monocytes of 23 healthy volunteers were quantified using the two-step TaqMan RT-PCR method as described in Materials and methods. The level of human resistin mRNA was normalized by that of human GAPDH mRNA in each sample for meaningful comparison, and the relative amounts of resistin mRNA were determined by calculating from the threshold cycles. Resistin mRNA levels corrected by GAPDH mRNA levels in undifferentiated THP-1 cells are defined as 1. (A) Resistin mRNA in healthy volunteers with each genotype. The data represent means ± SE using duplicate wells for each subject. ANOVA and Scheffe's test were used for statistical analysis. C/C ($n = 9$), C/G ($n = 11$), and G/G ($n = 3$). ANOVA: $F = 8.81$, $P = 0.0018$; Scheffe's test: $^*P < 0.05$ and $^{**}P < 0.005$ compared to C/C genotype. (B) Correlation between resistin mRNA in monocytes and its simultaneous serum resistin levels. Fasting serum resistin levels at the time of monocyte isolation were measured as described in Materials and methods. Simple regression analysis was used for statistical analysis. Fasting serum resistin level = 15.59 + 0.049 × resistin mRNA in monocytes ($R^2 = 0.268$). $R = 0.518$, $P = 0.011$.

analyzed. The resistin mRNA level in total RNA from human primary cultured adipocytes (Zen-Bio, NC) was quantitated as described above, but using three replicate wells, to compare resistin mRNA levels between human monocytes and adipocytes.

Statistical analysis. To examine the effect of the −420(G/G genotype on serum resistin levels, a single regression analysis involving the genotype, gender, age, age of onset, duration of T2DM, BMI, maximum body mass index in life (max BMI), or HbA1c as an independent variable was performed. A multiple regression analysis was then performed using only the significant factors of these variables. In these regression analyses, the genotypes for −420C/C, −420C/G, and −420G/G were denoted by two dummy variables (c1, c2) = (0,0), (1,0), and (0,1), respectively. To estimate the effects of serum resistin levels on T2DM, a multiple logistic regression analysis adjusted simultaneously for potentially confounding variables was performed. The variables considered in this model were age, gender, max BMI, and serum resistin. In the logistic regression analysis, the Wald test was used to assess statistical significance. Analysis of variance (ANOVA) followed by Sheffe's test is used in Figs. 1 and 2A. Student's t test is also used in Fig. 1 where indicated. Simple regression analysis is used in Fig. 2B.

Results

Serum resistin levels were higher in T2DM

We first compared serum resistin levels between 198 cases (SNP-420 genotype – n; C/C – 87, C/G – 87, and G/G – 24) and 157 controls (C/C – 80, C/G – 64, and G/G – 13) (Fig. 1). Serum resistin levels were significantly higher in T2DM than in controls (means ± SE, control vs T2DM; 11.2 ± 0.5 vs 15.1 ± 0.7 ng/ml, Student's t test, $P < 0.0001$). Fasting serum resistin levels increased with increasing number of G alleles in controls, T2DM, and both (both combined; C/C 10.2 ± 0.4; C/G 15.0 ± 0.7; and G/G 21.1 ± 1.7 ng/ml, ANOVA; $F = 38.3$, $P < 0.0001$, Scheffe's test; $P < 0.0001$ between each pair, see Fig. 1 legend for the other results).

SNP-420 genotype primarily determined serum resistin levels also increased with longer duration of T2DM and higher HbA1c

To examine which factors affect fasting serum resistin levels, we then analyzed 198 T2DM subjects (Table 2). A single regression analysis involving the genotype (C/G or G/G vs C/C), age, geader, age of onset, duration of T2DM, BMI, max BMI, or HbA1c as an independent variable revealed that only the genotype, duration of T2DM, and HbA1c were significantly associated with serum resistin levels.

A multiple regression analysis involving these three independent variables showed that serum resistin levels were ~4.4 ng/ml higher in C/G, and ~10.6 ng/ml higher in G/G than in C/C (Table 3). An increase in 1-year duration of T2DM and 1% of HbA1c was correlated with an increase in serum resistin at levels of 0.19 and 0.54 ng/ml, respectively.

A single regression analysis also revealed that serum resistin levels were determined by the genotype in 157 control subjects, whereas age, gender, BMI, max BMI, or HbA1c had no effects (data not shown). Neither BMI nor max BMI was associated with serum resistin levels, even when adjusted for genotype, age, gender, and HbA1c, either in the cases or the controls (data not shown). Therefore, serum resistin levels were strongly correlated with the SNP-420 genotype in both T2DM and controls. The duration of T2DM and HbA1c was positively correlated with these levels only in T2DM.

Table 2
Simple regression analysis involving fasting serum resistin level as a dependent variable in T2DM subjects

Variables	Parameter estimate	Standard error	P
CG	4.36	1.33	0.0012
GG	10.22	2.02	<0.0001
Gender (female)	0.93	1.33	0.488
Age	0.07	0.06	0.253
Age of onset	*−0.09*	*0.06*	*0.145*
Duration	0.24	0.08	0.002
BMI	−0.04	0.17	0.798
max BMI	0.14	0.16	0.373
HbA1c	0.86	0.38	0.023

Each of genotype of SNP-420, gender, age, age of onset of T2DM, duration of T2DM, BMI, max BMI, and HbA1c was involved in the analysis as an independent variable. Statistical analyses were performed as described in Materials and methods.

Table 3
Regression analysis for serum resistin in T2DM or T2DM as dependent variables

Variables	Estimate	Standard error	P
Serum resistin in T2DM			
Intercept	5.31	3.20	
C/G	4.42	1.36	0.0013
G/G	10.57	2.14	<0.0001
Duration of diabetes	0.19	0.07	0.0090
HbA1c	0.54	0.37	0.1486
T2DM (logistic regression)			
Intercept	−2.38	1.23	
Serum resistin	0.07	0.02	<0.0001
Age	−0.02	0.01	0.0725
Gender (female)	−0.29	0.24	0.2136
max BMI	0.13	0.03	0.0003

Each of serum resistin in T2DM, and T2DM was involved in the analysis as a dependent variable. The independent variables in each analysis are shown below each intercept. Statistical analyses were performed as described in Materials and methods.

Serum resistin level was an independent factor for T2DM

To determine whether serum resistin is associated with T2DM, a logistic regression analysis involving serum resistin level, age, gender, and max BMI was employed. Serum resistin level was found to be an independent determinant for T2DM (Table 3). Therefore, serum resistin levels, primarily determined by the SNP-420 genotype, could induce T2DM.

Resistin mRNA level in monocytes was higher in the G/G genotype and positively correlated with serum resistin levels

To determine whether the resistin SNP-420 genotype is associated with resistin gene expression in human monocytes, we analyzed its mRNA levels using RT-PCR (Fig. 2). To assess isolated effects of the SNP-420 genotype, 23 healthy volunteers were employed. Resistin mRNA was significantly higher in the C/G or G/G genotype than in the C/C genotype. Consistent with the data on serum resistin levels (Fig. 1), resistin mRNA in monocytes appears to be highest in the G/G genotype (means ± SE, C/C 62.6 ± 4.0; C/G 243.8 ± 54.0; and G/G 412.8 ± 87.5), although the difference did not quite reach the levels of significance when compared between G/G and C/G ($P = 0.07$) (Fig. 2A).

Finally, when these volunteers were analyzed together, resistin mRNA levels were positively correlated with *serum resistin levels* ($R = 0.518$, $P = 0.011$) (Fig. 2B). We also found that resistin mRNA level was more than ~100-fold higher in human monocytes than in human primary cultured adipocytes (resistin mRNA in human primary cultured adipocytes, means ± SE of three replicate wells; 0.61 ± 0.06). Therefore, the SNP-420 genotype determines resistin mRNA in monocytes and serum levels, which could induce T2DM.

Discussion

We report here that the resistin promoter SNP-420 genotype was associated with its monocyte mRNA and serum levels, and that T2DM subjects had higher serum resistin levels than controls. A logistic regression analysis revealed that serum resistin level was an independent factor for T2DM. Therefore, the SNP-420 determines monocyte mRNA and serum levels of resistin, which could induce T2DM.

We found that the SNP-420 genotype was a major determinant of serum resistin levels. Serum resistin levels were highest in the G/G genotype, followed by the C/G and C/C genotypes. This order was also confirmed in a report on Korean subjects [26]. Haplotypes including this SNP-420 showed a similar tendency in Japanese subjects [41]. We also found that resistin mRNA levels in monocytes were higher in healthy volunteers with the G/G genotype. Smith et al. [38] showed that obese human subjects with the G/G genotype also have higher resistin mRNA levels in their abdominal subcutaneous fat.

We found that resistin mRNA in monocytes was positively correlated with serum resistin levels. We also found that resistin mRNA was more than ~100-fold higher in monocytes than in primary cultured adipocytes in humans. Whereas it is dominantly expressed in adipose tissues of mice, resistin is most highly expressed in macrophages in humans [32–34]. Therefore, monocytes are promising candidates for the main source of serum resistin in humans, although other regulatory factors or secretory tissues could also affect serum resistin levels.

The association of resistin mRNA in adipose tissue *with serum resistin or insulin resistance has been reported* by other investigators. Heilbronn et al. [42] reported that serum resistin is positively correlated with resistin mRNA in the subcutaneous adipose tissue of obese subjects. The fat content in the liver and HOMA-IR has been also reported to be positively correlated with resistin mRNA in subcutaneous adipose tissues of obese subjects [38]. A total of four independent reports have shown that the activity of the mutant resistin promoter including −420G is higher than that of the wild type promoter including −420C [6,26,38,41]. Therefore, G of SNP-420 enhances resistin gene promoter activity, which could increase resistin mRNA levels in adipose tissues as well as monocytes, leading to whole body insulin resistance.

We have shown that serum resistin levels were associated with T2DM. The serum levels increased with the *number of G alleles in both T2DM and control subjects*. The duration of T2DM and HbA1c was also positively correlated with serum resistin in T2DM. Serum resistin levels have been reported to be increased or unchanged in human T2DM or obesity [14,26–31]. The discrepancy between previous reports may be resolved by considering the SNP-420 genotype as well as the duration of T2DM and HbA1c. It should be noted that serum resistin probably exists as a hexamer (major form) or trimer (a more biologically active form) in mice, which may also affect the assay results [43].

In summary, we elucidated factors correlated with serum resistin levels and effects of SNP-420 on resistin mRNA in monocytes. Fasting serum resistin was significantly higher in T2DM and its independent determinant. Resistin monocyte mRNA levels were positively correlated with their simultaneous serum levels. Therefore, the SNP-420 determines the monocyte mRNA and serum levels of resistin, which could induce T2DM. It is not presently clear how resistin induces insulin resistance in human subjects and whether adipocytes or macrophages are the main sources of serum resistin. Further experiments will be required to clarify these points.

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A novel alternative splice variant of nicastrin and its implication in Alzheimer disease

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Abstract

Nicastrin interacts with γ-secretase complex components predominantly via the N-terminal third of the transmembrane domain. The authentic transmembrane domain is critically required for the interaction with γ-secretase complex components and for formation of an active γ-secretase complex. In this study, we have identified a novel alternatively spliced transcript of nicastrin in human brain tissue. This transcript (NCSTN-ΔE16) lacks exon 16 of nicastrin mRNA, which leads to deletion of 71 amino acids just upstream of its transmembrane domain. Its expression pattern was analyzed in the hippocampus of patients with pathologically diagnosed Alzheimer disease (cases) and non-Alzheimer dementia (controls). In patients with the APOE-ε4 allele, the frequency of Alzheimer disease appeared to be increased in the NCSTN-ΔE16-positive group, but the association was not statistically significant. In conclusion, the expression of NCSTN-ΔE16 transcript may confer some additional risk for developing Alzheimer disease beyond the risk due to Apoε-ε4 allele. Further investigation in larger scale population would be necessary to address its potential implication in Alzheimer disease.

Keywords: Alzheimer disease; Nicastrin; Apolipoprotein E; Alternative splicing

Introduction

Accumulation of amyloid plaques in the brain is a key component of the pathology of Alzheimer disease (AD). Amyloid β-peptide (Aβ), the main component of amyloid plaques, is released from the β-amyloid precursor protein by β- and γ-secretases (Hardy and Selkoe, 2002). Recent studies revealed that nicastrin is a component of γ-secretase complex, which also contains presenilin-1/presenilin-2, APH-1 and PEN-2 (Takasugi et al., 2003).

Yu et al. first reported that artificial deletion mutants of the conserved hydrophilic DYIGS domain in nicastrin decreased Aβ production, whereas a double-missense mutation (D336A+Y337A) increased Aβ production (Yu et al., 2000). Capell et al. reported that a decrease of nicastrin expression by RNAi in HEK293 cells was accompanied by reduced expression of *presenilin-1, APH-1*aL, and *PEN-2* and reduced Aβ generation. Overexpression of wild-type nicastrin restored their reductions, while expression of nicastrin lacking the transmembrane domain did not (Capell et al., 2003). These results suggest that nicastrin plays an important role in activation of γ-secretase complex, production of Aβ peptide and onset of Alzheimer disease.

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Materials and methods

Subjects

All subjects were Japanese ($n = 23$, 74% female, all clinically demented, age range at death 69–98 years). They were inpatients at Fukushimura Hospital (Toyohashi, Aichi, Japan), and were cognitively evaluated by neuropsychological tests such as the Mini-Mental State Examination during hospitalization.

Treatment of autopsied brain

When they died, autopsy and pathological diagnosis were carried out according to the criteria of the Consortium to Establish a Registry for Alzheimer's disease (Mirra et al., 1991). Written consent of the patients' guardians for diagnosis and biochemical, molecular biological and genomic research was obtained. The autopsied brain was weighed, and cut midsagittally. One half of the brain was divided into several portions (frontal, temporal, parietal, occipital cortex, hippocampus, etc.), snapped frozen in liquid nitrogen, and stored at −80 °C. The other half was fixed and used for pathological diagnosis, as described previously (Akatsu et al., 2002). Based on this pathological diagnosis, subjects were divided into AD group and non-Alzheimer dementia (non-AD) group.

Genotyping

APOE genotyping was performed using DNA samples extracted from dissected brain tissues, according to the procedure described previously (Yoshiiwa et al., 1997).

Screening for novel splicing variants and sequencing

Total RNA was extracted from the frozen hippocampus using Trizol (Invitrogen, Carlsbad, CA, USA.), according to the manufacturer's protocol, and first strand cDNAs were synthesized from 5 μg total RNA with an oligo(dT)$_{12-18}$ primer using 50 units superscript II RNase H$^-$ reverse transcriptase (Invitrogen) in a total volume of 20 μl, according to the manufacturer's protocol. The cDNAs were diluted at *1:5* with distilled water, and then 2 μl was used as a template for PCR with Platinum Taq DNA polymerase (Invitrogen) and the sense and anti-sense primers listed in Table 1. Sequencing was performed by direct sequencing method with a dye terminator cycle sequencing FS kit (PE Biosystems) following the manufacturer's protocol.

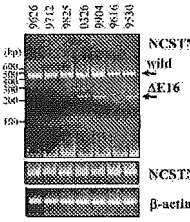

Fig. 1. Identification of a novel alternatively spliced variant of NCSTN. Upper panel: RT-PCR from human hippocampus with primers, NCSTN4-F and NCSTN4-B. Note the 427 bp band (wild-type) exists in all patients, while the 214 bp band (ΔE16) exists in some patients (9626, 9904, and 9530). Middle panel: RT-PCR from hippocampus with primers, NCSTN5-F and NCSTN5-B (see Table 1) as intra-molecular control. Lower panel; RT-PCR from hippocampus with β-actin primers as external control.

Reverse-transcription PCR (RT-PCR)

RT-PCR was performed with the cDNAs from the hippocampus, the primers; NCSTN4-F and NCSTN4-B, and Platinum Taq DNA polymerase at 95 °C for 0.5 min, 55 °C for 1 min, at 72 °C for 1 min, for 40 cycles.

Statistical analysis

AD group (cases) and non-AD group (controls) were further divided by the presence of APOE-ε4 allele into APOE-ε4-positive and APOE-ε4-negative groups. The frequency of *NCSTN-ΔE16* transcript was compared between *AD* and non-AD groups by χ^2 analysis. Differences with p values of <0.05 were considered significant.

Results

With the primers, NCSTN4-F and NCSTN4-B, two major bands were detected in some brain samples (Fig. 1). The most frequent was a 427 bp band (wild-type), followed by a 214 bp band. Sequencing analysis revealed that this latter transcript was an in-frame splicing variant that lacks exon 16, and it was designated "NCSTN-ΔE16". The exact result of sequencing analysis is described in Fig. 2A. The schematic structure of NCSTN-ΔE16 is illustrated in Fig. 2B.

Out of the 23 patients examined in this study, 10 were diagnosed pathologically with AD (mean age at onset of dementia: 72.2 ± 7.4 years, mean age at death: 81.7 ± 8.2 years, mean brain weight at death: 1024 ± 105 g) and 13 were diagnosed as non-AD (mean age at onset of dementia: 83.2 ± 8.4 years, mean age at death: 89.2 ± 7.5 years, mean brain weight at death: 1099 ± 109 g). Non-AD included normal physiological aging, multiple infarctions, diffuse Lewy-body disease, Parkinson disease, etc. (data not shown). 13 patients carried the APOE-ε4 allele (APOE-ε4-positive group), while

Table 1
Primers used for screening for splicing variants of nicastrin

	Name	Sequences	Position
Sense primer	NCSTN1-F	GCTAACAGACAGCAGGCCGAACG	94–115
	NCSTN2-F	TGGGCAATGGTTTGGCTTATG	642–662
	NCSTN3-F	GAGAAGAGTGGTGCTGGTGTCC	1346–1367
	NCSTN4-F	GCCCCACCAACACCACTTATG	1818–1838
	NCSTN5-F	TGGACTGAGAGCCGCTGGAAAG	2084–2105
	NCSTN6-F	GGGTTCCTGATTAAGCCAACAAC	1712–1735
	NCSTN7-F	TCATGGTTCCAGTCTATCCTCAGG	1736–1759
	NCSTN8-F	GCCTTGTCTCCTGCCTTTGAAC	2030–2051
Anti-sense primer	NCSTN1-B	CTTCATAAGCCAAACCATTGCC	665–644
	NCSTN2-B	TGAGGATGACAGCAGGGACATC	1383–1361
	NCSTN3-B	AAGTGGTGTTGGTGGGCTGGTAGTAC	1835–1811
	NCSTN4-B	GGAGCATGAAAGGACATCAGC	2244–2222
	NCSTN5-B	AGCACCCCCACCCTCTAATGTG	2800–2787
	NCSTN6-B	GCATTGATGCAGTAGGTGACGATG	2317–2194
	NCSTN7-B	CAGTGGGACAGATGCTCTAGGAAG	2333–2310
	NCSTN8-B	CTGAAGGGCAAATTAGGGTGG	2384–2364
	NCSTN9-B	AAAAGTAGAAGGGTCCTGAAGGG	2680–2572

The number depicts the position of the sequence in NCSTN cDNA (Genbank Accession # AF349468).

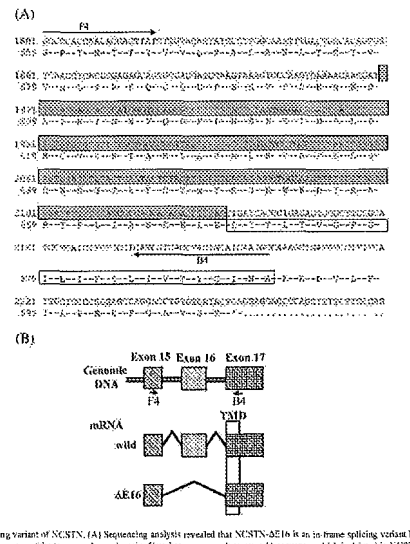

Fig. 2. Structure of the splicing variant of NCSTN. (A) Sequencing analysis revealed that NCSTN-ΔE16 is an in-frame splicing variant lacking exon 16. Open box corresponds to amino acid sequence of the transmembrane domain. Grey box corresponds to exon 16 sequence, which is deleted in NCSTN-ΔE16. F4 (NCSTN4-F) and B4 (NCSTN4-B) are the primers used to detect NCSTN-ΔE16. (B) Schematic representation of NCSTN gene and its wild-type (wild) and alternatively spliced transcript (ΔE16).

Table 2
Summary of the characteristics of AD and non-AD subjects by detection of NCSTN-ΔE16 transcript

	Pathological diagnosis		
	Overall	AD	Non-AD
Age at onset of dementia			
NCSTN-ΔE16(−)	79.5±9.9(11)	71.5±9.1(4)	84.0±7.4(7)
NCSTN-ΔE16(+)	75.6±9.3(9)	72.7±7.0(6)	81.3±12.1(3)
P value	0.38	0.84	0.75
Age at death			
NCSTN-ΔE16(−)	85.8±9.5(12)	79.5±9.3(4)	88.9±8.4(8)
NCSTN-ΔE16(+)	86.2±7.8(11)	83.2±7.9(6)	89.8±6.7(5)
P value	0.90	0.54	0.83
Brain weight at death			
NCSTN-ΔE16(−)	1071±96(12)	1073±138(4)	1071±89(8)
NCSTN-ΔE16(+)	1060±131(11)	991±75.6(6)	1143±144(5)
P value	0.81	0.34	0.35

Values are means±S.D. (number of cases). No significant difference was detected between NCSTN-ΔE16(−) and NCSTN-ΔE16(+) groups. AD: Alzheimer disease, non-AD: non-Alzheimer dementia.

10 patients did not (APOE-ε4-negative group). RT-PCR analysis with the primers, *NCSTN4-F* and *NCSTN4-B*, detected the wild-type NCSTN transcript in the hippocampus in all 23 patients, while it also detected NCSTN-ΔE16 in 11 patients.

The age at onset of dementia, age at death and brain weight at death were not significantly different between NCSTN-ΔE16(−) group and NCSTN-ΔE16(+) group in overall, AD, and non-AD patients, as described in Table 2. NCSTN-ΔE16 transcript was detected in 6 out of 10 AD patients and 5 out of 13 non-AD patients. The difference in the frequency of NCSTN-ΔE16 transcript between AD cases and non-AD controls was not significant ($p=0.55$) (Table 3). When analysis was limited to APOE-ε4-negative patients, the NCSTN-ΔE16 transcript was detected in 1 out of 2 AD patients, and 4 out of 8 non-AD patients. The frequency of NCSTN-ΔE16 transcript was not significantly different between AD patients and non-AD patients, either ($p=1.00$). Likewise, when analysis was limited to APOE-ε4-positive patients, the NCSTN-ΔE16 transcript was detected in 5 out of 8 AD patients, and 1 out of 5 non-AD patients. The frequency of NCSTN-ΔE16 transcript was not significantly different between AD patients and non-AD patients, either ($p=0.35$).

Discussion

Assembly of nicastrin into γ-secretase complex is essential for activation of γ-secretase and generation of Aβ. In molecular and cellular biological studies, Capell et al. reported that nicastrin interacts with γ-secretase complex components predominantly via the N-terminal third of the transmembrane domain (670–692 amino acids). The authentic transmembrane domain of nicastrin is critically required for the interaction with γ-secretase complex components and for formation of an active γ-secretase complex (Capell et al., 2003).

In this study, in the human hippocampus, we identified a novel alternatively spliced transcript lacking exon 16, which encodes the 71 amino acid sequence just upstream of this functional transmembrane domain (see Fig. 2A). This transcript was detected in some patients, but not in others. The cause of this dissociation is unknown. It is not clear if this endogenous deletion may affect the function of nicastrin and the activity of γ-secretase in the human brain or even in vitro. Change in the activity of γ-secretase may influence the risk of AD. Accordingly, the implications of the expression of this transcript and AD pathology were examined here.

When we analyzed overall patients, the difference in the frequency of NCSTN-ΔE16 transcript between AD cases and non-AD controls was not significant. As described in most other studies, APOE-ε4 allele is a major risk factor for developing AD. It is estimated to account for about 40–50% of the genetic variation in late-onset AD (Roses, 1996). To examine the association between the existence of NCSTN-ΔE16 transcript and the development of AD independently of APOE genotype, we further categorized AD and non-AD patients by the presence of APOE-ε4 allele into APOE-ε4-negative and APOE-ε4-positive groups. In APOE-ε4-negative group, the difference between AD cases and non-AD controls was not significant, either. In APOE-ε4-positive group, the frequency of NCSTN-ΔE16 transcript appeared to be higher in AD cases than in non-AD controls. However, the association was not statistically significant because of the small population size. This suggests the possibility of interaction between NCSTN-ΔE16 and APOE-ε4, so that NCSTN-ΔE16 only influences risk if an individual carries APOE-ε4; however, statistical tests for interaction were not significant.

Several genetic studies have focused on the association between nicastrin polymorphisms and the onset of AD. Helisalmi et al. reported that one haplotype of nicastrin significantly increased the risk of AD in patients without an APOE-ε4 allele in the Finnish population (Helisalmi et al., 2004). Dermaut et al. reported that one SNP haplotype of nicastrin is increased in patients with familial early-onset AD without the APOE-ε4 allele in the Dutch population (Dermaut et al., 2002). On the contrary, Orlacchio et al. (2004) and Cousin et al. (2003) reported no such associations. Thus, there is disagreement in opinion, and further investigation of this matter is necessary.

In conclusion, the expression of NCSTN-ΔE16 transcript may confer some additional risk for developing Alzheimer disease beyond the risk due to ApoE-ε4 allele. Further

Table 3
The number of subjects with or without NCSTN-ΔE16 transcript in overall AD and non-AD patients, and by ApoE genotype subgroups

	Overall[a]		APOE-ε4-negative[b]		APOE-ε4-positive[c]	
	AD	Non-AD	AD	Non-AD	AD	Non-AD
	($n=10$)	($n=13$)	($n=2$)	($n=8$)	($n=8$)	($n=5$)
NCSTN-ΔE16(−)	4	8	1	4	3	4
NCSTN-ΔE16(+)	6	5	1	4	5	1

a: $\chi^2=0.36$, $p=0.55$. b: $\chi^2=0.00$, $p=1.00$. c: $\chi^2=0.85$, $p=0.35$.

investigation in larger scale population would be necessary to address its potential implication in AD.

Acknowledgements

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Lymphocyte-specific protein tyrosine kinase is a novel risk gene for Alzheimer disease

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Abstract

Lymphocyte-specific protein tyrosine kinase (LCK) is a lymphoid-specific, Src family protein tyrosine kinase that is known to play a pivotal role in T-cell activation and interact with the T-cell coreceptors, CD4 and CD8. It has been shown to be significantly down-regulated in Alzheimer disease (AD) hippocampus compared with non-demented controls. Furthermore, it is located in a previously identified genetic linkage region (1p34-36) associated with AD. Therefore, we consider it to be a candidate gene for AD. We examined the relationship between AD and the LCK and apolipoprotein E (APOE) genes in 376 AD (including 323 late-onset AD (LOAD) cases and 53 early-onset AD (EOAD) cases) and 378 non-demented controls using a single nucleotide polymorphism (SNP). The polymorphism in intron 1 (+6424 A/G) was significantly associated with AD risk. The odds ratio (OR) for total AD associated with the GG genotype was 1.41 (95% CI=1.06–1.87) and that for LOAD was 1.37 (95%CI=1.02–1.85), while that for APOE-ε4 was 5.06 (95% CI=3.60–7.12). In the APOE-ε4 non-carrier subgroup, the GG genotype also showed significant association (OR=1.66; 95% CI=1.16–2.38). These results indicate that the LCK is a novel risk gene for AD regardless of the APOE genotype.

Keywords: Alzheimer disease; Lymphocyte-specific protein tyrosine kinase (LCK); Polymorphism; Association study; ApoE; Risk factor

1. Introduction

Alzheimer disease (AD) is a progressive neurodegenerative disorder characterized by multiple cognitive deficits and progressive memory impairment in mid- to late-life. Both genetic and environmental factors have been implicated in the development of AD, but it is still unclear how these factors combine and ultimately lead to the neurodegenerative process [1–3]. A number of chemokines, as well as their related receptors, have been shown to be up-regulated in AD brain, supporting the hypothesis that lymphocytes are related to its pathogenesis [4–7].

Lymphocyte-specific protein tyrosine kinase (LCK) is a lymphoid-specific, Src family protein tyrosine kinase that is known to play a pivotal role in T-cell activation and interact with the T-cell coreceptors, CD4 and CD8 [8–10]. In situ hybridization and immunohistochemical studies indicate that the LCK gene is expressed in neurons throughout the brain in distinct regions, including hippocampus and cerebellum [11]. Immunohistochemical examination of brain tissue in mice revealed that its expression was highest in the hippocampus, particularly in dendrites of pyramidal cells [12]. It has also been shown to be significantly down-regulated in the hippocampus in Alzheimer disease (AD)

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patients compared with non-demented controls [13]. Furthermore, human LCK is located in a previously identified genetic linkage region (1p34-36) associated with AD [14]. It has 13 exons distributed across 35 kb of genomic DNA. Its expression is driven by two promoters (distal and proximal) that are active at different stages of development [15]. All of these data suggest that LCK contributes to the pathogenesis of AD. To date, the potential roles for LCK have been reported in T-cell leukemia, colon cancer, type 1 diabetes, systemic lupus erythematosus, relapsing–remitting multiple sclerosis, and rheumatoid arthritis [16–23]. However, there are no reports regarding the association of LCK gene polymorphism with AD. In this study, we investigated whether LCK gene polymorphism could contribute to the risk of sporadic AD.

2. Subjects and methods

The Ethics Committee of Ehime University School of Medicine approved the study protocol. Patients were selected using NINCDS-ADRDA criteria for definite or probable AD, and non-demented controls were rigorously evaluated for cognitive impairment using the Mini-Mental State Examination (MMSE)[24,25]. Brain and blood samples were obtained with informed consent from the patients (or their guardians) in the Chubu, Kansai and Ehime areas of Japan [26,27]. A total of 376 unrelated AD patients had been diagnosed previously, and 376 controls (outpatients or healthy volunteers) were selected and matched for age and place of residence for each patient. The mean age±SD (years) at the time of this study was 78.2±8.3 for late-onset AD and 75.5±4.9 for controls. Genomic DNA was extracted from the brain or peripheral blood using the phenol-chloroform method [28].

During screening for LCK gene mutation and polymorphism, we detected a common single nucleotide polymorphism (SNP) of +6424 A/G (C/T) (hCV1895446) in the intron 1 region (minor allele frequency: 0.34). It was consistent with the SNP database, NCBI build 34 Genome (Caucasian 0.14, African–American 0.01, Japanese 0.33, and Chinese 0.30). Genotyping of SNPs was performed using the TaqMan-PCR method. The primers and probes were obtained by ABI assay-on-demand C_1895446_10. Amplification was performed according to the manufacture's protocol. The fluorescent intensity of the PCR products was measured using an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). The person who assessed the genotype was blinded to the clinical data of the subjects from whom the samples originated. To investigate the contribution of the gene to sporadic LOAD, we compared allele frequencies between LOAD and control subjects. Because APOE-ε4 is a risk factor for AD, we stratified the population by ε4 carrier status. APOE genotyping was performed as described previously [26]. Allelic and genotypic distribution were analyzed by the usual Chi-squared test of association. The genotypic frequencies were compared by Chi-squared test with the values predicted under the assumption of Hardy–Weinberg equilibrium in the sample. Values of $p<0.05$ were considered significant. Odds ratios were calculated with two-tailed p values and 95% confidence intervals. The relation of genotypic factors and the effect of APOE-ε4 on AD were assessed by logistic regression analysis. Statistical analyses were performed with SPSS software version 11.0 (SPSS Inc., Chicago, IL).

3. Results

Table 1 shows the distribution of the three genotypes (GG, GA, AA). The distribution obtained for the patients and controls were in Hardy–Weinberg equilibrium. The GG genotype was found in 53% of the 376 total AD patients (57% of early-onset AD (EOAD) and 52% of late-onset AD (LOAD)) and 44% of the 378 control subjects. A significant association was observed between the +6424 A/G polymorphism and total AD ($p<0.02$), and LOAD ($p<0.05$). The odds ratio (OR) for AD associated with the GG genotype was 1.41 (95% CI=1.06–1.87; Table 2) and that for LOAD was 1.37 (95%CI=1.02–1.85). Stratifying AD patients by sex, no statistically significant differences in allele distribution were observed (data not shown). As expected, APOE-ε4 conferred an increased risk for AD (OR=5.06, 95% CI: 3.60–7.12; Table 2). After the logistic regression analysis, a co-dominant model (ε4 dose–effect)

Table 1
Genotype and allele numbers and frequencies for G/A polymorphism in LCK

Group	Genotype (frequency)				Allele (frequency)	
	GG	GA	AA	AA+GA	G	A
Control (378)	167 (0.44)	168 (0.44)	43 (0.12)	211 (0.56)	502 (0.66)	254 (0.34)
Total AD (376)	198 (0.53)	138 (0.37)	40 (0.10)	178 (0.47)**	534 (0.71)	218 (0.29)
EOAD (53)	30 (0.57)	15 (0.28)	8 (0.15)	23 (0.43)	75 (0.71)	31 (0.29)
LOAD (323)	168 (0.52)	123 (0.38)	32 (0.10)	155 (0.48)*	459 (0.71)	187 (0.29)

EOAD: early-onset AD, LOAD: late-onset AD.
* $p<0.05$.
** $p<0.02$.

Table 2
Relative risk for interaction between APOE-ε4 and 16424GG

		AD cases	Controls	Odds ratio	95%CI
	16424G/A				
	Non-GG	178	211	Reference	
	GG	198	167	1.41	1.06–1.87
APOE-ε4					
−		195	320	Reference	
+		185	60	5.06	3.60–7.12
APOE-ε4	GG				
−	−	86	181	Reference	
−	+	108	137	1.66	1.16–2.38
+	−	92	30	6.45	3.97–10.5
+	+	93	30	6.31	3.88–10.3

APOE-ε4 (+), one or two copies of ε4; APOE-ε4 (−), no copies of ε4; 95% CI, confidence interval at 95% level.

provided the best fit (P=0.024; Exp(β)=2.78; 95% CI=1.14–6.77), but a dominant model could not be rejected (P=0.054; Exp(β)=2.50; 95% CI=0.98–6.34). After logistic regression analysis, a combination of a recessive model of LCK and a co-dominant model of APOE-ε4 provided the best fit (P=0.014; Exp(β)=3.01; 95% CI=1.24–7.30). We then examined the GG genotype as a risk factor for AD, considering the APOE status. To quantify possible interactions between APOE-ε4 and LCK-GG, we analyzed the data with respect to various carrier status combinations, taking subjects who had neither APOE-ε4 nor LCK-GG as a reference (Table 2). Four categories were defined by the presence (+) or absence (−) of an ε4 or GG genotype. The GG genotype alone showed an increased risk (OR=1.66; 95% CI=1.16–2.38), and OR for APOE-ε4 and the GG genotype was 6.31. As for the interaction between the APOE-ε4 and LCK-G alleles for the risk of AD, logistic regression analysis did not indicate a significant effect (P=0.61). The synergistic effect of G allele in patients having APOE-ε4 was weak. Interestingly, the allele distribution was similar among the AD patients regardless of age at onset (EOAD and LOAD) in the APOE-ε4 non-carrier subgroup. The LCK +6424G allele frequency was also significantly higher in AD patients than in controls (0.66 vs. 0.70–0.73) (Table 3). The results showed that the LCK gene was associated with AD regardless of the APOE genotype.

4. Discussion

We carried out an association analysis of LCK polymorphism with AD. Our data showed that LCK GG homozygosity was associated with significantly increased risk of AD, especially in patients without the APOE-ε4 allele. Patients with the G allele had a higher risk of AD than those with the A allele. The association was obvious not only between total AD patients and controls but also between LOAD patients and controls, even excluding the effect of APOE-ε4. The APOE gene is the only established genetic risk factor for LOAD. However, 50% of LOAD cases carry no APOE-ε4 alleles, suggesting that there must be additional risk factors. Our preliminary data suggest that the LCK gene, or a nearby gene (1p35), is one of the additional risk factors, independent of the APOE gene in AD. We can also suppose that the GG genotype in intron 1 may influence the expression of LCK and could be involved in the selective vulnerability of neurons in AD. The LCK gene consists of 13 exons. The proximal promoter, like that of Src family members, is TATA-less and contains multiple start sites for initiation of transcription. Muise-Helmericks and Rosen determined a potentially important sequence located at positions −474 to −466 acts as a strong repressor of transcription [29]. Although the SNP studied here is located in intron 1, it lies only 7 kb downstream from the critical region of transcription regulation site. According to the SNPbrowser Version 2.0 (Applied Biosystems), strong linkage disequilibrium is shown around the LCK gene. Therefore, it is reasonable to think that +6424A/G polymorphism in intron 1 can contribute to promoter activity. +6424A/G may be the representative marker that influences gene expression. In our data, EOAD patients with the GG genotype did not show a significant difference compared with controls, but the P values are near the threshold. This may be due to a small sample size.

The results of this study support the hypothesis that immunological response contributes to the selective vulnerability of neurons in the entorhinal cortex in AD, and altered patterns of LCK immunoreactivity may play an important role in the pathophysiological processes of AD [13]. Although the detailed mechanism of the involvement of LCK in AD is unknown, our data raise the possibility that LCK contributes to the pathogenesis of AD.

Table 3
Genotype and allele numbers and frequencies for G/A polymorphism in LCK without APOE-ε4

Group	Genotype (frequency)				Allele (frequency)	
	GG	GA	AA	AA+GA	G	A
Control (318)	137 (0.43)	146 (0.46)	35 (0.11)	181 (0.57)	420 (0.66)	216 (0.34)
AD (194)	108 (0.56)***	65 (0.33)	21 (0.11)	86 (0.44)***	281 (0.72)*	107 (0.28)
EOAD (35)	20 (0.57)	9 (0.26)	6 (0.17)	15 (0.43)	49 (0.70)	21 (0.30)
LOAD (148)	88 (0.55)	56 (0.35)	15 (0.10)	71 (0.45)**	232 (0.73)*	86 (0.27)

EOAD: early-onset AD, LOAD: late-onset AD.
* $p<0.05$.
** $p<0.02$.
*** $p<0.01$.

LCK might be involved in a new signal transduction pathway. Five of the Src family members, lck, lyn, fyn, src, and yes, have been reported to be expressed in the CNS [30–32]. The adult fyn-deficient brain exhibits abnormal hippocampal development and impairment of long-term potentiation. Although lck knock-out mice have no obvious neurological disorder, a complementation mechanism which expresses a consistent increase in the amount of Src protein may mask its actual effect [33,34]. Furthermore, there is evidence that members of the Src PTK family play important roles in synaptic transmission and plasticity at excitatory synapses in the CNS [35]. In particular, src itself has been shown to up-regulate the activity of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptor in the hippocampus and spinal cord [36,37]. The efficiency with which N-methyl-D-aspartate receptors (NMDARs) trigger intracellular signaling pathways governs neuronal plasticity, development, senescence, and disease [38]. To date, the potential roles for LCK have been reported in T-cell leukemia, colon cancer, type 1 diabetes, systemic lupus erythematosus, relapsing–remitting multiple sclerosis, and rheumatoid arthritis [16–23]. However, there are no reports regarding the association of LCK gene polymorphism with AD. Our data should be further examined by functional analysis of LCK polymorphisms in AD. A systematic survey in a larger cohort of subjects and family studies are required to evaluate the functional relevance of all SNPs, alone or in combination, in patients. Our study also provides a direction for further investigation of the function of p56lck in the central nervous system.

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Original Article

Association of Dopamine β-Hydroxylase Polymorphism with Hypertension through Interaction with Fasting Plasma Glucose in Japanese

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Dopamine-β-hydroxylase (DBH) catalyzes the conversion of dopamine to norepinephrine and is released from sympathetic neurons into the circulation. Several lines of evidence, including the finding of elevated plasma DBH activity in essential hypertension, suggest an important role of DBH in hypertension. Recently, a novel polymorphism (−1021C/T) in the 5′ flanking region of the DBH gene has been shown to account for 35–52% of the variation in plasma DBH activity. We therefore investigated the possible association between the DBH −1021C/T polymorphism and hypertension in a large Japanese population. Moreover, because the development of hypertension is considered to be due at least partly to gene-environmental interactions, we also investigated the possible interactions between the DBH −1021C/T polymorphism and environmental factors. Consequently, we found a significant interaction between the DBH −1021C/T polymorphism and fasting plasma glucose (FPG) in the association with hypertension. CC homozygotes showed a steeper increase in probability of hypertension with FPG than T allele carriers. We also found a marginally significant trend suggesting the presence of an interaction between the DBH −1021C/T polymorphism and FPG in the association with blood pressure. Consistent with the presence of the interaction, we found that a 19 bp sequence containing the DBH −1021C/T polymorphism includes two palindromic non-canonical E boxes separated by 5 bps, and closely resembles the glucose response element of the L-type pyruvate kinase gene. These findings could be helpful in conducting further molecular and biological studies on the relationship among glucose metabolism, the sympathetic nervous system, and hypertension. (*Hypertens Res* 2005; 28: 215–221)

Key Words: dopamine-β-hydroxylase, essential hypertension, genetics, polymorphism, glucose

Introduction

Hypertension is considered to be a complex trait to which genetic, environmental, and demographic factors contribute interactively (*1–5*). Dopamine-β-hydroxylase (DBH) catalyzes the conversion of dopamine to norepinephrine and is released from sympathetic neurons into the circulation. Because the sympathetic nervous system is intimately involved in both the origin and the perpetuation of a hypertensive state (*6, 7*), DBH may play an important role in the pathogenesis of essential hypertension. Indeed, neonates with DBH deficiency show episodic hypotension (*8*). DBH activity, derived largely from sympathetic nerves, can be measured in human plasma (*9, 10*), and elevated plasma DBH activity has also been shown in essential hypertension (*11, 12*), although the conclusions have not been completely consistent (*13*). Moreover, DBH inhibitors have been shown to produce a dose-dependent decrease in mean arterial blood pressure (*14, 15*).

The DBH gene, approximately 23 kb in length, is composed of 12 exons (*16*). Recently, a novel polymorphism (−1021C/T) in the 5′ flanking region of the DBH gene has been shown to account for 35–52% of the variation in plasma DBH activity in several ethnically different populations, including Japanese (*17*). The strong association of the DBH −1021C/T polymorphism with plasma DBH activity has also been replicated in a native Western European population (*18*). Thus, considering several lines of evidence for the relation between DBH and blood pressure, the DBH −1021C/T polymorphism appears to be an attractive candidate variable contributing to hypertension. Nevertheless, there have been few reports investigating the possible association between the DBH gene and hypertension. We therefore investigated the possible association between the DBH −1021C/T polymorphism and hypertension. Moreover, because the development of hypertension is considered to be due at least partly to gene-environmental interactions, we also investigated the possible interactions between the DBH −1021C/T polymorphism and environmental factors.

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Table 1. Characteristics of Participants According to Hypertension Status

Variable	Normotensive (*n*=547)	Hypertensive (*n*=275)
Sex (male %)	78.8	88.1
Age (years)	52.7±8.6	57.3±8.5
Body mass index (kg/m^2)	22.6±2.8	23.8±2.9
SBP (mmHg)	112.6±10.7	143.2±17.4
DBP (mmHg)	72.0±9.1	89.1±9.9
Total cholesterol (mg/dl)	198.0±30.6	202.4±37.2
HDL cholesterol (mg/dl)	54.2±14.5	51.9±14.0
Triglyceride (mg/dl)	116.7±81.7	150.9±127.7
Fasting plasma glucose (mg/dl)	101.2±17.3	106.0±19.2

Data are mean±SD. Blood pressure readings before the start of antihypertensive medication were not available for 118 hypertensive subjects whose values were measured under treatment. SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high density lipoprotein.

Methods

Subjects

According to the criteria described below, 275 hypertensive subjects and 547 normotensive subjects were selected from a population in the Hyogo region of Japan (Table 1) (*19*). All subjects were Japanese urban residents. They had participated in a medical check-up, and the mean values of variables in their personal health records were used in the analyses. All subjects gave their informed consent. The ethics committee of Ehime University approved the study.

Diagnostic Categories

Each subject was assigned to one of the blood pressure diagnostic categories defined by the following criteria. Hypertensive subjects had a previous diagnosis of hypertension and were being treated with antihypertensive medication, or their *systolic/diastolic blood pressure (SBP/DBP)* was ≥140/90 mmHg. Normotensive subjects had never been treated with medication for hypertension, and their SBP/DBP was <140/90 mmHg.

Subjects were considered to have impaired fasting glycemia (IFG) if their fasting plasma glucose (FPG) concentration was ≥110 mg/dl. Subjects were considered to have diabetes mellitus (DM) if their FPG was ≥126 mg/dl.

DNA Analysis

The TaqMan chemical method, which is an established and frequently used method (*20–23*), was used to detect the DBH −1021C/T polymorphism. The forward primer was 5′-GGATCAAGCAGAATGTCCTGAAG-3′, the reverse primer was 5′-GGCACCTCTCCCTCCTGTC-3′, the T-allele specific probe was 5′-Fam-CTCTCCCACAAGTAGA-MGB-3′, and the C-allele specific probe was 5′-Vic-CTC CCGCAAGTAGA-MGB-3′. The person who assessed the genotype was blinded to the clinical data of the subjects from whom the samples originated.

Statistical Methods

Statistical analysis was performed with SPSS statistical software. Comparisons of categorical variables were performed using the χ^2 test. Analysis of variance was used to assess differences in means and variances of continuous variables. Logarithmically transformed plasma triglyceride (TG) and FPG values were used in the analysis. Logistic regression models were used to assess whether the DBH −1021C/T polymorphism made a statistically significant contribution to prediction of hypertension, with consideration of interactions between the polymorphism and confounding factors. General linear regression models were used to assess whether the DBH −1021C/T polymorphism made a statistically significant contribution to prediction of blood pressure, with consideration of interactions between the polymorphism and confounding factors. *p* values less than 0.05 were considered statistically significant.

Table 2. DBH Genotype and Allele Frequencies in Hypertensive and Normotensive Subjects

Genotype and allele	Genotype frequency		*p* value	OR	95% CI
	Normotensive	Hypertensive			
DBH genotypes					
CC (%)	378 (69.1)	184 (66.9)			
CT (%)	153 (28.0)	86 (31.3)			
TT (%)	16 (2.9)	5 (1.8)	0.52*	0.60*	0.66–1.23*
DBH alleles					
C (%)	907 (83.1)	454 (82.5)			
T (%)	185 (16.9)	96 (17.5)	0.78	0.96	0.73–1.26

*p value, OR and 95% CI are for CC vs. CT+TT. DBH, dopamine-β-hydroxylase; OR, odds ratio; CI, confidence interval.

Table 3. Logistic Regression Model of FPG in the Association with Hypertension According to DBH Genotype

Genotype	Coefficient	Constant	*p* value for regression	OR	95% CI	*p* value for interaction
CC	3.12	−15.14	5.4×10^{-5}	22.59	5.90–86.55	
CT+TT	0.20	−1.53	0.82	1.22	0.22–6.78	0.0086

DBH, dopamine-β-hydroxylase; FPG, fasting plasma glucose; OR, odds ratio; CI, confidence interval.

Results

Association of DBH −1021C/T Polymorphism with Hypertension

A total of 822 Japanese individuals from the Hyogo region were categorized as hypertensive or normotensive and genotyped for the DBH −1021C/T polymorphism (Tables 1 and 2). The relative frequencies of the CC, CT and TT genotypes were 68%, 29% and 3%, respectively. The allele frequencies were 83% and 17% for the C and T alleles, respectively. These results are consistent with the Hardy-Weinberg equilibrium ($p>0.25$). Because of the relatively small number of subjects with the TT genotype, we analyzed differences between subjects with the CC genotype and those with the CT and TT genotypes. Statistical analysis failed to show a significant difference in the frequencies of the alleles ($p=0.52$) and genotypes ($p=0.78$ for CC *vs.* CT+TT) between the hypertensive and normotensive subjects (Table 2).

Interaction of DBH −1021C/T Polymorphism with FBS in the Association with Hypertension

We next analyzed possible interactions of the DBH −1021C/T polymorphism with confounding factors in the association with hypertension in logistic regression models, because the development of hypertension is attributable at least partly to gene-environmental interactions. The DBH −1021C/T polymorphism did not interact with sex, age, body mass index (BMI), plasma total cholesterol, high density lipoprotein (HDL)-cholesterol, or TG. In contrast, the DBH −1021C/T polymorphism significantly interacted with FPG ($p=0.0086$) (Table 3). The interaction was significant even after adjustment for sex and age ($p=0.014$), and for sex, age, BMI, plasma total cholesterol, HDL-cholesterol, and TG ($p=0.031$). Subjects with the CC genotype showed a steeper increase in probability of hypertension with FPG than those with the CT and TT genotypes (Fig. 1).

Because the distribution of logarithmically transformed FPG was still slightly skewed, we also examined this interaction using stratification of FPG by quartiles (first quartile <94 mg/dl, second quartile 94 to 99 mg/dl, third quartile 100 to 106 mg/dl, and fourth quartile >106 mg/dl). Consequently, the *p* value for the interaction was 0.014. The *p* value was 0.019 after adjustment for sex and age, and 0.037 after adjustment for sex, age, BMI, plasma total cholesterol, HDL-cholesterol, and TG. Moreover, stratified analyses showed that subjects with the CT and TT genotypes had a significantly higher probability of hypertension than those with the CC genotype in the first quartile (FPG <94 mg/dl) ($p=0.0056$; OR=2.58, 95% CI=1.32–5.05, where OR indicates odds ratio and 95% CI indicates 95% confidence interval).

Interaction of DBH −1021C/T Polymorphism with FBS in the Association with Blood Pressure

We next analyzed possible interactions of the DBH −1021C/T polymorphism with FPG in the association with blood pressure in general linear models. Analysis only of subjects not on current antihypertensive treatment showed that the DBH −1021C/T polymorphism significantly interacted with FPG ($p=0.045$) in the association with DBP (Table 4). The *p* value was 0.056 after adjustment for sex and age, and 0.055 after adjustment for sex, age, BMI, plasma total cholesterol, HDL-cholesterol, and TG.

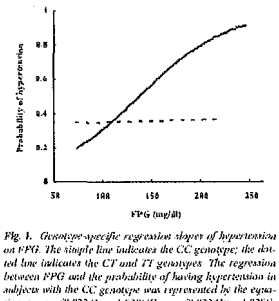

Fig. 1. Genotype-specific regression slopes of hypertension on FPG. The single line indicates the CC genotype; the dotted line indicates the CT and TT genotypes. The regression between FPG and the probability of having hypertension in subjects with the CC genotype was represented by the equation: y = exp(0.0224x − 3.938)/(1 + exp(0.0224x − 3.028)). The equation was: y = exp(0.00864x − 0.685)/(1 + exp (0.00864x − 0.685)); in subjects with the CT and TT genotypes. Subjects with the CC genotype showed a steeper slope than those with the CT and TT genotypes (p = 0.0086).

Discussion

The present study provided evidence for the interaction between the DBH −1021C/T polymorphism and FPG in the association with hypertension in a large Japanese population. There was also a marginally significant trend suggesting the presence of an interaction between the DBH −1021C/T polymorphism and FPG in the association with blood pressure. This lack of significance was possibly due to the unstable nature of blood pressure (*19*). In addition, the inclusion or exclusion of subjects who were receiving antihypertensive treatment influenced the distribution of blood pressure, and blood pressure readings before the start of antihypertensive medication were not available for 118 hypertensive subjects in our population.

In theory, the DBH −1021C/T polymorphism might be associated with hypertension, because this polymorphism is associated with plasma DBH activity (*17, 18*) and plasma DBH activity is associated with hypertension (*11, 12*). However, in practice, the present study failed to show a significant association between the DBH −1021C/T polymorphism and hypertension. This failure was possibly due to the interaction between the DBH −1021C/T polymorphism and FPG in the association with hypertension. However, evidence for this possibility is insufficient, because data on plasma DBH activity were not available in our population. In addition, the previous reports showing that the DBH −1021C/T polymorphism is associated with plasma DBH activity did not analyze the interaction between the DBH −1021C/T polymorphism and FPG in the association with plasma DBH activity (*17, 18*).

Supporting the interaction between the DBH gene and FPG, there is biological evidence showing that glucose and other sugars induce an increase of DBH (*24*). Indeed, rats with experimental diabetes have increased plasma DBH activity (*25*). Thus, the most important physiological influence on plasma DBH activity is considered to be the plasma glucose level (*26*). In addition, DBH-containing neurons in the hindbrain that innervate the hypothalamus have been implicated in the feeding response to glucose deprivation (*27*). In humans, the difference in sympathetic response to glucose ingestion related to family history of hypertension suggests the existence of genetic factors influencing the sympathetic response to glucose ingestion (*28*). The DBH gene may be one such genetic factor.

The precise mechanism of the interaction between the DBH −1021C/T polymorphism and FPG in the association with hypertension remains elusive, a simple explanation may be that the CC genotype or a genotype in linkage disequilibrium with it might produce a controlled amount of DBH in association with the plasma glucose level, leading to increased blood pressure. In contrast, the CT and TT genotypes or genotypes in linkage disequilibrium with them might produce a constant amount of DBH irrespective of the plasma glucose level, leading to relatively stable blood pressure. This explanation may be in line with the observation in a previous study that all 19 chimpanzees were homozygous for the C allele (*29*).

Alternatively, depending on the genotype, glucose level could influence plasma insulin level, which in turn could influence blood pressure. However, the previous observation that insulin administration lowered plasma glucose level, but not plasma DBH activity, challenges this possibility (*24*). Moreover, in humans, activation of the sympathetic nervous

Table 4. General Linear Model for Regression of FPG to the Association with Blood Pressure According to DBH Genotype

BP	Genotype (*n*)	Coefficient	Constant	*p* value for regression	Determination coefficient	*p* value for interaction
SBP	CC (562)	12.1	23.5	0.00916	0.035	
	CT+TT (260)	2.9	106.7	0.75	0.00056	0.057
DBP	CC (562)	11.8	22.1	0.0034	0.021	
	CT+TT (260)	−3.1	91.0	0.65	0.0011	0.045

FPG, fasting plasma glucose; DBH, dopamine-β-hydroxylase; BP, blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure.

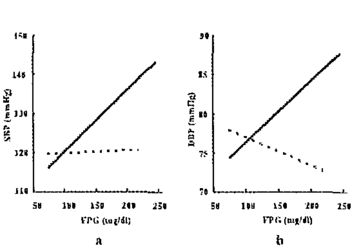

Fig. 2. *Genotypic variations in the relationship between FPG and blood pressure. a: The simple line indicates the CC genotype; the dotted line indicates the CT and TT genotypes. The regression between FPG and SBP in subjects with the CC genotype was represented by the equation: y = 0.1558x + 104.71. The equation was: y = 0.0071x + 119.15; in subjects with the CT and TT genotypes. Subjects with the CC genotype showed a steeper slope than those with the CT and TT genotypes (p = 0.057). b: The simple line indicates the CC genotype; the dotted line indicates the CT and TT genotypes. The regression between FPG and DBP in subjects with the CC genotype was represented by the equation: y = 0.16x − 4.53. The equation was: y = 0.22x − 6.10; in subjects with the CT and TT genotypes. Subjects with the CC genotype showed a steeper slope than those with the CT and TT genotypes (p = 0.045).*

system is related to plasma glucose level but not hyperinsulinemia or insulin hypersecretion in essential hypertension (*30*). However, because the etiology of hypertension, the effects of glucose, and the regulation of the sympathetic nervous system are all complicated, the above explanation remains completely speculative. Epidemiological studies in large populations with information on plasma DBH activity and plasma insulin level as well as biological studies could test this hypothesis.

With respect to the possible functionality of the DBH −1021C/T polymorphism, transient-transfection assays of the reporter gene construct in human neuroblastoma cell lines designed to assess whether this polymorphism directly alters transcriptional activation of the DBH gene have been negative to date (*31*, *32*). In this context, we found that a 19 bp sequence containing the DBH −1021C/T polymorphism (CCCTCAGTCTACTTGYGGG, where Y indicates the C/T polymorphism) includes two palindromic non-canonical E boxes separated by 5 bps, and closely resembles the glucose response element of the L-type pyruvate kinase gene (*33*). The DBH −1021C/T polymorphism resides in a critical 6-bp area. This suggests that the DBH −1021C/T polymorphism may alter the responsiveness to glucose, consistent with the interaction between the polymorphism and FPG, although direct molecular evidence is lacking.

In conclusion, the present study revealed a significant interaction between the DBH −1021C/T polymorphism and FPG in the pathogenesis of hypertension in a large Japanese population. This interaction was partly supported by other epidemiological and molecular biological evidence. Despite several limitations of this study, if our findings are confirmed, they could be helpful in conducting further molecular and biological studies on the relationship among glucose metabolism, the sympathetic nervous system, and hypertension.

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Regular Article

Effect of Genetic Polymorphism of OATP-C (SLCO1B1) on Lipid-Lowering Response to HMG-CoA Reductase Inhibitors

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Summary: The effect of genetic polymorphism of human organic anion transporting polypeptide C (OATP-C) on the lipid-lowering response to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitors was assessed.

A retrospective study was conducted on 66 patients who underwent treatment of hyperlipidemia with HMG-CoA reductase inhibitors in a municipal hospital in a community-based cohort of Ehime prefecture in the southern part of Japan. Plasma lipid concentrations before and after administration were analyzed in patients in relation to the 521T/C (Val-174→Ala) polymorphism in the OATP-C gene (TT: n = 44 (66.7%), TC: n = 20 (30.3%), CC: n = 0 (0.0%), undetermined: n = 2 (3.0%)). Total cholesterol level was significantly lowered after treatment with HMG-CoA reductase inhibitors in all patients ($p<0.001$); moreover, subjects with the 521C allele showed an attenuated total-cholesterol-lowering effect compared with those homozygous for the 521T allele ($-22.3\pm8.7\%$ vs. $-16.5\pm10.5\%$, $p<0.05$).

These data suggest that the 521T/C polymorphism of the OATP-C gene modulates the lipid-lowering efficacy of HMG-CoA reductase inhibitors.

Key words: HMG-CoA reductase inhibitor; genetic polymorphism; transporter; OATP-C; cholesterol; individualized medicine

Introduction

The treatment of common diseases as typified by hyperlipidemia and hypertension gives first priority to lifestyle regimens such as smoking cessation, dietary therapy, kinesitherapy, and maintenance of optimal body weight. However, pharmacotherapy is combined with these measures in patients showing low effectiveness or compliance. Hydoxymethylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins) are now the most widely prescribed drugs worldwide and are established as the first-line treatment for hyperlipidemia. Inhibition of HMG-CoA reductase, which catalyzes the rate-limiting step of cholesterol biosynthesis, causes a decrease in intracellular cholesterol levels, resulting in upregulation of low density lipoprotein (LDL) receptors, increasing clearance of LDL-cholesterol, and leading to a further lipid-lowering effect. The statins decrease blood levels of total cholesterol, LDL-cholesterol, very low density lipoprotein (VLDL)-cholesterol and triglyceride. High-density lipoprotein (HDL) level is increased to a moderate degree.[1)] The clinical significance of statins has been established as the class of drug that most effectively lowers LDL-cholesterol at present. Recent primary and secondary prevention trials have evidenced that statins also reduce the risk of coronary heart disease (CHD).[2-12)]

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Pravastatin, one of the statins, is widely used in the treatment of hyperlipidemia. After oral administration, it is absorbed from the gastrointestinal tract, and then taken up from the circulation by the liver through organic anion transporting polypeptide C (OATP-C).[13,14)] OATP-C, encoded by the gene SLCO1B1 and also referred to as liver-specific transporter 1 (LST-1) or OATP2, is a liver-specific multispecific organic anion transporter that plays a major role in the hepatic uptake of a variety of endogenous and foreign chemicals.[15-17)] In addition to pravastatin, it also plays a major role in the hepatic uptake of pitavastatin,[18)] and an inhibition study suggested that lovastatin, simvastatin and atorvastatin are potential substrates of OATP-C.[19)] Recently, a number of single nucleotide polymorphisms (SNPs) have been identified in the human OATP-C gene by different groups, and some nonsynonymous SNPs have been found to alter its transport activities.[18-21)] The distribution of OATP-C haplotypes varies among ethnic groups. The T521C polymorphism is strongly associated with the A388G variant in Japanese subjects,[22)] while in European Americans, the A388C521 (OATP-C*5) allele occurs at a considerable frequency of 14-15%.[20,23)] An *in vivo* pharmacokinetic study in healthy Japanese subjects showed reduced total and nonrenal clearance of pravastatin in subjects with the G388C521(OATP-C*15) allele as compared with individuals homozygous for the G388T521 (OATP-C*1b) allele.[22)] The reduced hepatic uptake due to this gene polymorphism may be associated with a lower hepatic concentration, resulting in attenuation of the lipid-lowering effect of statins, since the liver is the target organ of statins. In this retrospective study performed in Japanese patients with hyperlipidemia in whom a statin was prescribed, the effect of genetic polymorphism of OATP-C (T521C) on the lipid-lowering response to statins was assessed.

Methods

Subjects: This retrospective cohort study included 3071 subjects in a rural district of Ehime prefecture in the southern part of Japan. Of these subjects, 101 were prescribed HMG-CoA reductase inhibitors between July 1, 2003 and August 28, 2003.

Follow-up survey was based on the medical records of the municipal hospital. The date of first administration of an HMG-CoA reductase inhibitor was confirmed, and the data of total cholesterol, HDL-cholesterol and triglyceride before and after the first administration were transcribed. LDL-cholesterol concentration was calculated using Friedewald's formula. Subjects who showed low or no drug compliance in their medical record were excluded from the analysis. Sixty six subjects were finally available for analysis.

All subjects gave informed consent, and the study was approved by the ethics committee of Ehime University.

DNA analysis: Genomic DNA was extracted from blood lymphocytes using an extraction kit (QIAGEN GmbH, Hilden, Germany). DNA was amplified by degenerate oligonucleotide-primed PCR (DOP-PCR). DOP-PCR amplification was performed as previously described,[24)] with slight modifications as follows. The PCR reactions contained 4 μM DOP-PCR primer (5'-CCGACTCGAGNNNNNNATGTGG-3'), 400 μM dNTPs, 2×GC buffer I, 2.5 mM $MgCl_2$, and 2.5 U Taq polymerase (TaKaRa LA Taq, TAKARA BIO Inc.) in a final volume of 50 μL. The reaction mixture was subjected to an initial denaturation step of 5 min at 95°C; then 10 cycles of 94°C for 30 sec, 30°C for 2 min, and 68°C for 7 min (a ramping step of 0.08°C/sec to 68°C); and then 25 cycles of 94°C for 30 sec, 60°C for 1 min, and 68°C for 7 min. Amplification was carried out in a GeneAmp PCR System 9700 (Applied Biosystems Inc.). Then the DOP-PCR-preamplified DNA samples were subjected to ExoSAP-IT (Amersham Biosciences Inc.) according to the manufacturer's protocol to remove unincorporated primers and dNTPs and used to determine the gene polymorphism. The TaqMan chemical method was used to detect the OATP-C T521C (Val174Ala) polymorphism. The forward primer was 5'- AGG TTG TTT AAA GGA ATC TGG GTC ATA C -3', the reverse primer was 5'- CTC CCC TAT TCC ACG AAG CAT ATT -3', the T allele-specific probe was 5'- FAM- CCC ATG AAC ACA TAT AT-MGB -3', and the C allele-specific probe was 5'- VIC-CCATGAACGCATATAT-MGB -3'.

Statistical analysis: All values are expressed as mean ± SD. Statistical comparisons among genotypes were performed by ANOVA. Chi-squared tests were used to compare the prevalence among genotypes and to verify Hardy-Weinberg equilibrium. The effect of statin treatment on lipid values was analyzed by *t* test for dependent samples. Analysis of variance for repeated measurements was used to determine the significance of differences in serum lipid concentrations. Probability values less than 0.05 were considered to be significant. Statistical analysis was performed with SPSS statistical software (SPSS Inc.).

Results

Baseline characteristics of the subjects are shown in **Table 1.** Out of the 66 subjects, 22 were treated with pravastatin, 11 with atorvastatin and 33 with simvastatin. The allele frequencies of the OATP-C T521C polymorphism were 0.85 and 0.15, respectively, and agreed with the results of previous reports in Japanese.[21,22)] Genotype frequencies were: TT, 66.7%; TC, 30.3%; CC, 0%; undetermined, 3.0%.

Lipid concentrations in patients treated with statins are shown in **Table 2.** The mean serum concentrations of total cholesterol, LDL-cholesterol, and triglyceride

decreased from their mean baseline concentrations of 259 to 203, 167 to 119, and 177 to 126 mg/dL, respectively. The mean serum HDL-cholesterol concentration increased slightly from the baseline of 58.7 mg/dL to 59.9 mg/dL. The mean percent changes in total cholesterol, LDL-cholesterol, triglyceride, and HDL-cholesterol concentrations between pre- and post-treatment were −20.9%, −28.3%, −7.6%, and +4.6%, respectively. There were significant differences in the concentration of total cholesterol ($p<0.001$), LDL-cholesterol ($p<0.001$), and triglyceride ($p<0.01$) between pre- and post-treatment. No statistically significant difference was found in HDL-cholesterol ($p=0.275$).

Then the differences in the effect of three kinds of statins; pravastatin, atorvastatin, and simvastatin, were examined. There was no significant difference in the patterns of change of total cholesterol, LDL-cholesterol, and HDL-cholesterol levels. In contrast, the triglyceride-lowering pattern differed (repeated measures ANOVA; $p=0.040$). Out of the three statins, a significant difference between simvastatin and atorvastatin was found by subsequent Tukey's multiple comparison

Table 1. Baseline characteristics (n = 66)

Age (years)			70.4 ± 8.4
Sex (male/female)			17/49
Body mass index:BMI (kg/m^2)			23.7 ± 2.6
Drug (n)	Pravastatin		22
	Atorvastatin		11
	Simvastatin		33
Polymorphism of OATP-C (n)	V174A	VV	44 (66.7%)
		VA	20 (30.3%)
		AA	0 (0%)
		N.D.	2 (3.0%)

N.D.; not determined

Table 2. Lipid concentrations in patients treated with statins

	n		Pre (mg/dL)	Post (mg/dL)	% Change (95% CI, LL/UL)*	p
Total	66	TC	259.2 ± 33.6	203.7 ± 28.7	−20.9 (−23.3/−18.5)	<0.001
	59	LDL-C	167.0 ± 39.3	119.1 ± 24.5	−28.3 (−32.4/−24.3)	<0.001
	62	TG	176.9 ± 131.7	126.1 ± 63.9	−7.6 (−21.6/6.4)	<0.01
	59	HDL-C	58.7 ± 19.6	59.9 ± 14.8	4.6 (0.1/9.2)	0.275
Pravastatin	22	TC	253.6 ± 33.5	208.3 ± 28.5	−17.5 (−21.3/−13.6)	<0.001
	21	LDL-C	161.2 ± 32.3	122.9 ± 29.1	−23.0 (−29.0/−17.0)	<0.001
	21	TG	159.1 ± 83.8	148.2 ± 86	6.8 (−20.3/33.9)	0.555
	20	HDL-C	59.0 ± 12.8	57.5 ± 12.2	−2.0 (−68.0/2.8)	0.302
Atorvastatin	11	TC	249.5 ± 36.9	198.5 ± 31.9	−20.3 (−24.4/−16.1)	<0.001
	8	LDL-C	139.2 ± 54.2	102.2 ± 19	−34.8 (−41/−28.5)	<0.05
	10	TG	282.9 ± 266.1	139.7 ± 69.8	−7.9 (−58.9/43.1)	0.152
	9	HDL-C	56.2 ± 16.0	64.9 ± 12.5	10.7 (−1.43/22.8)	0.059
Simvastatin	33	TC	266.1 ± 32	202.4 ± 28.2	−23.4 (−27.2/−19.6)	<0.001
	30	LDL-C	180.2 ± 33.0	122.2 ± 21.1	−30.2 (−36.5/−23.9)	<0.001
	31	TG	154.8 ± 69.9	106.8 ± 33.1	−17.2 (−33.8/−0.7)	<0.001
	30	HDL-C	58.8 ± 24.4	60.0 ± 17.3	7.2 (−0.4/14.9)	0.582

TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol
*CI, confidence interval; UL, upper limit; LL, lower limit.
p value: significant difference between pre- and post-treatment.

Table 3. Association of lipid-lowering effect by statins and OATP-C polymorphism

	T521C	N	Pre (mg/dL)	Post (mg/dL)	% Change (95% CI, LL/UL)*	p
TC	TT	44	259.4 ± 35.4	200.3 ± 28.7	−22.3 (−25.0/−19.7)	<0.05
	TC	20	256.8 ± 31.4	213.1 ± 28.3	−16.5 (−21.4/−11.6)	
LDL-C	TT	39	170.2 ± 36.1	118.6 ± 26.8	−29.0 (−33.6/−24.4)	0.094
	TC	20	158.4 ± 46.3	122.6 ± 20.3	−12.4 (−33.4/8.6)	
HDL-C	TT	38	56.1 ± 15.4	57.0 ± 13.7	1.2 (−6.6/9.0)	0.745
	TC	20	63.0 ± 26.0	64.9 ± 16.7	11.1 (−5.3/27.4)	
TG	TT	40	170.7 ± 89.0	125.8 ± 68.0	−10.8 (−28.0/6.4)	0.492
	TC	19	152.8 ± 97.3	127.6 ± 61.2	3.4 (−24.7/31.5)	

TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol
*CI, confidence interval; UL, upper limit; LL, lower limit.
p value: significant difference of lipid-lowering effect of statins in T521C variant.

($p=0.010$). The percent changes in total cholesterol, LDL-cholesterol, triglyceride, and HDL-cholesterol concentrations between pre- and post-treatment showed no significant difference among the three statins.

The effect of the T521C polymorphism of the OATP-C gene on the lipid-lowering response to the statins is shown in **Table 3.** The serum concentration of total cholesterol significantly decreased in subjects with both 521TC and 521TT genotype, from the baseline concentration of 256.8 ± 31.4 to 213.1 ± 28.3 mg/dL and 259.4 ± 35.4 to 200.3 ± 28.7 mg/dL, respectively. Moreover, 521TC heterozygous subjects showed a smaller decrease than 521TT homozygous subjects. A significant effect of the T521C variant was observed in the total-cholesterol-lowering effect of statins (repeated measures ANOVA; $p=0.041$). No statistically significant effect of the T521C variant was found in the other lipid-lowering responses to the statins (LDL-cholesterol, HDL-cholesterol, and triglyceride).

Discussion

Cholesterol-lowering therapy is the central approach in the primary and secondary prevention of CHD. HMG-CoA reductase inhibitors (statins) are currently the most widely used cholesterol-lowering drugs. Large-scale clinical trials have unequivocally demonstrated the efficacy of statin treatment in reducing the risk of CHD.[2-12)] On the other hand, an adequate reduction in CHD events is not necessarily achieved in all patients treated with statins.[25)] Pharmacogenomic variability is an important determinant of drug response. Assessment of polymorphic genes involved in the pharmacokinetics and pharmacodynamics of statins prior to initiation of treatment may help to identify patients at risk of a low response. Choosing an appropriate therapeutic approach for individual patients may be of great advantage not only from the therapeutic standpoint, but also in relation to cost effectiveness, since therapeutic drugs for lifestyle-related diseases such as statins are prescribed over the long term. In this study, the association of genetic polymorphism of liver-specific organic anion transporter OATP-C, which is concerned with the pharmacokinetics of statins, with the lipid-lowering effect of statins was examined in a community-based cohort.

Previous large scale clinical trials of statins reported 18-27%, 25-46%, 10-16%, and 5-8% reductions on average in serum concentrations of total cholesterol, LDL-cholesterol, triglyceride, and HDL-cholesterol, respectively.[2-12)] Our results essentially agree with these results. Serum concentrations of total cholesterol, LDL-cholesterol, and triglyceride significantly decreased after administration of statins, but HDL-cholesterol did not change significantly. The major effect of statins is considered to be the upregulation of LDL receptors. This effect increases the clearance of LDL-cholesterol and leads to a further lipid-lowering effect. Suppression of the synthesis and secretion of VLDL by a reduction of cholesterol synthesis in the liver also decreases serum triglyceride. In contrast, the increase in HDL-cholesterol by statins is moderate.[1,27)]

Statins are well tolerated apart from two uncommon but potentially serious adverse effects: (i) elevation of liver enzymes in less than 2% of patients and (ii) skeletal muscle abnormalities, which range from benign myalgia, which may occur in 0.5 to 2.5% of patients, to myopathy (10-fold elevation of creatine kinase with muscle pain or weakness) in up to 0.3% of patients to life-threatening rhabdomyolysis. These serious adverse effects were not recorded in the medical records of the subjects in this study.

The frequency of the CC genotype of the OATP-C T521C polymorphism is very low in Japanese (previous studies reported 0.8% (ref. 22) and 3% (ref. 21)), although the 521C allele occurs at a considerable frequency (16% (ref. 22), 11% (ref. 21)). In the total 3701 subjects in this cohort study, genotype frequencies were: TT; 2175 (70.8%), TC; 750 (24.4%), CC; 80 (2.6%), and undetermined; 66 (2.1%), consistent with previous reports.[21,22)] However, no individuals homozygous for the 521C allele were ultimately included in the subjects for analysis.

The therapeutic efficacy of statins for total-cholesterol lowering was compared in subjects with and without the 521C allele. The therapeutic effect was attenuated in subjects with the 521C allele compared with those homozygous for the 521T allele. Therefore, it is possible that the reduced hepatic uptake due to the gene polymorphism is associated with the therapeutic effect of statins. This tendency is expected to be more profound in patients homozygous for the 521C allele according to the results of Nishizato *et al.*[22)] and Mwiniyi *et al.*,[23)] On the other hand, Niemi *et al.* recently reported no gene-dose effect of the 521T>C variant on the systemic exposure to pravastatin.[25)] Haplotype analysis revealed that the haplotype containing the −11187G>A, 388A>G and 521T>C SNPs had a particularly pronounced effect on the $AUC_{0\text{-}12h}$ of pravastatin. This result suggests that the 521T>C variant is not the only predictable SNP of the OATP-C phenotype, and haplotype analysis is more informative than single SNPs analysis. Further study is required to elucidate the most effective SNP or haplotype for predicting OATP-C phenotype.

Unlike pravastatin, atorvastatin and simvastatin have not been shown to be a substrate of OATP-C. Since atorvastatin is administered to patients as the acid form, it is possible that OATP-C accounts for its hepatic uptake. Simvastatin is administered as the lactone form, and it is generally considered that it crosses the plasma

membrane by passive diffusion. However, simvastatin undergoes conversion to the acid form, which is the active form, in the body. A substantial amount of the active form was detected in the blood circulation. Therefore, the acid form may be taken up by the liver by a transporter, presumably by OATP-C. This may account for the attenuated cholesterol-lowering effect of simvastatin treatment in subjects with the 521C allele.

Genetic polymorphisms in drug-metabolizing enzymes, transporters, receptors, and other drug targets have been linked to individual differences in the efficacy and toxicity of many drugs. Therapeutic effect is determined by the interplay of several genes encoding proteins involved in multiple pathways of drug metabolism, disposition, and effects.[7,8] To optimize the benefits of medication for individual patients, it is necessary to accumulate clinical data on the association between genotypes and phenotypes for the target drug. Currently, no genetic polymorphisms that are useful for the prediction of effects and adverse drug reactions to statin therapy are available.[7,8] Our results indicated that the T521C polymorphism in the OATP-C gene, which is one of the transporters related to the pharmacokinetics of statins, affected the therapeutic effects of statins on hyperlipidemia. Assessment of the OATP-C T521C polymorphism could be useful for the prediction of therapeutic efficacy and the risk of statin treatment in individualized medicine.

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