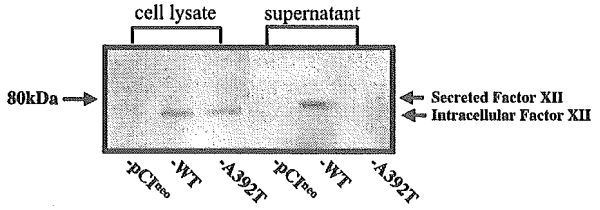

Figure 3 Western blot of recombinant FXII detected in the culture medium and cell lysates. Plasmid constructs (PCI^{neo}, PCI^{neo}/FXII-WT, PCI^{neo}/FXII-A392T) were transiently transfected into CHO cells. Supernatants containing secreted recombinant FXII were immunoprecipitated by anti-FXII antibodies. Transfected cells were solubilized with SDS sample buffer. All samples were subjected to SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes and probed with anti-FXII antibody. Only FXII-WT antigen was detected in the supernatant. In contrast, both the FXII-WT and A392T antigens were detected in the cell lysates.

antigen levels between WT and A392T. Using three anti-FXII antibodies, FXII antigen secreted into the supernatant of the culture medium was immunoprecipitated with Protein A beads and the transfected cells were simultaneously solubilized. The immunoprecipitates and cell lysates were separated by SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and used for Western blotting (Fig. 3). In the supernatant, the FXII antigen was detected only in WT, and not in A392T, a finding compatible with the CRM-negative phenotype. We observed the FXII antigen in cell lysates of both WT and A392T. The difference in recombinant FXII molecular weights between supernatants and cell lysates is likely due to a posttranslational modification, such as carbohydrate addition.

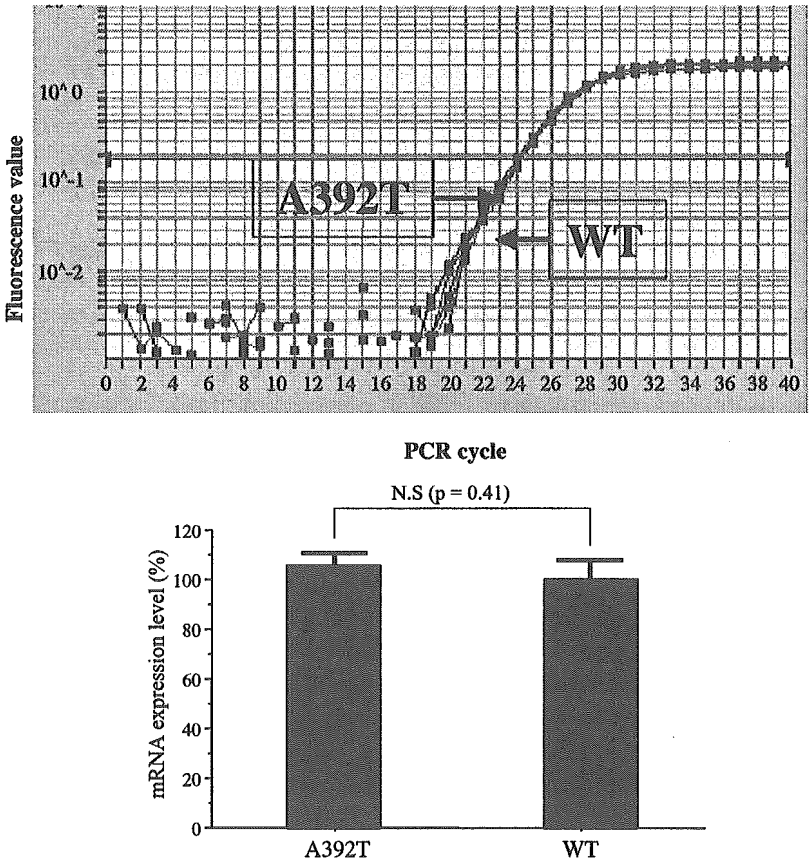

Figure 4 Comparison of mRNA expression levels between A392T and WT. Total RNA was purified from CHO cells transfected transiently by plasmid constructs (PCI^{neo}, PCI^{neo}/FXII-WT, PCI^{neo}/FXII-A392T). Quantitative RT-PCR was performed using a TaqMan probe and appropriate PCR primers using Platinum quantitative RT-PCR ThermoScript one-step system (Invitrogen) and ABI PRISM 7700 (Applied Biosystems). Total RNA from WT was serially diluted and used as a template of quantitative RT-PCR to generate the standard curve. An adequate fluorescent intensity was chosen and the number of required PCR cycles to reach the point was determined as the threshold cycle. The quantitative mRNA expression level of A392T was calculated from the standard curve and depicted as a percentage of WT. The result is from three independent RNA samples. There was no difference in the mRNA expression level between A392T and WT in CHO cells (p=0.41, Student's t-test).

Evaluation of FXII mRNA expression

We investigated the mRNA expression levels of WT and A392T in transient transfectants by quantitative RT-PCR. There was no significant difference in the threshold cycle between WT and A392T (p=0.41, Student's t-test; Fig. 4).

Discussion

We investigated a patient with CRM-negative FXII deficiency and discovered a homozygous mutation in exon 10 of FXII gene. This is a novel mutation, not yet reported, resulting in the amino acid substitution Ala to Thr at position 392 (A392T) in the catalytic domain, and was designated FXII Shizuoka. Transient expression study revealed the absence of A392T protein secretion to the culture medium, whereas the mutant protein and mRNA were present inside of the cells at an equivalent level compared to WT, suggesting that the A392T protein is normally transcribed and synthesized at the endoplasmic reticulum.

Only a few gene mutations related to CRM-negative FXII deficiency have been reported. Screening of mutations in the human FXII gene of 31 unrelated FXII-deficient patients identified six mutations associated with CRM-negative FXII deficiency [14], although expression analysis was not performed. Four CRM-negative mutations were determined by expression analysis. FXII Tenri [5] exhibited a Tyr34Cys substitution. The additional Cys in the NH_2-terminal type II domain is related mostly to proteasome-mediated degradation, and a small amount of residual FXII Tenri was secreted to the plasma combined with α_1-microglobulin, which was connected to the mutated Cys34. Two other CRM-negative FXII mutations, Gln421Lys and Arg123Pro, were examined by in vitro expression analysis [6]. Gln421Lys is likely to accumulate in the endoplasmic reticulum due to an impaired transport system, whereas Arg123Pro is susceptible to proteasome degradation.

In the present case, the amount of intracellular FXII Shizuoka protein detected by Western blot was no more than that of WT; that is, there was no accumulation of FXII Shizuoka in the cells (Fig. 3). Moreover, the mRNA expression level of FXII Shizuoka was equivalent to that of WT (Fig. 4). We and another group previously reported another CRM-negative FXII deficiency, Trp486Cys [7,8]. The recombinant Trp486Cys protein accumulated in the cells more than in WT, with a slight increase in mRNA expression. In the endoplasmic reticulum, the signal of aberrant protein accumulation is transduced to the nucleus by transcription factors, the control of which is related to protein expression for long-term adaptation or apoptosis of the cells [15]. The lack of recombinant FXII Shizuoka protein accumulation and constant mRNA expression in the cells suggest that the molecular etiology of FXII deficiency induced by Ala392Thr is an increased susceptibility to proteasome rather than endoplasmic reticulum retention due to impaired transportation.

As a member of serine proteases, the structure formed by the catalytic triad His393–Asp442–Ser544 is critical for the FXII enzyme activity [14]. It is noteworthy that the present mutation Ala392Thr is located close to the active site His393. Threonine has a hydroxyl group in its side chain. It is possible that the hydroxyl group at residue 392 makes the new hydrogen bond with other amino acid residues and affects the geometry of the active site. It is reported that the missense mutations Leu395Met and Arg398Gln close to His393 also cause CRM-negative FXII deficiency [14]. All of these Ala392, Leu395 and Arg398 residues are conserved for human, bovine and guinea pig FXII [16,17].

It is interesting that FXII mutations in the catalytic domain could cause both CRM-negative and -positive phenotypes. FXII Washington DC (Cys571Ser) [9], FXII Locarno (Arg353Pro) [10], FXII Asp442Asn, and Gly570Arg are CRM-positive phenotypes [14]. Whether the mutants were degraded or secreted would depend on the misfolded conformation induced by the mutation, which would be recognized by the protein quality control system in the endoplasmic reticulum [18–20].

In conclusion, we identified a novel homozygous FXII mutation, Ala392Thr, and clarified that the mutation was an independent molecular basis of the CRM-negative phenotype. Elucidation of the protein degradation mechanism requires further investigation of the intracellular protein quality control system.

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T280M and V249I polymorphisms of fractalkine receptor CX3CR1 and ischemic cerebrovascular disease

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Abstract

The contribution to atherosclerosis of two CX3CR1 single nucleotide polymorphisms, V249I and T280M has been recently reported. The atherosclerosis of intracranial vessels is thought to be the major pathological mechanism of ischemic stroke. In this study, we investigated the risk of ischemic stroke associated with fractalkine receptor CX3CR1 polymorphisms.

We investigated the T280M and V249I mutations in the CX3CR1 gene in 235 Japanese patients with ischemic cerebrovascular disease (CVD) and 306 age- and sex-matched healthy controls. Polymerase chain reaction and restriction fragment length polymorphism were used for genotyping.

There was no significant difference in both polymorphisms between patients with ischemic CVD and controls (VV versus II + VI, $p = 0.83$; TT versus MM + TM, $p = 0.66$). The I and M allele frequencies were not significantly different between CVD patients and controls: odds ratio (OR) = 0.89 (95% confidence interval (CI) = 0.50–1.60, $p = 0.70$) and OR = 1.19 (95% CI = 0.71–2.00, $p = 0.51$), respectively. We found eight of nine possible combined genotypes, including a new haplotype V249-M280, in Japanese.

Our results show that these CX3CR1 gene polymorphisms are not associated with an increased risk for ischemic CVD in the Japanese population.
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Keywords: Ischemic stroke; Atherosclerosis; Polymorphism; CX3CR1; Fractalkine

In 2001, Moatti et al. [12] reported that the I249 allele of the CX3CR1 gene had the ability to reduce the risk of coronary artery disease. Two CX3CR1 single nucleotide polymorphisms, V249I and T280M, are located in the coding sequence, and a recent study demonstrated that I249-M280 homozygotes have lower fractalkine binding affinity on primary peripheral blood mononuclear cells (PBMC) compared with V249-T280 homozygotes [3]. These results prompted researchers to examine the correlation between these alleles and the pathogenesis of atherosclerotic disease.

Imai et al. [7] identified a novel seven-transmembrane receptor for fractalkine, which is expressed mainly on NK cells and monocytes, and partly on T cells, and named it CX3CR1. Fractalkine, a CX3C motif protein, is a transmembrane, mucin/chemokine hybrid molecule, which is induced on activated primary endothelial cells [1]. Fractalkine is captured on the cell surface of inflammatory factor-activated leukocytes, so interaction between fractalkine and CX3CR1 may contribute to atherogenesis, which consists of inflammation, adhesion between leukocytes and the endothelium, and leukocyte migration into the endothelium [4].

Some studies have examined the correlation between these polymorphisms and atherosclerotic disease, but no study has focused on the association between these polymorphisms

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and stroke. The primary aim of this study was to determine whether these CX3CR1 single nucleotide polymorphisms are associated with cerebrovascular disease.

We analyzed 235 unrelated Japanese patients with ischemic cerebrovascular disease (CVD) and 306 age- and sex-matched controls. All CVD patients had attended the outpatient clinic of Keio University Hospital, Tokyo for regular follow-up examinations. We selected CVD patients aged ≦70 years at the onset of CVD. On the basis of the Classification of Cerebrovascular Diseases III report from the committee established by the National Institute of Neurological Disorders and Stroke, CVD patients diagnosed with atherothrombotic infarction, lacunar infarction, or transient ischemic attack (TIA) were enrolled in this study. Those with cardioembolic cerebral infarction or cerebral hemorrhage were excluded. Controls were patients who had had regular check-ups. Those with a clinical history of cerebrovascular disease, myocardial infarction, or peripheral vascular disease were excluded. Written informed consent was obtained from all subjects after a full explanation of the study and a guarantee of total confidentiality. Brain CT and/or MRI was performed on all patients with CVD. Hypertension, hypercholesterolemia, and smoking were defined as described previously [8].

To analyze the T280M and V249I polymorphisms of CX3CR1, polymerase chain reaction (PCR) was carried out as described previously [8]. Briefly, amplification of a 307 bp fragment of the CX3CR1 gene was performed with the 5′ primer 5′-GCTACTTCAGAATCATCCAG-3′ and 3′ primer 5′-AGGCATTTCCCATACAGGTG-3′. PCR consisted of 1 cycle of 15 min at 80 °C and 4.5 min at 94 °C, 44 cycles of 30 s at 94 °C, 45 s at 50 °C, and 1 min at 72 °C, followed by 7 min at 72 °C in a Gene Amp PCR system 2400 (Perkin-Elmer, Foster City, CA, USA). The PCR product (5 μl) was cleaved with 20 U BsmBI restriction enzyme (New England Biolabs, Beverly, MA, USA) or with 20 U Psp1406I restriction enzyme (Takara Shuzo, Ohtu, Shiga, Japan). To analyze the T280M polymorphism, BsmBI digestion of the PCR product yielded bands of 126, 106, and 75 bp in TT homozygotes, 181 and 126 bp in MM homozygotes, and all bands in heterozygotes. For the V249I polymorphism, Psp1406I digestion of the PCR product yielded bands of 192, and 115 bp in VV homozygotes, 307 bp in II homozygotes, and all bands in heterozygotes.

The differences in genotype frequencies and other risk factors were analyzed by χ^2 test. Mean age and allele frequencies in the two groups were compared by Student's *t*-test. Multiple logistic regression methods were used to control for possible confounding factors. All statistical analyses were performed using Statview software (ver 5.0 for Windows, SAS Institute, CA, USA).

The 235 CVD patients and 306 controls were well matched in terms of age and sex (Table 1). The risk factors hypertension, diabetes mellitus, and smoking were significantly more common in CVD patients than in controls ($p < 0.001$). The frequencies of the V249I and T280M polymorphisms showed no deviation from Hardy–Weinberg equilibrium: V249I ($p = 0.59$) and T280M ($p = 0.59$), respectively.

Table 1
Clinical characteristics of CVD patients and controls

	Controls	CVD patients	
	($n = 306$)	($n = 235$)	p^a
Male (%)	77.5	77.9	NS[b]
Age, mean ± S.D., years	58.6 ± 4.3	58.3 ± 7.8	NS[b]
Hypertension (%)	25.1	56.2	<0.001
Hypercholesterolemia (%)	33.0	38.6	0.177
Diabetes mellitus (%)	6.54	25.8	<0.001
Smoking (%)	37.6	53.3	<0.001
Body mass index >27.3 kg/m^2 (%)	5.19	9.02	0.737
Family history (%)	24.2	29.3	0.298

[a] χ^2 test was used to compare values of all parameters in CVD patients and controls, except for age, which was compared by Student's *t*-test.
[b] Not significant.

The I and M allele frequencies were not significantly different between CVD patients and controls: 4.3% versus 4.7%, odds ratio (OR) = 0.89 (95% confidence interval (CI) = 0.50–1.60, $p = 0.70$) and 6.2% versus 5.2%, OR = 1.19 (95% CI = 0.71–2.00, $p = 0.51$), respectively (Table 2). There was also no deviation in the frequency of carriers (II + VI and MM + TM) between CVD patients and controls: 7.7% versus 8.2%, OR = 0.93 (95% CI = 0.49–1.76, $p = 0.83$) and 10.6% versus 9.5%, OR = 1.14 (95% CI = 0.65–2.00, $p = 0.66$), respectively. Eight of the nine possible combined genotypes were observed in our population (Table 3). The frequency of the VV–TT combined genotype in the three groups of CVD patients, the AT, the lacunar and the TIA group, was 88.4, 86.6, and 95.8, respectively, but there was no statistic difference in the frequency between these subgroups of CVD patients and controls.

There were no carriers (II + VI) of the I allele in TIA patients with the V249I genotype. In contrast, there was no significant deviation of genotype between stroke subtypes and controls. In logistic regression analysis, sex, age, hypertension, hypercholesterolemia, diabetes mellitus, current smoking, body mass index, family history of stroke and CX3CR1 genotypes (VV versus VI + II or TT versus TM + MM) were included as independent variables. This analysis revealed that presence of the I allele or M allele was independent of these acquired risk factors.

The present study examined the relation between CVD and two CX3CR1 single nucleotide polymorphisms. There was no significant difference in T280M and V249I polymorphisms between patients with CVD and controls.

In two animal studies on atherogenesis, CX3CR1$^{-/-}$Apolipoprotein E$^{-/-}$ (ApoE) double knockout mice given a western diet showed a reduction in macrophage infiltration in the vessel wall and decreased size of atherosclerotic lesions compared with CX3CR1$^{+/+}$ApoE$^{-/-}$ littermates [2,10]. These results indicate that CX3CR1 may play a critical role in the first step of atherogenesis;

Table 2
Genotype and allele frequencies of V249I and T280M polymorphisms of CX3CR1 gene in CVD patients and controls

Genotype	CVD patients ($n=235$)	AT ($n=69$)	Lacunar ($n=142$)	TIA ($n=24$)	Controls ($n=306$)
II+VI (%)	7.7 (18)	8.7 (6)	8.5 (12)	0.0 (0)	8.2 (25)
VV (%)	92.3 (217)	91.3 (63)	91.5 (130)	100.0 (24)	91.8 (281)
p^a	0.828	0.886	0.920	0.145	–
MM+TM (%)	10.6 (25)	8.7 (6)	12.7 (18)	4.2 (1)	9.5 (29)
TT (%)	89.4 (210)	91.3 (63)	87.3 (124)	95.8 (23)	90.5 (277)
p^a	0.655	0.840	0.304	0.384	–
I (%)	4.3	5.1	4.6	0.0	4.7
V (%)	95.7	94.9	95.4	100.0	95.3
p^a	0.705	0.868	0.916	0.123	–
M (%)	6.2	5.8	7.0	2.1	5.2
T (%)	93.8	94.2	93.0	97.9	94.8
p^a	0.506	0.788	0.280	0.336	–

Number of subjects (X).
[a] χ^2 tests were used to compare genotype and allele frequencies between controls and all CVD patients and between controls and individual groups of CVD patients. AT: atherothrombotic infarction, Lacunar: lacunar infarction, TIA: transient ischemic attack.

that is, interaction between vascular endothelial cells and macrophages.

In two human studies on the association between these two polymorphisms and coronary artery disease, the I allele had the capability to reduce the risk of coronary artery disease [11,12]. In contrast there was no association with peripheral artery disease of Fontaine stage IIa or greater in white healthy controls [6]. Our study failed to reveal an association between ischemic stroke and two CX3CR1 single nucleotide polymorphisms. The frequency of the two single nucleotide polymorphisms, V249I and T280M, (4.7 and 5.2%) in the Japanese population was significantly lower than that in the Caucasian population (25.7 and 13.5%, respectively) [3]. We cannot exclude the possibility that the difference in frequency of these single nucleotide polymorphisms between Caucasian and Japanese people may have influenced our results. The relevance of these polymorphisms should be investigated in other populations and by prospective and family studies. On the other hand, recent Italian study on the association between the two polymorphisms and internal carotid artery (ICA) occlusive disease showed the presence of the M280 haplotype reduced risk of ICA occlusive disease and the I249 haplotype was associated with increased stability of carotid plaques [5]. This study, suggested the two polymorphisms were associated with extracranial atherosclerosis. The first step of atherosclerosis, the fatty streak formation, developed in intracranial arterial wall significantly later than in extracranial arterial wall in rabbits [9]. The mean age of our enrolled subjects was about 10 years younger than the mean age of the Italian study's subjects [5]. Our protocol cannot exclude contribution of extracranial atherosclerosis, i.e., artery to artery embolism, and these factors may have influenced our results. Nevertheless, our findings strongly suggest that the T280M and V249I polymorphisms in the CX3CR1 gene may have less effect on the occurrence of CVD than on coronary artery disease or ICA occlusive disease.

Table 3
Combined genotype frequencies of V249I and T280M polymorphisms of CX3CR1 gene in CVD patients and controls

Combined genotype	V249I	T280M	CVD patients % ($n=235$)	Controls % ($n=306$)
1	VV	TT	88.1 (207)	90.5 (277)
2	VV	TM	4.3 (10)	1.0 (3)
3	VV	MM	0.0 (0)	0.3 (1)
4	VI	TT	1.3 (3)	0.0 (0)
5	VI	TM	4.7 (11)	6.9 (21)
6	VI	MM	0.9 (2)	0.0 (0)
7	II	TT	0.0 (0)	0.0 (0)
8	II	TM	0.0 (0)	0.7 (2)
9	II	MM	0.9 (2)	0.7 (2)

Number of subjects (X).

Our other important finding is the detection of single nucleotide polymorphisms of the CX3CR1 gene in the Japanese population, and a new haplotype, V249-M280. A previous report documented that these polymorphisms were found only in a white population, but not in Vietnamese or West Africans [3]. Therefore, the I and M alleles had been thought to be unique to Caucasians. The frequency of the combined genotype VV–TT in the Japanese population was high, 88.1 and 90.5% in CVD patients and in controls, respectively, while the other combinations appeared to have very low frequencies. These findings suggest that the V249-T280 haplotype has a high proportion of the nine possible combined genotypes in Asians, including Japanese. In addition, only six of the nine possible combined genotypes were reported in the white population [3,6,11,12], and only three haplotyes, V249-T280, I249-T280, and I249-M280, of the four possible haplotypes have been detected. In this study, we identified eight combined genotypes including VV + TM and VV + MM, demonstrating the presence of a new haplotype, V249-M280, is present in Japanese.

In conclusion, this study revealed no association between both polymorphisms in the CX3CR1 gene and ischemic cerebrovascular disease. Although further studies are needed to evaluate whether CX3CR1 may play a role in the pathogenesis of CVD, our results indicate that CX3CR1 gene polymor-

phisms may have less effect on the occurrence of ischemic CVD.

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Genetic analysis of hereditary factor X deficiency in a French patient of Sri Lankan ancestry: *in vitro* expression study identified Gly366Ser substitution as the molecular basis of the dysfunctional factor X

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We investigated a new family with cross-reactive material-positive factor X (FX) deficiency. The proband was an 11-year-old French girl from Sri Lanka with a tendency towards severe bleeding. The FX antigen level was 67%, although the activity with extrinsic pathway was 1 U/dl. The complete nucleotide sequences of all exons and exon/intron junctions of the patient's genomic DNA revealed a homozygous G → A substitution in exon 8, which would result in replacement of Gly366 with Ser. The proband is the first reported case of homozygote for the FX Gly366Ser mutation. Heterozygosity for Gly366Ser substitution was previously reported in a Japanese patient (FX Nagoya 2). We studied the functional consequences by expressing mutant FX Gly366Ser protein in HEK293 cells. FX Gly366Ser was secreted into the culture media at levels similar to wild-type FX; however, mutant FX activities were only 0.04, 1.05, and 0.75% of wild-type FX upon activation by the extrinsic system, the intrinsic system, and Russell's viper venom, respectively. Moreover, the activity of FX Gly366Ser was undetectable when analyzed with chromogenic-activated FX and thrombin generation assays. These data suggest that the Gly366Ser substitution would cause a major defect in function of the FX molecule. *Blood Coagul Fibrinolysis* 16:9–16 © 2005 Lippincott Williams & Wilkins.

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Keywords: cross-reactive material-positive factor X deficiency, factor X Nagoya 2, Gly366Ser factor X mutant

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Introduction

Factor X (FX) is a vitamin K-dependent protein that is synthesized by hepatocytes and circulates in plasma as a two-chain zymogen composed of a light chain (molecular weight, 16.2 kDa) and a heavy chain (molecular weight, 42.1 kDa) joined by a disulfide linkage [1,2]. FX is a primary target of serine proteases activated factor VII (FVIIa) and activated factor IX (FIXa). Once activated by these enzymes, activated factor X (FXa) is the main enzyme involved in conversion of prothrombin to thrombin [1].

The human FX gene (F10) is located on the terminal portion of chromosome 13 in q34 [3–5]. Human FX is highly homologous to other vitamin K-dependent coagulation factors, such as factor VII, factor IX, and protein C, and has a similar structure–function relation [6,7]. The gene consists of seven introns and eight exons [6]. Exon I encodes the signal sequence, exon II encodes the propeptide and γ-carboxyglutamic-acid-rich domain, exon III encodes a short stretch of aromatic amino acid, exons IV and V encode the epidermal-growth-factor-like domains, exon VI encodes the activation peptide, and exons VII and VIII encode the catalytic domains.

FX plays a key role in the process of blood coagulation and is activated at the point of convergence of the intrinsic and extrinsic pathways. Activators of the intrinsic pathway include activated factor VIII, factor IXa, Ca^{2+}, and acidic phospholipids. The extrinsic pathway is activated by membrane-bound VIIa and tissue factor with Ca^{2+} [8]. Conversion of factor X to factor Xa involves cleavage of an alanine-isoleucine peptide bond in the heavy chain that liberates a small activation peptide of 52 amino acids. This leads to a conformational change and exposure of the active site triad of His236-Asp282-Ser379, which plays a critical role in the characteristic catalytic function of serine proteases.

In the intrinsic pathway of blood coagulation, this reaction is catalyzed by FIXa in the presence of activated factor VIII, Ca^{2+}, and phospholipids. The same peptide bond is also cleaved by FVIIa in the

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presence of tissue factor in the extrinsic pathway of blood coagulation. FX is also converted to FXa by a specific enzyme in Russell's viper venom (RVV-X) [9]. The N-terminal catalytic domain of FX, which contains His236 and Asp282 of the catalytic triad, is an anion-binding exosite. The C-terminal domain, which contains Ser379, forms the oxyanion hole as the specific pocket for substrate binding. FXa combines with activated factor V in the presence of phospholipid and Ca^{2+} to form prothrombinase, which activates prothrombin to thrombin [10]. The crystal structure of human Des (1–45) FXa has been determined, and the active site is similar to that of thrombin [11].

Congenital FX deficiency, which was first described more than 40 years ago, was named 'Stuart/Prower deficiency' from the names of first two patients in whom it was identified [12]. To date, more than 50 gene mutations related to FX deficiency have been identified [13–24]. The cross-reactive material (CRM)-negative phenotype of FX deficiency is characterized by significant decreases of both FX activity and antigen levels, whereas the CRM-positive phenotype shows decreased FX activity but normal FX antigen levels.

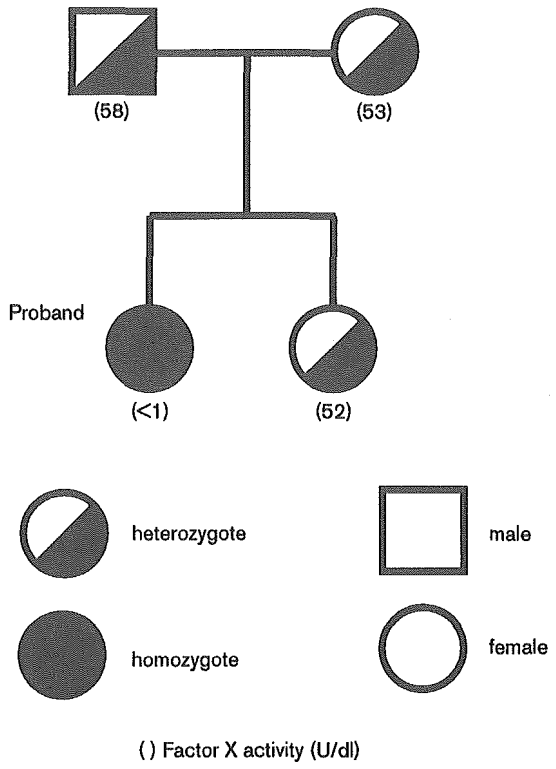
Here we report the molecular analysis and functional consequence of the CRM-positive FX deficiency (Gly366Ser) in a French girl of Sri Lankan origin.

Materials and methods

Patient

The proband was an 11-year-old French female of Sri Lankan descent. She was diagnosed with FX deficiency on the basis of results from a preoperative hemostasis test prior to tonsillectomy when she was 4 years old. Her prothrombin time (PT) was 156 s (control, 12.8 s), and her activated partial thromboplastin time (aPTT) was 140 s (control, 34 s). She had a FX activity level of 1 U/dl (normal range, 70–138 U/dl), but her antigen level was 67% (normal range, 70–130%). At 9 years of age, she had a large hematoma in her right thigh after trauma and received prothrombin complex concentrate (PPSB) by transfusion for 8 days. At 10 years of age, she experienced a prolonged bleeding after a tooth extraction and required PPSB treatment for 3 days. At 11 years of age, she had menorrhagia with anemia (hemoglobin, 7 g/dl). The bleeding was stopped after antifibrinolytic therapy and PPSB transfusion. At that time, a hormonal substitutive treatment was started, and the gynecological bleeding was controlled. Her father, mother, and sister have FX activity levels of 58, 53, and 52 U/dl, and antigen levels of 89, 88, and 80%, respectively (Fig. 1).

Fig. 1



Pedigree of the factor X (FX)-deficient family in the present study and plasma FX activity data. The proband is homozygous for a mutation causing FX deficiency. The father, mother, and sister are heterozygous for this position. FX activity was measured by a one-stage clotting assay based on the prothrombin time.

Isolation and characterization of the mutant F10 gene

The patient's parents gave their informed consent for blood sample collections and family genetic studies. The complete nucleotide sequences of all eight exons and the exon/intron junctions of the F10 gene were determined in the proband. Polymerase chain reaction (PCR) primers flanking the 5′ and 3′ ends of each exon of the F10 gene were described previously [19,20]. Direct sequencing was done for all eight exons with an ABI Prism 377 (Applied Biosystems, Foster City, California, USA). For restriction fragment length polymorphism (RFLP) analysis of a single *Alu*I site, the 163 base pair fragment of exon 8 was amplified with mutated primers 5′-GCAAGTTGTCCACGAGTTT CATC and 5′-GCACAGCCCTCTCCCCAACTGACG (underlined bases are altered).

Site-directed mutagenesis

The expression vector pCMV4/FX wild type, which contains FX cDNA including the complete FX coding sequence [22], a translation initiation sequence, and part of the 3′ untranslated region, was a gift from Dr K. A. High (Children's Hospital of Philadelphia, Pennsylvania, USA). The point mutation responsible for the

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amino acid change from Gly (GGC) to Ser (AGC) was introduced into the FX cDNA with a PCR-based technique. The mutagenic primers were forward primers 5′-GCGACAACAACCTCACGCGTATC and 5′-CTGT GCTAGCTACGACACC, and reverse primers 5′-GTC GTAGCTAGCACAGAAC and 5′-TGCAGGCATGCA AGCTTTGGCTG (underlined bases are altered). These primers create an *Nhe*I site adjacent to the point mutation without changing the encoded amino acid. The presence of the mutation and the orientation of the insert DNA were confirmed by direct sequencing with the ABI Prism 377. PCR amplification was performed for 25 cycles of denaturation (94°C, 15 s), annealing (58°C, 30 s), and extension (68°C, 60 s) using Platinum Pfx polymerase (Gibco BRL, Rockville, Maryland, USA).

Cell culture and transfection for *in vitro* expression

Human embryonic kidney cells (HEK293) were grown at 37°C in 5% CO_2 with Dulbecco's modified Eagle's medium (DMEM) media (Gibco BRL) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Gibco BRL) and vitamin K_1 (Sigma-Aldrich Japan, Tokyo, Japan) to a final concentration of 6 μg/ml. Before transfection, the culture media were removed and replaced with serum-free DMEM including vitamin K_1. Plasmid constructs [wild type, Gly366Ser, and 'mock' (pCMV4 vector only); 7.5 μg each] were transiently transfected into the cells with cationic lipid LipofectAmine™ 2000 reagent (Invitrogen, Carlsbad, California, USA). The cells were incubated for 24 h after transfection, and supernatants were collected for analysis.

Determination of FX antigen levels in culture media by enzyme-linked immunosorbent assay and analysis of FX molecular size by western blotting

Concentrations of FX antigen in the patient's plasma and culture media were determined with a FX enzyme-linked immunosorbent assay (ELISA) Kit (Stago Diagnostica, Paris, France) according to the manufacturer's instructions. Data are from three independent measurements. For western blot analysis, the culture media were subjected to 5–20% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis under non-reducing conditions. Proteins were transferred to nitrocellulose membranes, blocked with 5% powdered non-fat milk in TBS-T (20 mmol/l Tris–Cl, 500 mmol/l NaCl, pH 7.4, 0.5% Tween-20), and then incubated with horseradish peroxidase-conjugated rabbit anti-human FX antibody (DakoCytomation, Glostrup, Denmark). After two washes with TBS-T, the bound antibody was detected with a chemiluminescent kit (Amersham Bioscience, Piscataway, New Jersey, USA).

FX functional assays

Clotting assays of recombinant wild-type and mutant Gly366Ser FX secreted into the culture media were performed with a CA-50 coagulometer (Sysmex, Kobe, Japan). FX antigen levels of wild-type and Gly366Ser FX in culture media were adjusted to 150 ng/ml by ELISA.

For analysis of FX activity in the extrinsic pathway (the PT), 50 μl supernatant from culture media diluted serially with serum-free DMEM and 50 μl FX-deficient plasma (Dade Behring Inc., Liederbach, Germany) were mixed and incubated for 3 min at 37°C. At the end of the incubation time, 100 μl thromboplastin C plus (Dade Behring) was added, and the clotting time was measured.

For analysis of FX activity in the intrinsic pathway (the aPTT), 50 μl supernatant diluted serially with serum-free DMEM and 50 μl FX-deficient plasma (Dade Behring) were mixed and incubated for 1 min at 37°C. At the end of the incubation time, 100 μl Actin® Activated Cephaloplastin Reagent (Dade Behring) was added, and the mixture was incubated for 2 min at 37°C. Then 50 μl of 20 mnol/l $CaCl_2$ was added, and the clotting time was measured.

For analysis of FX activity after activation with RVV, 50 μl supernatant diluted serially with serum-free DMEM and 50 μl FX-deficient plasma (Dade Behring) were mixed and incubated for 1 min at 37°C. At the end of the incubation time, 100 μl LA Test Gradipore (MBL, Tokyo, Japan) was added, and the clotting time was measured. For each analysis, the data from serially diluted wild-type supernatant were analyzed and curve fitting was performed using computer software (Statview 5.0; SAS International, Cary, North Carolina, USA) to generate a standard curve. The activity of Gly366Ser FX was expressed as a percentage of that of 1 × wild-type FX.

Chromogenic assay of FXa

The FX chromogenic activity against synthetic and macromolecular substrates was estimated with the FXa fluorogenic substrate S-2222 (50%/50% mixture of Bz-Ile-Glu (γ-OH)-Gly-Arg-pNa-HCl and Bz-Ile-Glu (γ-OCH_3)-Gly-Arg-pNa-HCl; Chromogenix Instrumentation Laboratory SpA, Milan, Italy). Absorbance at 405 nm was measured with a multilabel counter for quantitative detection of light emission (Wallac1420; Perkin-Elmer, Turku, Finland).

Before chromogenic assays, we investigated an activation of recombinant FX with RVV. Fifty microliters of supernatant containing 2 nmol/l recombinant FX (wild type and Gly366Ser) was incubated for 5 min at 37°C with 0.01 U RVV-FX activator (Pentapharm, Basel, Switzerland). They were then subjected to sodium dodecyl sulfate-polyacrylamide gel electophoresis under non-reducing conditions, and estimated through wes-

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tern blot analysis. Wild-type and mutant FX were equally digested under this condition (data not shown).

Fifty microliters of supernatant containing 2 nmol/l recombinant FX (mock, wild type, or Gly366Ser) was incubated for 5 min at 37°C with 0.01 U RVV-FX activator (Pentapharm). Then 1 mmol/l FXa fluorogenic substrate was added, and the absorbance at 405 nm was measured.

Thrombin generation assay

For the thrombin generation assay, FX-depleted plasma as a source of other essential factors for the coagulation cascade was diluted 1:25 in supernatant containing 2 nmol/l recombinant FX (mock, wild type, or Gly366-Ser) and incubated for 2 min at 37°C with thromboplastin extracted from human placenta (Thromborel S; Dade Behring). Reactions were quenched with 25 mmol/l ethylene glycol bis(2-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid, and absorbance at 405 nm was monitored with a multilevel counter for quantitative detection of light emission (Wallac1420; Perkin-Elmer).

Molecular modeling and comparative sequence analysis

The high-resolution crystal structure of wild-type FXa [11] was obtained from the protein structure databank (1XKA) and used as the basis to model the structure of mutant FXa (Gly366Ser). Comparative analyses of the mutant and wild-type FXa structures were performed with interactive graphics in a molecular operating environment (MOE; Chemical Computing Group, Inc., Montreal, Canada).

Results

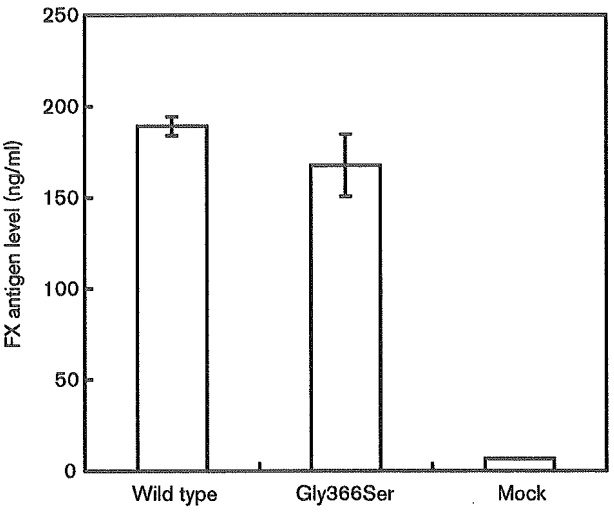
Characterization of the mutant F10 gene

The nucleotide sequences of all eight exons and exon/intron junctions of the F10 gene were determined for the proband. Sequence analysis revealed a single homozygous mismatch at position 363 (numbering according to GenBank accession number L29433) of exon 8, which results in the codon change Gly366 (GGC) to Ser (AGC). This mutation is similar to one reported previously (FX Nagoya) [14]. RFLP analysis confirmed that the proband was homozygous for this mutation, and all other family members (father, mother, and sister) tested were heterozygous (data not shown).

In vitro expression of FX Gly366Ser

Wild-type and mutant FX Gly366Ser were expressed using HEK293 cells, and the levels of secreted FX antigen in the culture media were determined by ELISA. The concentration of wild-type FX in culture media was 189.4 ± 5.1 ng/ml and that of FX Gly366Ser was 167.6 ± 17.0 ng/ml (Fig. 2). There was no significant difference between the levels of wild-type FX and mutant FX ($P = 0.1007$, unpaired Student's t test, performed in triplicate).

Fig. 2



Factor X (FX) antigen levels as determined by enzyme-linked immunosorbent assay in supernatants of HEK293 cells transfected with plasmid encoding wild-type FX or mutant FX Gly366Ser. 'Mock' indicates supernatants of HEK293 cells transfected with empty vector (pCMV). There was no significant difference between antigen levels of wild-type FX and FX Gly366Ser ($P = 0.1007$, unpaired Student's t test, performed in triplicate).

On western blots, both wild-type FX and FX Gly366-Ser present in culture media were detected as a single band under non-reducing conditions, and the molecular size was 59 kDa (Fig. 3).

Functional assays of FX Gly366Ser

The clotting activities of wild-type FX and FX Gly366-Ser in culture media were evaluated with three types of activation procedures; extrinsic pathway (PT), intrinsic pathway (aPTT), and RVV activation (RVV time). The clotting activity of FX Gly366Ser was expressed as a percentage of the wild-type FX clotting activity. FX Gly366Ser clotting activities were only 0.04 ± 0.00%, 1.05 ± 0.04%, and 0.75 ± 0.09% of those of wild-type FX in the PT, aPTT, and RVV time assays, respectively (Table 1).

We next investigated the catalytic activity of recombinant FX Gly366Ser directly with a low molecular weight chromogenic substrate for FXa. After activation of wild-type FX or FX Gly366Ser by RVV in culture media, fluorogenic substrates of FXa were added, and the cleaved fluorescent peptides were detected quantitatively. The wild-type FXa catalyzed the substrate in a time-dependent manner; however, FXa Gly366Ser had no catalytic activity (Fig. 4).

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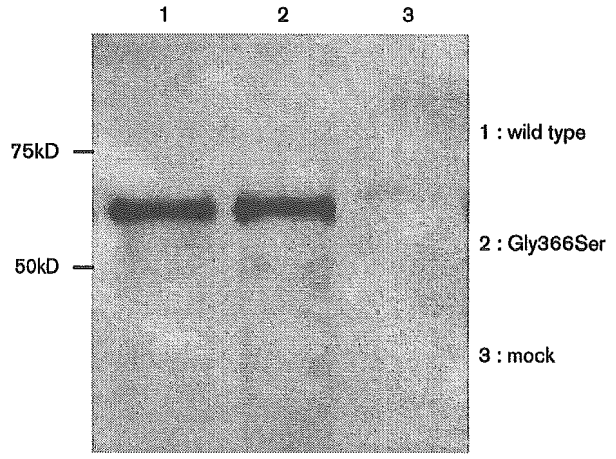
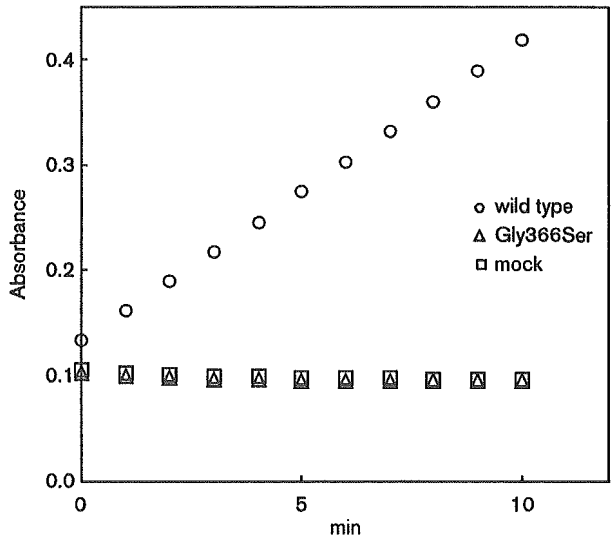
Fig. 3



Western blot analysis of supernatants of HEK293 cells transfected with plasmid encoding wild-type factor X (FX) or mutant FX Gly366Ser. 'Mock' indicates supernatants of HEK293 cells transfected with empty vector (pCMV4). Under non-reducing conditions, wild-type FX and FX Gly366Ser were each detected as a single band (59 kDa).

Fig. 4



Activity of recombinant factor X (FX) as measured with the chromogenic substrate of activated factor X (FXa). Supernatants containing wild-type FX or FX Gly366Ser were reacted with Russell's viper venom-FX activator and FXa fluorogenic substrate, and the absorbance at 405 nm was measured. Wild-type FX catalyzed the substrate in a time-dependent manner, whereas Gly366Ser had no catalytic activity. Mock, media from cells transfected with empty vector.

We then performed a thrombin generation assay to confirm the enzymatic activity of FX Gly366Ser. Wild-type FX or FX Gly366Ser in culture media were mixed with FX-depleted plasma, activated with thromboplastin, and added to thrombin fluorogenic substrates. The cleaved fluorogenic peptides were then detected as markers of thrombin generation. The thrombin-generating activity of FX Gly366Ser was undetectable (Fig. 5).

Molecular modeling

The molecular modeling of FXa suggested that replacement of Gly366 with Ser in the catalytic domain could affect the interaction between the surrounding residues. The predicted distance between the C_β atom of Ser366 and the O atom of Ala365 is so narrowed (1.9 Å) as to ensure the collision of the two atoms (Fig. 6). This collision can be considered inevitable because the side chain of the C_β atom of Ser366 cannot rotate, and thus the Gly366Ser mutation would cause a conformational change of the domain that leads to loss of catalytic activity.

Discussion

Genetic analysis of the present case of congenital FX deficiency revealed the homozygous missense mutation in exon 8 of the F10 gene that results in substitution of serine for glycine at position 366. Two heterozygous patients have been previously described for this mutation [14,19], named FX Nagoya 2. In one report [14], a Japanese woman was a heterozygote and had FX activity and antigen levels of 34 and 80%, respectively. The present study is the first to describe an expression study of this mutation *in vitro*. The proband showed a severe hemorrhagic tendency and markedly prolonged PT and aPTT. The patient's FX antigen level was 67% of the normal value, and FX activity was only 1 U/dl, suggesting that this variant is not functional. RFLP analysis revealed that the proband's father, mother, and sister were heterozygous for the

Table 1 The clotting assay of recombinant wild-type factor X (FX) and FX Gly366Ser in culture media

	Prothrombin time (s)	Activated partial thromboplastin time (s)	Russell's viper venom time (s)
rFX wild-type	37.0 ± 0.3	66.3 ± 0.3	166.1 ± 0.6
rFX Gly366Ser	284.4 ± 1.5	185.4 ± 1.0	537.9 ± 9.6
(% wild type)	(0.04 ± 0.00)	(1.05 ± 0.04)	(0.75 ± 0.09)

Data presented as mean ± standard deviation, and all assays were performed in triplicate.

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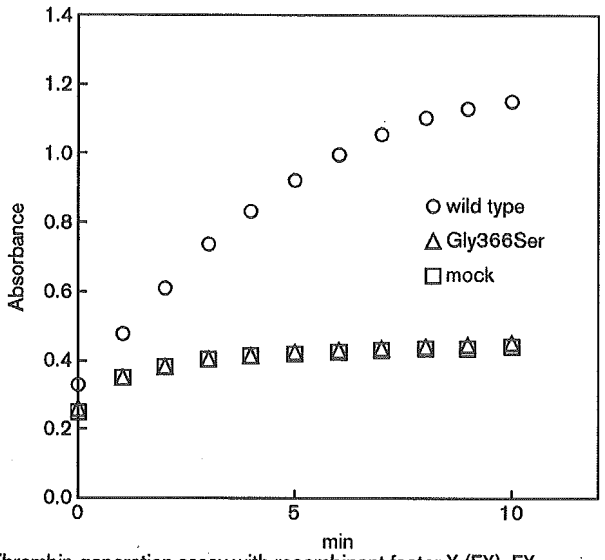
Fig. 5



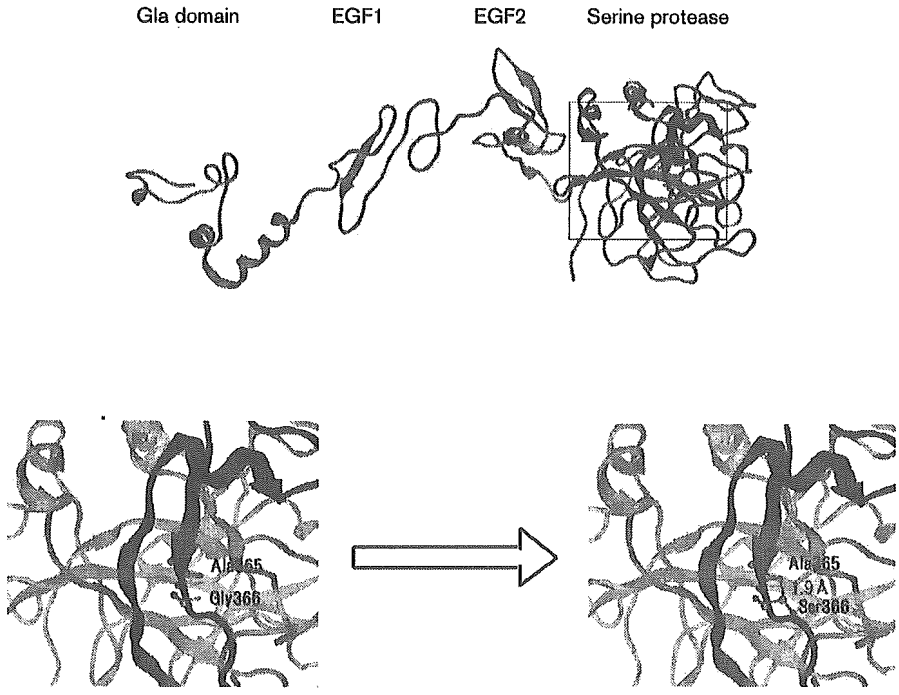
Thrombin generation assay with recombinant factor X (FX). FX-depleted plasma was diluted 1 : 25 in supernatant containing 2 nmol/l recombinant FX (mock, wild type, or Gly366Ser) and incubated for 2 min at 37°C with thromboplastin. Then, 300 μmol/l thrombin fluorogenic substrates was added, and the fluorescence at 405 nm was monitored. The thrombin-generating activity of FX Gly366Ser was undetectable.

mutation, and the results of their phenotypic coagulation tests were consistent with the results of the genetic analyses.

In vitro expression study of FX Gly366Ser conferred significant findings to understand the proband's phenotype. Western blot analysis showed that both of the wild-type and Gly366Ser transfectants secreted FX antigen of the same molecular weight into culture media, indicating that this mutation would not have a major effect on the molecular size of the protein. The quantitative analysis of secreted FX antigen by ELISA showed a statistically equivalent level between Gly366-Ser and wild type ($P = 0.1007$), although there was a tendency of slightly lower level of Gly366Ser secretion. This result of ELISA might be related to the proband's slightly lower plasma FX antigen level (67%). Because our *in vitro* studies utilized a transient expression system, however, the meaning and significance of the FX antigen expression level should not be overestimated.

We performed functional characterization of FX Gly366Ser with the recombinant protein. The activities of the mutant in PT, aPTT, and RVV time assays were only $0.04 \pm 0.00\%$, $1.05 \pm 0.04\%$, and $0.75 \pm 0.09\%$ of

Fig. 6



Molecular modeling of activated factor X (FXa). Upper: three-dimensional structure of FXa. The structure of the Gla domain was predicted with 1DAN (activated factor VII with human recombinant soluble tissue factor) from a homology search of the Protein Data Bank. The structure of the Gla domain was linked to 1XKA (three-dimensional structure of FXa from the EGF domain to the serine protease domain), described previously [11], and the entire structure of FXa was then developed. Lower: close-up of the FXa Gly366Ser catalytic domain. Gly366Ser substitution results in an inevitable collision (1.9 Å) between the O atom of Ala365 and the C_β atom of Ser366.

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those of wild-type FX. Moreover, both the chromogenic FXa substrate and thrombin generation assays could not show any FX activity in the Gly366Ser mutant.

Gly366 is located in the serine protease subdomain 2 of the FX catalytic domain. When FX is activated, cleavage of the Arg194–Ile195 bond initiates the reaction and then the N-terminal residue of Ile195 forms an ion-pair with Asp378, which generates the oxyanion hole and exposure of the catalytic His236–Asp282–Ser379 triad [13]. Several mutations associated with CRM-positive FX deficiency are reported near Gly366. Among them, FX Roma (Thr317Met) [23], FX Marseille (Ser334Pro) [24], FX Friuli (Pro343Ser) [21], and FX Gly381Asp [18] have been well characterized. In these deficiencies, it is interesting that mutation to Ser occurs in both FX Friuli and FX Gly366Ser. In FX Friuli, a novel hydrogen bond between mutated Ser343 and Thr318 is thought to be formed, leading to perturbation of the structure of the substrate-binding pocket [21]. Molecular modeling of the FXa Gly366Ser by MOE predicted that the mutation would disrupt the native conformational structure of the catalytic domain (Fig. 6). Moreover, the Cys364–Ala365–Gly366 sequence in the catalytic domain is conserved in mammalian serine proteases, suggesting that these positions in the catalytic domain are important for the structure of mammalian serine proteases [25]. Taken together, these findings indicate that the Gly366Ser change would cause partial disruption of the catalytic domain, possibly through impaired protein folding, leading to catalytic dysfunction.

The severity of bleeding tendency in homozygous patients with CRM-positive FX deficiency was controversial. Most of them were affected mildly or moderately; and even in patients with severe hemorrhagic symptoms with the same homozygous mutation, the onset was markedly different [18]. Our case exhibited relatively severe hemorrhagic symptoms compared with other homozygous patients. This might be attributed to the extremely low activity of FXaGly366Ser suggested by RVV activation assay, FXa chromogenic assay and thrombin generation assay. Moreover, since FX forms a complex with factor V in the presence of Ca^{2+} and phospholipids, the clinical bleeding feature could vary according to the effect of FX mutation on other components.

In conclusion, we presented here a new case of homozygous FX deficiency and data concerning the relations between structural and functional aspects of CRM-positive FX deficiency due to a Gly366Ser substitution. Our findings suggest that position 366 plays a critical role in wild-type FX catalytic function and that substitution with Ser results in loss of FX catalytic activity in the coagulation pathway.

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Original Article

Increased Risk of Obesity Resulting from the Interaction between High Energy Intake and the Trp64Arg Polymorphism of the β3-adrenergic Receptor Gene in Healthy Japanese Men

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BACKGROUND: Few studies have investigated the interaction between the Trp64Arg polymorphism of the β3-adrenergic receptor gene (*ADRB3*) and environmental factors. This study aimed to investigate whether energy intake affects the relationship between this polymorphism and obesity.
METHODS: Healthy Japanese men (n=295; age 46.1±11.5 years (mean ±standard deviation); waist circumference 83.9±9.3 cm; body mass index (BMI) 23.3±3.3 kg/m²) recruited in a Japanese chemical industry firm were eligible for analysis. Daily energy intake, protein, fat, and carbohydrate (PFC) ratio and daily physical activity were assessed by self-reported questionnaires. Genotyping for the polymorphism was performed with written informed consent.
RESULTS: When the subjects were classified into two groups according to presence of the polymorphism, the groups were not significantly different in waist circumference or BMI. Quartile classification of energy intake, however, demonstrated a significantly larger ratio of obese subjects to non-obese subjects in the group with the polymorphism in the highest 4th quartile alone. Multiple logistic regression analysis also revealed that the presence of the polymorphism increased the risk of obesity significantly in the 4th quartile alone (adjusted odds ratio=3.37, 95% confidence interval=1.12-10.2).
CONCLUSION: Presence of the polymorphism alone does not significantly increase the risk of obesity. However, high energy intake interacts with the polymorphism and leads to a significant increase in risk of obesity. The Trp64Arg polymorphism of *ADRB3* warrants consideration, along with other polymorphisms involved in the development of obesity, for tailor-made prevention of obesity.
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Key words: Obesity; Receptors, Adrenergic, beta-3; Polymorphism, Single Nucleotide; Energy Intake; Questionnaires.

Obesity is attributable to the effects of genetic and environmental factors, and it affects human health.[1] Accumulation of visceral fat, in particular, may play an important role in the pathogenesis of glucose intolerance, hyperlipidemia, and hypertension, and the accumulation of visceral fat is closely linked with atherosclerosis.[2-4]

Many genes are suspected to be associated with obesity,[5] and recently the β3-adrenergic receptor gene (*ADRB3*) has become the center of attention. In humans *ADRB3* is predominantly expressed in visceral adipose tissue.[6] It contains seven transmembrane domains and is coupled with G proteins.[7] Stimulation of the *ADRB3* by β-adrenergic agonists has been demonstrated to activate adenylate cyclase, which increases the intracellular cyclic AMP level and leads to acceleration of lipid metabolism and thermogenesis.[7]

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In 1995, Pima Indians, an ethnic group with a very high prevalence of obesity and non-insulin-dependent diabetes mellitus (NIDDM), were found to have a high frequency of the Trp64Arg polymorphism due to the replacement of thymidine (T) by cytosine (C) at nucleotide position 190 in *ADRB3*, and those with the polymorphism were found to have early onset of NIDDM and a low resting metabolic rate.[8] Subjects with the polymorphism in France have been found to have increased capacity to gain weight,[9] and the polymorphism is associated with abdominal obesity in Finns and may cause insulin resistance and earlier onset of NIDDM in that population.[10]

The polymorphism has been detected at an allelic frequency of about 0.20 in Japanese, which is lower than in Pima Indians (0.31) and higher than in Mexican-Americans (0.13), Blacks (0.12) and Whites (0.08) in the United States.[8,11] The resting metabolic rate of obese Japanese women with the polymorphism is about 200 kcal/day lower than of those without the polymorphism, and women with the polymorphism have difficulty in losing weight.[12] Moreover, the polymorphism has been found to be associated with visceral-fat obesity and insulin resistance syndrome, and it may be possible to use the polymorphism as a genetic marker for these syndromes.[13] Other studies have also shown that the polymorphism is associated with obesity[14,15] and insulin resistance.[15]

A meta-analysis of the association between the polymorphism of *ADRB3* and body mass index (BMI) in 31 studies demonstrated that subjects with the polymorphism had a BMI that averaged 0.30 (95% confidence interval [CI]=0.13-0.47) higher than those without the polymorphism.[16]

Many studies have demonstrated that the polymorphism of *ADRB3* is associated with obesity, but few studies have shown that it is possible to prevent or treat obesity and its complications in persons with the polymorphism. Therefore, to achieve tailor-made prevention or treatment based on genotype, including *ADRB3*, we investigated the interaction between energy intake and the polymorphism in *ADRB3* in healthy Japanese men.

METHODS

This study was approved by the Institutional Review Board (IRB) of Keio University School of Medicine, and was carried out in concordance with the principles of the Declaration of Helsinki. We explained this study by means of a written document to healthy Japanese men and women working for a company in Kanagawa prefecture and obtained written informed consent from 363 workers (male: 326, female: 37) to participate in the study, which included genotyping. The following surveys and genotyping for the polymorphism of ADRB3 were conducted in October and November 2003, and we obtained complete replies to the food frequency questionnaire (FFQ) from 337 (male: 295, female: 32) of the 363 workers, who were genotyped for the polymorphism of *ADRB3*. Allowing for the fact that the Japan Society for the Study of Obesity established different criteria for 'obesity disease' in men and women,[17] and the report that men might have a higher risk of coronary heart disease than women due to the difference in body fat distribution,[18] we performed the analysis focused on the 295 male workers.

Their mean age, waist circumference, and BMI (mean ± standard deviation) were 46.1±11.5 years, 83.9±9.3 cm, and 23.3±3.3 kg/m^2, respectively. Height, weight, waist circumference, systolic and diastolic blood pressure, fasting plasma glucose level, and serum lipid levels were measured in all the subjects. A Body mass index (BMI) was calculated as weight (kg)/height^2 (m^2). Obesity was defined as BMI 25+ kg/m^2 or waist circumference 85+ cm, based on the criteria for 'obesity' of the Japan Society for the Study of Obesity.[17] In addition, information on the age, physical activity, smoking status, energy intake, and the protein, fat, and carbohydrate (PFC) ratio of all the subjects was obtained by means of a self-report questionnaire. The semi-quantitative food frequency questionnaire (FFQ), which was evaluated by comparing the FFQ with the 7-day dietary records of 66 subjects by Takahasi K, et al. [19], was used to calculate energy intake and PFC ratio. Takahasi K, et al. reported that the correlation coefficients between the FFQ and the 7-day dietary records for energy, protein, fat, and carbohydrate intake were 0.47 ($p<0.001$), 0.42 ($p<0.001$), 0.39 ($p<0.01$), and 0.49 ($p<0.001$), respectively.[19]

Daily physical activity was calculated based on hours per day subjects engaged in each activity. The intensity of each activity was quantified by assigning metabolic equivalents (METs) to each activity.[20] METs represent the ratio of energy expended during each specific activity to resting metabolic rate. The time spent in each activity was multiplied by the specific number of METs assigned to each activity, and the products were summed. The sum was defined as the daily physical activity (METs · minutes) of each subject.

The FFQ used in this study asked about the consumption of food items in 29 food groups during the previous one or two months. Basically, the subjects were asked to describe the quantities and frequencies of consumption of food items during breakfast, lunch, and dinner. Three portion size categories (small, medium, and large) were used to evaluate the quantities of consumption of food items. "Small" is half the size of "medium", and "large" is one and a half times the size of "medium". When the frequency of consumption of a food is less than once or twice a month, the subject is instructed to answer "never". The FFQ illustrated the food items of each food group in quantity equal to "medium", so as to evaluate the quantities of consumption correctly. When the frequency of consumption of a food was low, the subjects were asked to answer the quantity of consumed at one time and the frequency of consumption per week. Foods of this kind included sea vegetables, fruit, potatoes, butter, pickles, etc. The subjects were also asked to describe the quantity of consumption at one time and the frequency of consumption per week of alcoholic and non-alcoholic beverages. When the quantity of a unit of a food was almost identical among subjects, the subjects were asked to state only the frequency of consumption per week

as the number of consumed units. Foods of this kind included rice, milk, egg, etc. Daily nutrient intake was calculated by multiplying the frequency of consumption of each food by the nutrient content of the portion size and summing the products for all foods items. The energy intake and PFC ratio of each subject were determined in this manner.

A peripheral blood specimen was collected from each subject, and genotyping for the polymorphism of *ADRB3* was performed by polymerase chain reaction (PCR) and single nucleotide primer extension (SNuPE) assay with Ampdirect (Shimadzu Corporation, Kyoto, Japan), which eliminates the DNA extraction process and amplifies the genomic DNA directly from the whole blood.

A 367-bp fragment of the gene encompassing the polymorphism site was amplified by PCR using 5'-primer (5'TTC-CTTCTTTCCCTACCGCCC)[8] and 3'-primer (5'GCAGCCAGTG-GCGCCCAACGG).[8] The PCR reactions were carried out in the PCR mixture containing 10 μL of Ampdirect-G/C (Shimadzu Corporation, Kyoto, Japan), 10 μL of Ampdirect Addition (Shimadzu Corporation, Kyoto, Japan), 4 μL of dNTP mixture (TaKaRa Bio Inc., Shiga, Japan), 0.5 μM of 5'-primer,[8] 0.5 μM of 3'-primer,[8] 0.25 μL of *Taq* DNA Polymerase (Promega Corporation, Wisconsin, USA), up to 50 μL of distilled water, and 1.0 μL of whole blood. The PCR consisted of preheating at 80℃ for 15 minutes; denaturation at 94℃ for 4.5 minutes; 40 cycles of denaturation at 94℃ for 30 seconds, annealing at 60℃ for 1 minute, and extension at 72℃ for 1 minute; a final extension at 72℃ for 7 minutes. The SNuPE assay was then performed. The SNuPE assay was based on the incorporation of a single fluorescent-labeled ddNTP, which was correctly paired with the template DNA and caused chain-termination, to the 3' terminus of a primer annealed next to the polymorphic site.[21] The SNuPE primer (5'ATGGTCTGGAGTCTCGGAGTCC) was designed so that the primer ends immediately before the polymorphic site. The SNuPE reactions were carried out in the mixture; containing 2 μL of PCR product, 4 μL of SNuPE premix (ddATP, ddCTP, ddGTP, ddTTP, DNA polymerase), and 2 pM of SNuPE primer. The SNuPE consisted of 25 cycles of denaturation at 94℃ for 10 seconds, annealing at 53℃ for 5 seconds, and extension at 60℃ for 10 seconds. The products were analyzed with ABI 7700 (Applied Biosystems, California, USA).

For statistical analysis, Student's t-test was used to compare normally distributed variables between groups. Variables that were not normally distributed were log transformed. When the log transformations of the variables were effective, Student's t-test was used. When ineffective, the Wilcoxon rank-sum test was used. The χ^2 test was used to compare categorical variables. Multiple logistic regression analysis was performed on obesity defined as waist circumference 85+ cm,[17] with presence of the polymorphism of *ADRB3*, age, smoking, and physical activity as variables.

Differences were assessed by two-sided tests, with an alpha level of 0.05. All statistical analyses were performed with Statistical Package for the Social Sciences® (SPSS) for Windows, version 11 software (SPSS Inc., Illinois, USA).

RESULTS

Genotyping for the Trp64Arg polymorphism of *ADRB3* in the 295 healthy Japanese male subjects showed that 198 were homozygous for the wild-type allele (Trp/Trp), 94 were heterozygous for the variant allele (Arg/Trp), and 3 were homozygous for the variant allele (Arg/Arg) (allelic frequency=0.17). These results were in Hardy-Weinberg equilibrium and did not conflict with the results previously reported in another Japanese population (p=0.240).[13]

The main characteristics of the subjects are shown in Table 1. There were no significant differences between the subjects with and without the polymorphism with regard to age, height, weight, BMI, waist circumference, systolic or diastolic blood pressure, fasting plasma glucose, triglyceride, or HDL cholesterol levels, PFC ratio (fat, carbohydrate), energy intake, physical activity, or smoker/non-smoker ratio. The total cholesterol values and PFC ratio (protein) of the subjects with the polymorphism were significantly lower than the subjects without the polymorphism (p=0.016 and 0.026, respectively).

The subjects were classified into quartiles according to energy intake based on their replies to the FFQ. The mean waist circumference and BMI values in each quartile are shown in Table 2. There were no significant differences in waist circumference between the subjects in the 1st quartile and the subjects in the other quartiles. There was a significant difference in BMI between the subjects in the 1st quartile and in the 4th quartile (p=0.035), but not between the subjects in the 1st quartile and the subjects in the 2nd or the 3rd quartile. In addition, the trend test showed progressive increases in waist circumference and BMI in the quartiles that paralleled increased levels of energy intake (p=0.043 and 0.024, respectively).

We then divided each quartile into two groups according to presence of the polymorphism and calculated the ratio of the obese to the non-obese subjects in each group (Tables 3 and 4). When subjects with waist circumference 85+ cm[17] were defined as obese (Table 3) in the 2nd quartile, the ratio of the group with the polymorphism was significantly lower than that of the group without the polymorphism (odds ratio [OR]=0.278, 95% CI=0.10-0.78), and in the 4th quartile, the ratio of the group with the polymorphism was significantly higher than that of the group without the polymorphism (OR=3.490, 95% CI=1.24-9.85).

When subjects with BMI 25+ kg/m^2 [17] were defined as obese, no significant difference between the ratio of obese to non-obese subjects in the group with the polymorphism and the group without the polymorphism was seen in any of the quartile (Table 4).

The results of the multiple logistic regression analysis on obesity (defined as waist circumference 85+ cm [17]) in the 4th quartile (total: 74, without polymorphism: 48, with polymorphism: 26) are shown in Table 5, in which the presence of *ADRB3* polymor-

phism, smoking, age and physical activity were independent variables. Only the presence of the polymorphism of *ADRB3* was associated with increased risk of obesity (adjusted OR=3.37, 95% CI=1.12-10.16). The results of the multiple logistic regression analyses on obesity defined as waist circumference 85+ cm [17] and the same variables demonstrated that presence of the polymorphism of *ADRB3* was not associated with increased risk of obesity in the 1st, 2nd, and 3rd quartile.

Table 1. Clinical characteristics of the subjects, according to the ADRB3 polymorphism.

		ADRB3 genotype		
	All subjects	without polymorphism (Trp/Trp)	with polymorphism (Trp/Arg or Arg/Arg)	p value
n	295	198	97	
Age (years)*	46.1 ± 11.5	46.5 ± 11.3	45.3 ± 11.9	0.300
Height (cm)	168.6 ± 6.26	168.3 ± 6.19	169.1 ± 6.41	0.289
Weight (kg)	66.3 ± 10.4	66.3 ± 10.3	66.3 ± 10.6	0.999
Body mass index (kg/m^2)	23.3 ± 3.28	23.4 ± 3.20	23.2 ± 3.47	0.633
Waist circumference (cm)	83.9 ± 9.30	84.1 ± 9.19	83.5 ± 9.57	0.636
Systolic blood pressure (mmHg)	134.4 ± 18.0	134.0 ± 18.5	135.2 ± 16.8	0.582
Diastolic blood pressure (mmHg)	81.7 ± 12.7	81.4 ± 13.1	82.3 ± 11.8	0.576
Fasting plasma glucose (mg/dL)†	100.2 ± 34.0	99.2 ± 31.8	102.5 ± 38.5	0.481
Total cholesterol (mg/dL)	206.9 ± 37.0	210.6 ± 35.8	199.3 ± 38.6	0.016
Triglyceride (mg/dL)†	131.9 ± 83.1	134.2 ± 86.1	127.1 ± 76.6	0.664
HDL cholesterol (mg/dL)	55.5 ± 14.0	55.5 ± 12.8	55.4 ± 16.4	0.976
Protein, fat, and carbohydrate ratio (%)				
Protein (%)	13.9 ± 2.39	14.1 ± 2.48	13.5 ± 2.14	0.026
Fat (%)	27.6 ± 5.36	27.7 ± 5.48	27.6 ± 5.13	0.890
Carbohydrate (%)	58.5 ± 6.88	58.2 ± 7.06	59.0 ± 6.51	0.379
Energy intake (kcal/day)	1855.3 ± 485.0	1857.3 ± 509.2	1851.3 ± 433.9	0.920
Physical activity (METs · minites)	2457.0 ± 710.9	2479.2 ± 763.8	2409.6 ± 583.6	0.442
Smoker/Non-smoker‡	175/119	112/85	63/34	0.184

Values are means ± standard deviation. Subjects with polymorphism were compared with subjects without polymorphism. Basically, we used Student's *t*-test. As to variables with mark, we analyzed as follow.

* : Wilcoxon rank-sum test.

† : Variable was log transformed, and the Student's *t*-test was used. Data is pre-log transformed.

‡ : χ^2 test.

Table 2. The mean waist circumference and body mass index values in each quartile, according to the energy intake.

	Energy intake				
	1st quartile (-1515 kcal/day)	2nd quartile (1516-1795 kcal/day)	3rd quartile (1796-2131 kcal/day)	4th quartile (2132+ kcal/day)	trend test
n	73	74	74	74	
Waist circumference (cm)	82.6±9.53	82.9±8.33	84.9±9.02	85.2±10.1	0.043
Body mass index (kg/m^2)	22.6±3.28	23.2±3.24	23.6±3.33	23.8±3.24*	0.024

Values are means ± standard deviation. Subjects in the 2nd, 3rd, and 4th quartile were compared with subjects in the 1st quartile. We used Student's *t*-test and trend test.

* : $P<0.05$ for Student's *t*-test.

Table 3. The ratio of the obese (waist circumference 85+ cm) to the non-obese (waist circumference less than 85 cm) by the ADRB3 polymorphism.

	ADRB3 genotype		
	without polymorphism (Trp/Trp)	with polymorphism (Trp/Arg or Arg/Arg)	both
1st quartile			
n (the obese/the non-obese)	24/26	8/15	32/41
the ratio of the obese to the non-obese	0.92	0.53	0.78
2nd quartile			
n (the obese/the non-obese)	24/21	7/22*	31/43
the ratio of the obese to the non-obese	1.14	0.32	0.72
3rd quartile			
n (the obese/the non-obese)	31/23	9/10	40/33
the ratio of the obese to the non-obese	1.35	0.90	1.21
4th quartile			
n (the obese/the non-obese)	21/27	19/7*	40/34
the ratio of the obese to the non-obese	0.78	2.71	1.18
all subjects			
n (the obese/the non-obese)	100/97	43/54	143/151
the ratio of the obese to the non-obese	1.03	0.80	0.95

The ratio of the obese to the non-obese in the group with the polymorphism was compared with that in the group without the polymorphism in each quartile. We used χ^2 test.
* : $P<0.05$.

Table 4. The ratio of the obese (body mass index 25+ kg/m^2) to the non-obese (body mass index less than 25 kg/m^2) by the ADRB3 polymorphism.

	ADRB3 genotype		
	without polymorphism (Trp/Trp)	with polymorphism (Trp/Arg or Arg/Arg)	both
1st quartile			
n (the obese/the non-obese)	12/38	5/18	17/56
the ratio of the obese to the non-obese	0.32	0.28	0.30
2nd quartile			
n (the obese/the non-obese)	14/31	6/23	20/54
the ratio of the obese to the non-obese	0.45	0.26	0.37
3rd quartile			
n (the obese/the non-obese)	17/38	6/13	23/51
the ratio of the obese to the non-obese	0.45	0.46	0.45
4th quartile			
n (the obese/the non-obese)	16/32	11/15	27/47
the ratio of the obese to the non-obese	0.50	0.73	0.57
all subjects			
n (the obese/the non-obese)	59/139	28/69	87/208
the ratio of the obese to the non-obese	0.42	0.41	0.42

The ratio of the obese to the non-obese in the group with the polymorphism was compared with that in the group without the polymorphism in each quartile. We used χ^2 test.