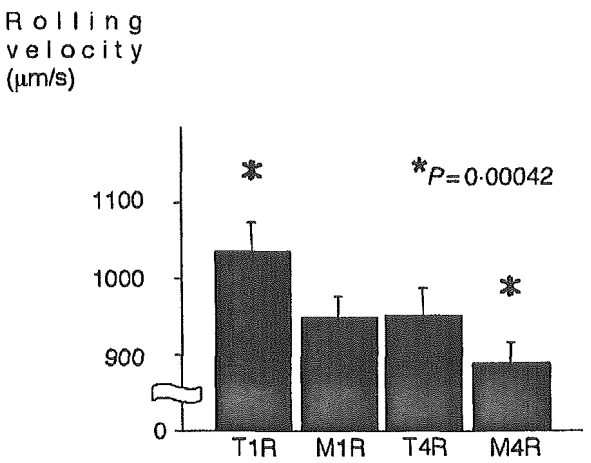

Fig 2. Interaction between immobilized VWF and CHO cells expressing GPIbαβIX complex was analysed under flow conditions (114/s). Each cell sequenced for the GPIbα polymorphism was applied to the flow chamber system with VWF-immobilized glass. Error bars indicate SDs. Results are mean value ± SD (μm/s) of four independent experiments.

($P = 0{\cdot}0574$). In GPIbβIX-expressing CHO cells, the rolling cell number was 5·2 ± 0·5. Under this flow condition, the cell rolling velocity was inhibited by GUR83–35; i.e. the velocities became faster and with soluble-VWF addition (Table III). Thus, the specificity of interaction between immobilized VWF and GPIbα-expressing CHO cells was confirmed under a shear condition of 114/s.

Discussion

The present study demonstrated that the ^{145}Met and 4R polymorphisms of GPIbα facilitate interaction with immobilized VWF under flow conditions, which is a highly adaptive physiologic response. To date, molecular mechanisms for the functional differences of the ^{145}Thr/Met and/or VNTR polymorphisms in GPIbα have not been fully understood whereas numerous epidemiologic data have been reported. We report the first experimental data obtained using recombinant proteins to determine the functional differences of ^{145}Thr/Met and VNTR GPIbα polymorphisms. Previously, ^{145}Met and/or 3R/4R polymorphisms were demonstrated to be associated with an increased risk for arterial thrombosis, such as coronary artery disease or stroke (Simmonds *et al*, 2001; Yamada *et al*, 2002). Because the ^{145}Thr/Met and VNTR polymorphisms are in linkage disequilibrium, focus on either ^{145}Met or 3R/4R allele was likely to be sufficient to examine the association between GPIbα polymorphisms and arterial thrombosis in the epidemiological study. We reported that the frequency of either ^{145}Met- or 4R-allele among patients with coronary artery disease was higher than that among control subjects and that the genotypes with the ^{145}Met-allele were more frequently found in the patients with cerebrovascular disease than in control subjects (Murata *et al*, 1997; Sonoda *et al*, 2000). A large case-cohort study (Afshar-Kharghan *et al*, 2004) showed the relationship of the 2R/2R genotype with a lower risk of coronary heart disease in African-Americans. However, conflicting data have also been published (Hato *et al*, 1997; Simmonds *et al*, 2001). In experimental studies of these polymorphisms, Boncler *et al* (2002) demonstrated that the inhibitory effect of the VWF antagonist on ristocetin-induced agglutination was higher in ^{145}Met/3R-positive platelets than in ^{145}Met/3R-negative platelets. Ulrichts *et al* (2003) reported that platelets with ^{145}Thr or recombinant GPIbα (residues 1–289) with ^{145}Thr had a higher VWF binding affinity than ^{145}Met. These findings are not consistent with our results although the experimental conditions of the present study differed from those of previous studies: use of ristocetin or botrocetin or use of an assay system. Other studies have also used various methods with inconsistent results (Mazzucato *et al*, 1996; Li *et al*, 2000; Jilma-Stohlawetz *et al*, 2003). These reports suggest that the functional analyses of GPIbα polymorphisms seem to be easily affected by several factors in relation to platelet activation or experimental conditions. Therefore, in this study, recombinant GPIbα and purified human VWF were examined under two experimental conditions to focus on the relationship between GPIbα polymorphisms and interactions with VWF. The first study, using soluble GPIbα lacking the VNTR polymorphism site, did not show the effect of the ^{145}Thr/Met polymorphism on the major conformation because the immunoreactivity to anti-GPIbα antibodies that recognize confirmation-specific epitopes were not significantly different between these polymorphisms. The ^{145}Thr/Met polymorphism did not affect the ^{125}I-VWF binding in the presence of ristocetin under static conditions. Although ristocetin provides a

Table III. Rolling velocity for GPIbα-expressing cells interacting with VWF under 114/s flow condition

	Soluble VWF (−)				Soluble VWF (+)	
	GUR83-35 (−)	*P*-value	GUR83-35 (+)	*P*-value	GUR83-35 (−)	*P*-value
T1R	1035·1 ± 40·5*		1291·1 ± 61·9	NS	1385·7 ± 82·5	NS
M1R	951·1 ± 26·4**	*0·00042	1486·8 ± 109·5		1165·4 ± 62·9	
T4R	952·5 ± 36·9**	**NS	1638·9 ± 384·6		1276·6 ± 86·5	
M4R	902·2 ± 30·8*		1521·5 ± 86·6		1293·5 ± 126·4	

Values for rolling velocity (μm/s) are mean ± SD. NS, not significant.

convenient method to investigate the VWF/GPIbα interaction *in vitro*, it is not a physiologic substance. Thus, the second study was designed with an alternative approach, an *in vitro* assay for VWF/GPIbα interaction under flow conditions. Cells expressing GPIbα were prepared as a GPIbαβIX complex because expression of a full-length GPIbα alone was unstable in the cell culture system (Lopez *et al*, 1992). Two types of cells with naturally occurring sequences (T1R and M4R) and two types of cells with artificial or extremely rare sequences (T4R and M1R) were used to determine which polymorphism was more closely related to the VWF/GPIbα interaction. We carefully measured the GPIbα expression level on each cell because these levels were reported to affect the VWF/GPIbα interaction under flow conditions (Nishiya *et al*, 2000). After using FACS to obtain cells expressing similar GPIbα levels, EIA assay was performed using GUR83–35 and GUR20–5. Because these two antibodies were shown not to be influenced by the ^{145}Thr/Met polymorphism (Table I), we used these antibodies in this assay. Perfusion analyses of the quantified cells indicated that M4R, which is a risk factor for arterial thrombosis, had a high ability to interact with VWF under a flow condition of 114/s, as compared with T1R. This flow condition of 114/s may correspond to wall shear rate for large veins *in vivo* (Bevan *et al*, 1995), where VWF-dependent platelet phenomena may not take place. Compared with platelets, however, CHO cells have 2·5- to threefold larger diameters, and the GPIbα-expressing CHO cells are approximately 20-fold higher in GPIbα density. The cell size and receptor density are likely to affect the sensitivity of cells to flow conditions. Also, we were unable to determine the order of effectiveness of the polymorphisms among the four sequences, ^{145}Thr, ^{145}Met, 1R, and 4R, in VWF/GPIbα interactions because T4R and M1R had a similar ability to interact with VWF. Although the synergistic effect of the ^{145}Thr/Met and VNTR polymorphisms on GPIbα function remains unclear, the present data are compatible with previous speculations (Lopez, 1994; Murata *et al*, 1997) that GPIbα with 4R is longer in size and thus places the VWF-binding global domain further away from the platelet plasma membrane. Thus, VWF would be more easily accessible to the binding site on the receptor under high shear conditions. Functional polymorphisms of GPIbα might be responsible for the increased prevalence of arterial thrombosis. Our observations might explain the molecular basis for the previous epidemiologic studies. Further studies to examine the interactions between GPIbα polymorphisms and other ligands are necessary. The present data support a potentially new therapeutic approach to arterial thrombosis by targeting specific GPIbα polymorphisms.

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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

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.

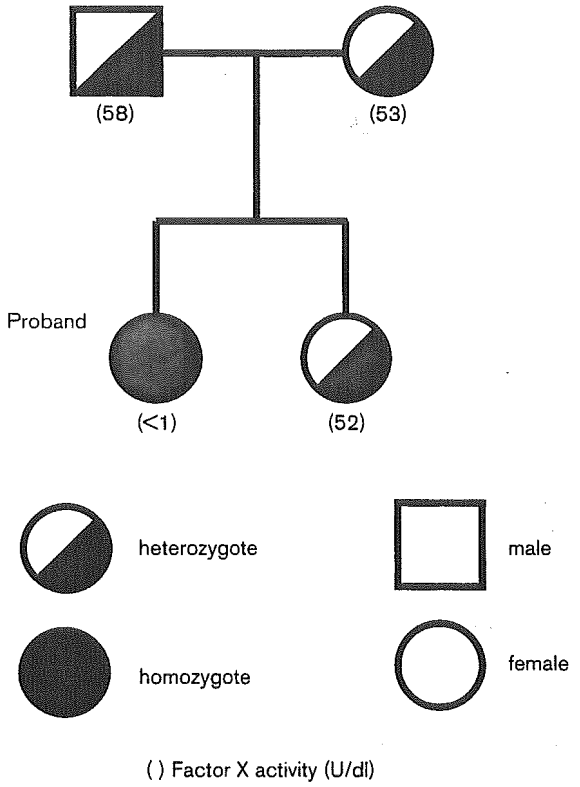
Here we report the molecular analysis and functional consequence of the CRM-positive FX deficiency (Gly366Ser) in a French girl of Sri Lankan origin.

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

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′-CTGTGCTAGCTACGACACC, and reverse primers 5′-GTCGTAGCTAGCACAGAAC and 5′-TGCAGGCATGCAAGCTTTGGCTG (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^{TM} 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-

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

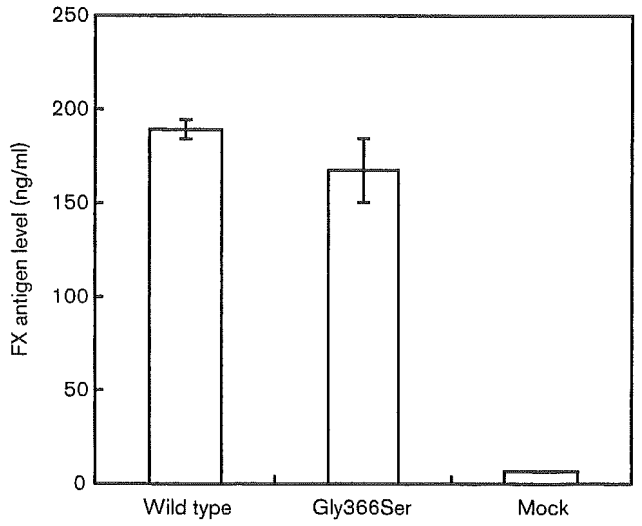
Characterization of the mutant F10 gene

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).

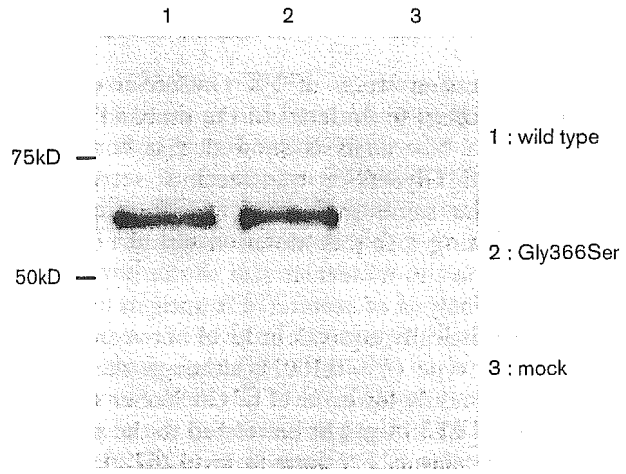
On western blots, both wild-type FX and FX Gly366-Ser present in culture media were detected as a single band under non-reducing conditions, and the molecular size was 59 kDa (Fig. 3).

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).

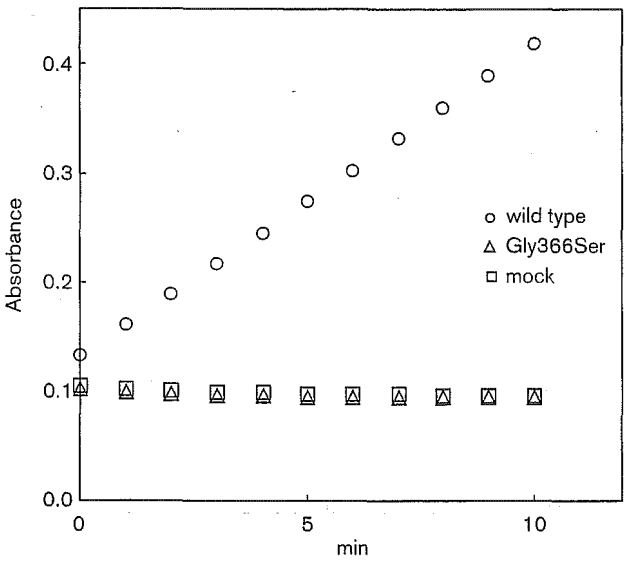
We next investigated the catalytic activity of recombinant FX Gly366Ser directly with a low molecular weight chromogenic substrate for FXa. After activation of wild-type FX or FX Gly366Ser by RVV in culture media, fluorogenic substrates of FXa were added, and the cleaved fluorescent peptides were detected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

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

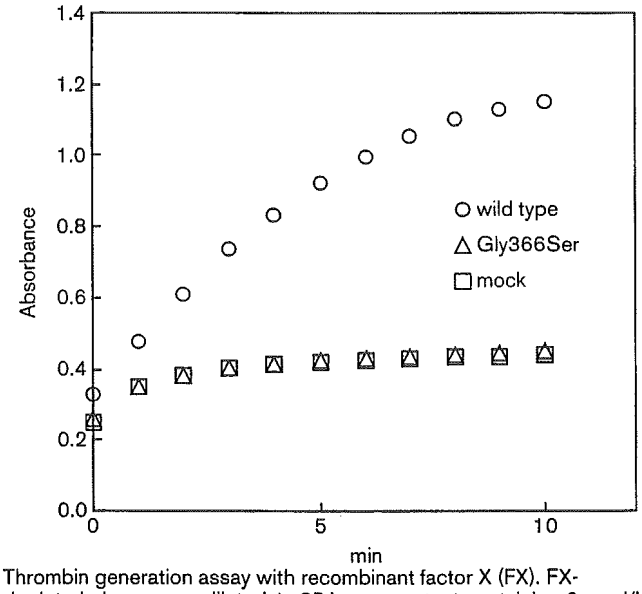
Genetic analysis of the present case of congenital FX deficiency revealed the homozygous missense mutation in exon 8 of the F10 gene that results in substitution of serine for glycine at position 366. Two heterozygous patients have been previously described for this mutation [14,19], named FX Nagoya 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.

Fig. 5



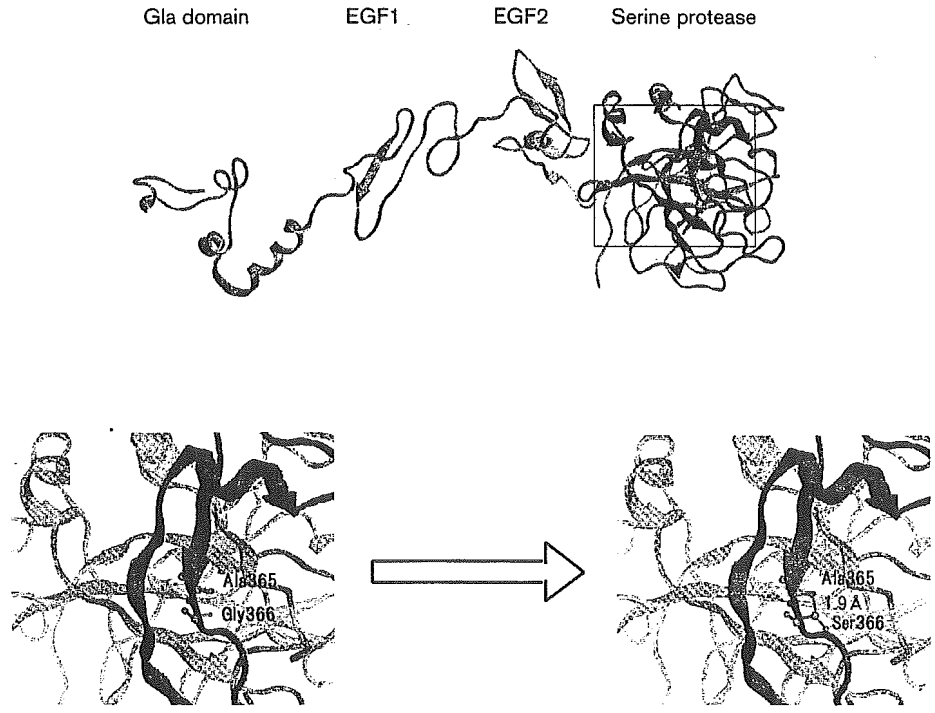
Thrombin generation assay with recombinant factor X (FX). FX-depleted plasma was diluted 1 : 25 in supernatant containing 2 nmol/l recombinant FX (mock, wild type, or Gly366Ser) and incubated for 2 min at 37°C with thromboplastin. Then, 300 μmol/l thrombin fluorogenic substrates was added, and the fluorescence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

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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Relation Between Development of Nephropathy and the p22phox C242T and Receptor for Advanced Glycation End Product G1704T Gene Polymorphisms in Type 2 Diabetic Patients

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OBJECTIVE — The development of diabetic nephropathy is considered to be associated with oxidative stress. NADPH oxidase and the receptor for advanced glycation end products (RAGE) have attracted attention as mechanisms of generating oxidative stress. We studied the relation between the genotypes of the NADPH p22phox C242T and RAGE G1704T polymorphisms and the development of diabetic nephropathy in type 2 diabetic patients.

RESEARCH DESIGN AND METHODS — Using a retrospective review of clinical data, we allocated 181 Japanese type 2 diabetic patients to one of two groups: patients without diabetic nephropathy (group N; $n = 108$) and patients developing diabetic nephropathy (group D; $n = 73$) for 10 years or more. The p22phox C242T and RAGE G1704T polymorphisms were examined by Taqman PCR methods.

RESULTS — The frequency of the p22phox CC genotype was significantly higher in group D than in group N (90 vs. 79%; $P = 0.0427$). The frequency of the RAGE GT + TT genotype was significantly higher in group D than in group N (26 vs. 13%; $P = 0.0313$). The frequency of the combination of p22phox CC and RAGE GT + TT genotypes was significantly higher in group D than in group N (22 vs. 8%; $P = 0.0057$). In multiple logistic regression analysis, systolic blood pressure, HbA_{1c}, triglycerides, and the combination of polymorphisms were shown to be independent variables.

CONCLUSIONS — These results suggest that assessment of the combination of NADPH p22phox C242T and RAGE G1704T polymorphisms may be useful in identifying the risk for developing diabetic nephropathy in type 2 diabetic patients.

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Diabetic nephropathy is the main cause of end-stage renal disease. Although poor glycemic control for a long period of time is a major factor in the development of diabetic nephropathy, hypertension, hyperlipidemia, and genetic factors are also associated with its development. The molecular mechanisms that underlie the pathogenesis of diabetic nephropathy remain unclear. Recent studies have highlighted the hyperglycemia-induced production of reactive oxygen species (ROS) as one of the mechanisms involved in the development of diabetic microangiopathy, via the following pathological pathways: activation of protein kinase C (PKC), formation of advanced glycation end products (AGEs), and activation of transcription factors such as nuclear factor-κB (NF-κB) (1). It has been proposed that genes associated with oxidative stress contribute to the risk of diabetic nephropathy (2–4). Recently, NADPH oxidase and the receptor for AGEs (RAGE) have received attention as mechanisms of generating oxidative stress. NADPH oxidase is a membrane-associated enzyme for superoxide production in vascular smooth muscle cells and endothelial cells (5). It has been reported that the NADPH oxidase p22phox C242T polymorphism is associated with vascular superoxide production in human blood vessels from coronary artery disease (CAD) patients (6). In addition, it has been suggested that AGEs might play a role in the pathogenesis of diabetic nephropathy and the progression to renal failure (7). The actions of AGEs are dependent on RAGE; thus, the AGE-RAGE system might participate in the development of diabetic microangiopathy (7). An intron polymorphism in the RAGE gene, G1704T, has been reported to be associated with microvascular dermatoses and antioxidant status (8,9). Moreover, a recent study has demonstrated that AGE-induced generation of ROS involves AGE-RAGE interaction through stimulation of membrane-bound NADPH oxidase (10). Therefore, in this study, we decided to investigate the association of genotypes of the p22phox C242T and RAGE G1704T

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Abbreviations: AGE, advanced glycation end product; CAD, coronary artery disease; NF-κB, nuclear factor-κB; PKC, protein kinase C; RAGE, receptor for AGEs; ROS, reactive oxygen species; UAI, urinary albumin index.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

polymorphisms and the combination of these polymorphisms with the susceptibility to developing nephropathy in Japanese type 2 diabetic patients.

RESEARCH DESIGN AND METHODS — The subjects were 691 unrelated Japanese patients diagnosed with type 2 diabetes according to World Health Organization criteria (11) who had been followed up on a regular basis at the outpatient clinic of Saitama Social Insurance Hospital since 1990. From among these subjects, we recruited all the patients who fulfilled the noted criteria (n = 181) and allocated them to one of two groups: group N, comprised of 108 patients without diabetic nephropathy, and group D, comprised of 73 patients with developing diabetic nephropathy. The stage of nephropathy was defined according to urinary excretion of albumin per gram of creatinine (urinary albumin index; UAI) and categorized as follows: normoalbuminuria, <20 mg/gCr, stage 0; microalbuminuria, 30–300 mg/gCr, stage 1; and macroalbuminuria, >300 mg/gCr, stage 2. Diabetic patients with UAI of 21–29 mg/gCr were assumed to be "borderline" and were therefore excluded from this study. Group N was defined using the following criteria: 1) >10 years after diagnosis of type 2 diabetes and 2) normoalbuminuria throughout the follow-up period. Group D was defined using the following criteria: 1) normoalbuminuria (stage 0) or microalbuminuria (stage 1) at the beginning of follow-up and 2) progression from normoalbuminuria (stage 0) to microalbuminuria (stage 1) or macroalbuminuria (stage 2), or progression from macroalbuminuria (stage 1) to macroalbuminuria (stage 2) during the follow-up period. To exclude patients with nondiabetic renal disease, patients with microscopic hematuria, clinical pyuria, or evidence of bacterial infection in a urine culture were excluded from this study. Diabetic retinopathy was diagnosed by independent diabetic ophthalmologists using standard fundus photographs at least every year; the stage of diabetic retinopathy was classified as simple, preproliferative, or proliferative retinopathy. HbA_{1c} levels, lipid profiles, blood pressure, and urinary excretion of albumin were measured at least every year. Clinical data, including onset age, BMI at the diagnosis of diabetes, disease duration since the diagnosis of type 2 diabetes, HbA_{1c} levels, blood pressure, lipid profiles, and the use of medication were obtained from the medical records of the patients. Written informed consent was obtained from each subject after full explanation of the purpose, nature, and risk of all procedures used. The protocol of this study was approved by the ethical review committee of the Saitama Social Insurance Hospital, Saitama, Japan.

Table 1—*Characteristics of patient groups*

	Group N	Group D	*P*
n	108	73	—
Sex (M/F)	52/56	39/34	NS
Onset age (years)	44.3 ± 7.2	45.2 ± 8.2	NS*
Disease duration (years)	18.4 ± 5.4	18.5 ± 5.5	NS*
BMI at onset (kg/m^2)	23.4 ± 3.7	24.0 ± 3.2	NS
HbA_{1c} (%)	7.3 ± 1.1	8.0 ± 1.2	0.0002
Total cholesterol (mmol/l)	5.11 ± 0.73	5.15 ± 0.78	NS
Triglyceride (mmol/l)	1.25 ± 0.73	1.53 ± 0.97	0.0137*
LDL cholesterol (mmol/l)	3.08 ± 0.71	3.06 ± 0.74	NS
Systolic blood pressure (mmHg)	134.0 ± 15.2	149.6 ± 18.0	<0.0001
Diastolic blood pressure (mmHg)	81.2 ± 8.8	86.2 ± 9.5	0.0004
Retinopathy (%)	43	67	0.0014
Medication (diet/OHA/insulin)	43/36/29	9/29/34	NS

Data are means ± SD unless otherwise indicated. *P values determined by Mann Whitney U test. OHA, oral hyperglycemic agents.

DNA preparation and detection of polymorphisms in p22phox and RAGE

Genomic DNA was isolated from peripheral blood leukocytes of the 181 patients, as previously described (12). The C242T transition polymorphism in the NADPH oxidase p22phox gene (GenBank M61107), exon 6 region, was determined by the TaqMan (Applied Biosystems, Tokyo, Japan) PCR method (13). The following primers and probes were included in the reaction: forward primer, 5′-TTC CTC CCT CCC CCA GG-3′; reverse primer, 5′-CCT GGT AAA GGG CCC GAA-3′; T allele–specific probe, 5′-Vic-ACA GAA GTA CAT GAC CG-3′; and C allele–specific probe, 5′-Fam-AGA AGC ACA TGA CCG-3′. The G1704T transversion polymorphism in the RAGE gene (GenBank D28769), intron region, was also determined by TaqMan PCR method. The following primers and probes were included in the reaction: forward primer, 5′-AGG ATG TGA GTG ACC TGG AGA GA-3′; reverse primer, 5′-TCA GCT CCT AGC CTG CCT TTC-3′; T allele–specific probe, 5′-Vic-CCA TAA CTA TCA ACA GGG-3′; and G allele–specific probe, 5′-Fam-CAT AAC TAG CAA CAG GG-3′. PCR was carried out using an ABI Prism 7700 (Applied Biosystems).

Statistical analysis

The sample size of this study was established on the basis of our pilot study. Regarding p22phox, our pilot studies indicated that the frequency of 242CT + TT would be 0.10 for Group D and 0.28 for Group N. It was calculated that the number of subjects needed to achieve 80% power to detect a difference between the two groups, with a significance level α (chance of a two-sided α error) of 0.05 was 59. Regarding RAGE, pilot studies indicated that the frequency of 1704GT + TT would be 0.30 for Group D and 0.13 for Group N. It was calculated that the number of subjects needed to achieve 80% power to detect a difference between the two groups was 72.

Data are expressed as means ± SD. The statistical significance of differences in mean values was analyzed by Student's t test or the Mann-Whitney U test. The frequencies of various alleles and genotypes were compared between the groups by χ^2 or Fisher's exact test, and multiple logistic regression analysis was performed to calculate the odds ratio and to evaluate the relation between the genotypes and other conventional risk factors. All statistical analyses were performed using Statview Software (version 5.0 for Windows; SAS Institute, Cary, NC).

RESULTS — Table 1 shows the clinical

Table 2—*Frequencies of genotypes of p22phox C242T and RAGE G1704T polymorphisms*

	Group N	Group D	χ^2	*P*
p22phox242				
CC	85 (79)	66 (90)		
CT + TT	23 (21)	7 (10)	4.318	0.0427
RAGE1704				
GG	94 (87)	54 (74)		
GT + TT	14 (13)	19 (26)	4.987	0.0313

Data are *n* (%) unless otherwise indicated.

characteristics of the enrolled patients. Clinical data, including HbA_{1c} levels, lipid profiles, blood pressure, and the use of medication were obtained from the medical records, and retinopathy was assessed in 2001. There were no significant differences in age at onset, disease duration since the diagnosis of type 2 diabetes, BMI at onset, total or LDL cholesterol, or the use of medication between groups N and D, whereas blood pressure, HbA_{1c} levels, triglyceride levels, and the proportion of subjects with retinopathy were significantly higher in group D than in group N.

The genotype distribution of these genes in each group is shown in Table 2. The genotype frequencies in both groups were in Hardy-Weinberg equilibrium. The frequency of the CC, CT, and TT genotypes of the p22phox gene were 79, 20, and 1% in group N compared with 90, 8, and 2% in group D, respectively. The allelic frequencies of the C and T alleles were 95 and 5% in group D versus 89 and 11% in group N, respectively. Because the frequency of the TT genotype was low, we divided the enrolled subjects into two groups: CC and CT + TT. The frequency of the CT + TT genotypes was significantly higher in group N than in group D ($\chi^2 = 4.318$, $P = 0.0427$; 2 × 2). In addition, multiple logistic regression analysis identified the HbA_{1c} level, systolic blood pressure, and triglyceride level as independent risk factors for the development of diabetic nephropathy ($P < 0.005$) within the following variables: age at onset, disease duration, BMI at onset, HbA_{1c}, total cholesterol, triglyceride, retinopathy, systolic blood pressure, and p22phox polymorphism; this was not the case for p22phox CT + TT ($P = 0.0991$) (data not shown). The frequencies of the GG, GT, and TT genotypes of the RAGE G1704T gene were 87, 12, and 1%, respectively, in group N compared with 74, 23, and 3% in group D. The allelic frequencies of the G and T alleles were 74 and 26% in group D versus 87 and 13% in group N, respectively. Because the frequency of TT was low, we divided the enrolled subjects into two groups: GG and GT + TT. The frequency of the GT + TT genotypes was significantly higher in group D than in group N ($\chi^2 = 4.987$, $P = 0.0313$; 2 × 2). Multiple logistic regression analysis identified HbA_{1c} level and systolic blood pressure as independent risk factors for the development of diabetic nephropathy ($P < 0.005$), whereas this was not the case for RAGE GT + TT ($P = 0.0522$) (data not shown).

Next, we analyzed the relation between the development of diabetic nephropathy and the combination of these gene polymorphisms in the patients (Table 3). According to the results in Table 2, we assigned the combination of these genotypes to three levels of predicted risk of developing diabetic nephropathy: high-risk genotype (p22phox CC and RAGE GT + TT), intermediate-risk genotypes (p22phox CC and RAGE GG, p22phox CT + TT and RAGE GT + TT), and low-risk genotype (p22phox CT + TT and RAGE GG). The frequency of p22phox CC and RAGE GT + TT (high-risk genotype) in group D was significantly higher than that in group N ($\chi^2 = 10.338$, $P = 0.0057$; 2 × 3). In multiple logistic regression analysis, HbA_{1c} level, triglyceride level, systolic blood pressure, and the combination of p22phox CC and RAGE GT + TT polymorphisms were shown to be independent variables ($P < 0.005$) (Table 4). These analyses revealed that the combination of these polymorphisms is a significant factor in the development of diabetic nephropathy.

CONCLUSIONS— NADPH oxidase is a critical enzyme for superoxide production in phagocytes, vascular smooth muscle cells, and mesangial cells (5). Recently, Guzik and colleagues (14,15) reported that superoxide production in human blood vessels from diabetic patients is mediated by upregulated NADPH oxidase activity through the PKC pathway. P22phox is an essential component of NADPH oxidase, and it has been reported that the C242T polymorphism is associated with vascular superoxide production in human blood vessels from CAD patients (5,6). The C242T polymorphism results in an amino acid polymorphism (His/Tyr) at residue 72, which is located in the putative heme-binding sites (16). Because the histidine residue is considered to be a candidate for the ligand of the heme prosthetic group of cytochrome *b*, it has been suggested that this polymorphism is directly associated with the function of p22phox (17). Inoue et al. (6) reported that the risk of CAD was lower in individuals carrying the 242T genotype in the Japanese population, whereas other investigations showed conflicting findings in patients with CAD and cerebrovascular disease (18–23). It has also been reported that this polymorphism is associated with significantly lower basal and NADPH-stimulated vascular superoxide production in human blood vessels from patients with atherosclerosis (14). Individuals with the 242T genotype might have lower oxidative stress as a result of lower O_2 production compared with individuals bearing the 242C genotype. In this study, we showed that patients with the 242T genotype have a lower risk of developing diabetic nephropathy. Our result was consistent with that of a previous

Table 3—*Distribution of p22phox C242T and RAGE G1704T genotypes in patient groups*

p22phox and RAGE	Group N	Group D	χ^2	*P*
CT + TT and GG	18 (17)	54 (5)		
CT + TT and GT + TT/CC and GG	81 (75)	53 (73)		
CC and GT + TT	9 (8)	16 (22)	10.338	0.0057

Data are *n* (%).

Table 4—*Multiple logistic regression analysis of candidate variables for risk factors for diabetic nephropathy*

Variables	OR (95% CI)	*p*
HbA_{1c}	1.54 (1.13–2.10)	0.0034
Triglyceride	1.01 (1.00–1.01)	0.0388
Systolic blood pressure	1.06 (1.03–1.09)	<0.0001
Retinopathy	1.78 (0.83–3.80)	0.067
p22phox CC and RAGE GT + TT	2.93 (1.34–6.41)	0.0073

Variables included in the model: onset age, disease duration, BMI at onset, HbA_{1c}, total cholesterol, triglyceride, retinopathy, p22phox and RAGE polymorphisms, and systolic blood pressure. $r^2 = 0.267$, $P < 0.0001$ for retinopathy (none = 0, retinopathy = 1) and for p22phox and RAGE polymorphisms (CT + TT and GG = 0, CT + TT and GT + TT/CC and GG = 1, CC and GT + TT = 2).

study of NADPH oxidase function (14). The T allele frequency in control subjects in our study was 0.11, which was similar to that in previous studies of Japanese populations, whereas it was ~33% of that in Caucasian populations (6,20,23).

AGEs by themselves have been shown to generate ROS and, conversely, the generation of AGEs is enhanced by oxidative stress (24). In vivo and in vitro studies indicate that AGEs play a role in the pathogenesis of diabetic nephropathy and the progression of renal failure (4). It has been reported that aminoguanidine and OPB-9195 are effective in preventing diabetic nephropathy (25,26). AGEs can bind to several binding sites, including RAGE. In RAGE-overexpressing mice, it has been demonstrated that the AGE-RAGE system is essential for the development of diabetic nephropathy (27). The AGE-RAGE interaction in mesangial and endothelial cells causes enhanced formation of oxygen radicals, with subsequent activation of NF-κB and release of proinflammatory cytokines and adhesion molecules, leading to collagen gene expression in mesangial cells (28). It has been reported that the A-374T polymorphism is associated with diabetic nephropathy in type 1 diabetic patients with poor metabolic control (4). The G1704T gene polymorphism has been reported to show a significant association with the complication of microvascular dermatoses and antioxidant status (8,9). Subjects bearing the RAGE 1704T polymorphism had significantly lower plasma levels of several antioxidants (total carotenoids, lutein, lycopene, and tocopherol) than those bearing RAGE 1704G, suggesting that this polymorphism may relate to oxidative stress and supporting our present results that patients with the 1704T genotype have a higher risk of developing diabetic nephropathy. The functional impact of the intron site is not clear, but possible mechanisms include alternative splicing, a change in mRNA stability, or a linkage of a nearby coding single nucleotide polymorphism.

We also examined the combination of the p22phox C242T and RAGE G1704T polymorphisms in relation to susceptibility to diabetic nephropathy. We showed that patients with the combination of p22phox CC and RAGE GT + TT genotypes had a significantly higher risk of diabetic nephropathy than those with the combination of p22phox CT + TT and RAGE GG genotypes. In this study, the RAGE G1704T gene polymorphism alone was weakly related to the susceptibility to diabetic nephropathy, but the combination of p22phox and RAGE gene polymorphisms was more significantly related to diabetic nephropathy. Both NADPH oxidase and RAGE are known to be expressed in mesangial cells, and recently, NADPH oxidase was reported to be a central target of RAGE; ROS generated by this mechanism could significantly impact cellular properties (10).

In conclusion, we propose that the combination of NADPH p22phox C242T and RAGE G1704T polymorphisms is associated with the development of diabetic nephropathy. It is obvious that microalbuminuria is the best documented predictor of the development of diabetic nephropathy (29). However, we propose that risk assessment based on gene polymorphisms, such as in this study, is also important. Further prospective studies are needed to clarify whether these polymorphisms can predict the development of diabetic nephropathy. Several studies have suggested that antioxidant treatment such as with ACE inhibitors (30), probucol (30), vitamin E (31), or α-lipoic acid (32) might be beneficial in preventing the development of diabetic nephropathy. It may be useful to identify the risk of the development of diabetic nephropathy and to select a medication such as an antioxidant.

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血栓性素因遺伝子の網羅的検索と脳卒中リスク

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脳卒中，とくに虚血性脳血管障害の危険因子としては，高血圧や動脈硬化の進展を早める因子の他に血栓性素因が重要である．候補遺伝子アプローチ（candidate gene approach）により，脳卒中と関係する血液凝固因子や血小板の因子が解明されてきた．一方近年，ゲノムワイドスキャン（genome-wide scan）による原因遺伝子同定が急ピッチにおこなわれており血栓症についても注目すべき報告がみられる．脳卒中については今後多くのデータが蓄積されると思われる．血栓性素因遺伝子の網羅的検索によって得られた情報は脳血管障害の予防ならびにテーラーメイド医療に大きく貢献するであろう．

Key Words
虚血性脳血管障害，危険因子，血栓症，遺伝子多型，ゲノムワイドスキャン

はじめに

虚血性脳血管障害の病態は，粥状硬化が原因であるアテローム血栓性梗塞とTIA，穿通枝の細動脈硬化に起因するラクナ梗塞，心疾患が原因である心原性塞栓症など，それぞれの病型により異なるが，一般には動脈硬化がその基盤に存在する．脳梗塞の危険因子としては，高血圧や従来から知られている動脈硬化の進展を早める因子，糖尿病や高脂血症の他に，血栓形成を助長させる血液凝固能や血小板機能の亢進を考慮せねばならない．実際，脳卒中患者のなかで動脈硬化の危険因子をもたない若年患者では，血栓性素因が虚血性脳血管障害の発症に関与している可能性がある．

1 血栓性素因の遺伝的要因

健常人の血液凝固因子の血中濃度を測定してみると非常にバラつきが多く，少なくともこの一部は遺伝的に決定されており，しかも単純な点変異（多型）で説明可能である．たとえばフィブリノゲン，プロトロンビン，凝固VII因子，XII因子などには，それぞれの血中濃度を左右する点変異が知られている．また血小板機能の個体差も多くが遺伝的に決定されるといわれる．たとえば健常人の血小板凝集能を測定してみると，同胞では男-男，男-女，女-女，いずれの組み合わせでも凝集率はよく相関するが，同じ環境に生活する夫婦間では凝集率の相関はまったくみられない[1]．

2 脳卒中と関連する遺伝子の研究方法

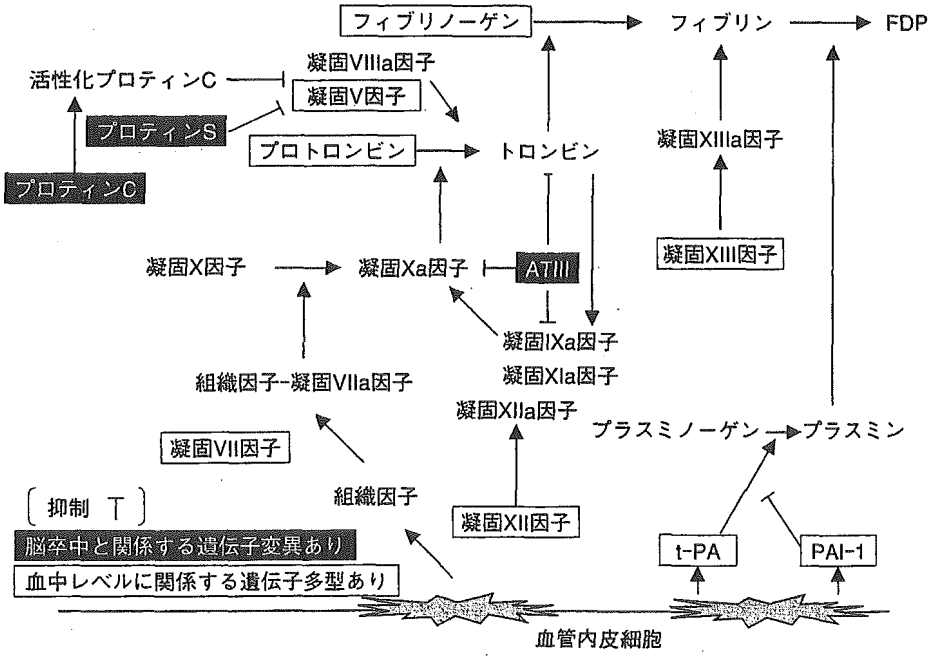
一般に，疾患と関連する遺伝子の同定にはいくつかの方法があるが，その一つは，過去の病態研究から疾患の成因に関与している可能性が高い物質に対し，その遺伝子を順次検索し変異（多型）を同定し，疾患の罹病率や病型との関係を比較してゆく候補遺伝子アプローチ（candidate gene approach）法であり，いま一つは全ゲノム上の遺伝的多型をまんべんなくタイピングし，疾患と連鎖する座位を機械的に決定してゆく全ゲノムスキャン（whole genome scanまたはgenome-wide scan）である．

3 候補遺伝子アプローチ：易血栓性と関係する因子

血栓症と関係する多くの多型が候補遺伝子アプローチ法でみつかった．とくに冠状動脈疾患（CAD）でその成果は著しい．特筆すべきものとして，Yamadaらの候補遺伝子アプローチ法を用いた心筋梗塞と関係する遺伝子多型の同定があげられる[2]．しかし，遺伝子変異と表現型（phenotype）の関連が不明のもの，動脈血栓と静脈血栓での関与の違い，研究間での結果の不一致など，問題点は多い．脳卒中については，心筋梗塞のような大規模な研究は見当たらないが，易血栓性と関係する因子の多型との関係がしだいに明らかにされてきている．

◉1．血液凝固に関係する因子の先天異常

血液凝固系の制御因子であるアンチトロンビンIII（AT III），プロテインC，プロテインSの遺伝子変異はこれら物質の量的・質的異常が血栓傾向をもたらすこと，そして脳動脈血栓や脳静脈血栓と関係することが明らかとなっている．AT IIIはヘパリンを補因子として血液凝固系の活性化の結果生じたトロンビン，活性化第X因子，あるいは活性化第IX因子の作用を阻害する（図❶）[3]．よってAT IIIの遺伝子変異による量的・質的異常は過剰な血液凝固反応の進行を招く．AT III欠損症は一般人口において，2,000～5,000人に1人の割合で存在する．虚血性脳血管障害（Large artery）患者群では約8％にAT III欠損が見出されている．この他，プロテインCやプロテインS遺伝子変異によるこれら因子の欠乏・減少が認められている．プロテインCはトロンビンと血管内皮細胞上のトロンボモジュリンとの結合により活性化され，活性化プロテインC（APC）となる．APCはプロテインSを補酵素としながら第V因子や凝固第VIII因子を特異的に分解，失活化することで凝固系を抑制する．したが



図❶ 血液凝固カスケードと脳卒中の遺伝的危険因子（松原由美子ほか，2002[3]より引用）

って，プロテインCやプロテインSの欠乏症では生体内の抗凝固作用が減少し，血栓傾向が招来する．これらの異常は，以下に述べる血液凝固や血小板因子の遺伝的多型にくらべればまれである．

◍2．健常者における血液凝固，血小板因子の遺伝的多様性

1．凝固V因子Leiden変異とプロトロンビン変異

凝固V因子Leiden変異はアミノ酸^{506}Arg→Gln変異をきたすことによって，APCによる分解が障害されると説明されている．APC抵抗性（APCR）は欧米での静脈血栓症の主要な原因であるが，また動脈血栓の原因にもなりうる．しかし日本人では^{506}Gln点変異をもつ症例は一例も報告されていない[4]．一方，同様に白人でプロトロンビン遺伝子の^{20210}G/A変異が静脈血栓症や動脈血栓症に関係すると報告されている．^{20210}Aをヘテロ接合体で有する個体では^{20210}Gホモ接合体にくらべ血中プロトロンビン活性が高い．上述の凝固V因子Leiden変異と同様，この変異は日本人では認められない[5]．

2．トロンボモジュリン（TM）

TMはトロンビンを向凝固因子から抗凝固因子へと変換する．TMの先天的欠損／異常は向血栓性となると予想されるが，実際ヘテロ欠損症や分子異常症で肺梗塞を発症した例が報告されている．$^{-133}$C/A，$^{-33}$G/A，^{25}Ala/Thr，^{61}Gly/Ala，^{468}Asp/tyr，^{483}Pro/Leuなどが知られている．^{25}Ala/Thrは若年の心筋梗塞と関係するといわれるが脳血管障害との関連は確立されていない．

3．フィブリノゲン

高フィブリノゲン血症は従来から心筋梗塞の危険因子とされていたが複数の遺伝子多型が報告されており，とくにβ鎖^{453}G/Aは運動後の血中フィブリノゲン増加率と関係し，冠動脈疾患の発症率に関与しているという説もある．しかしわれわれの検討ではこの遺伝子多型は虚血性脳血管障害に関係しなかった．

4．凝固第VII因子，XII因子とXIII因子

第VII因子には数ヵ所の遺伝子多型があり，血中第VII因子活性や冠動脈疾患や脳血管障害との関連について多くの報告がある．凝固XII因子の開始コドン直前にある^{46}C/T多型では，C型で翻訳効率が高く，また実際にC型を有する個体では血中XII因子抗原量が高い．われわれの検討でも血中XII因子活性や活性化XII因子血中濃度（XIIa）もこの多型に強く依存していることが判明した[6)7)]．しかしこの多型と脳血管障害の有病率の関係は非常に微妙なものであり，唯一アテローム血栓性脳梗塞と弱い関連を認めたのみであった．日本人は白人にくらべT型（翻訳効率の悪いタイプ）の頻度が高いことが知られている．日本人の平均凝固XII因子活性が白人のそれにくらべ低いことの一つの原因と思われる．また凝固第XIII因子の一塩基置換（一アミノ酸置換）が第XIII因子濃度を変化させ，冠状動脈疾患や脳血管障害の発症率と関係していると報告されている．

5．血小板膜糖蛋白

動脈血栓の形成過程の初期過程は，速い血流の状態下，動脈硬化プラークの破綻に伴って露呈する内皮下組織のコラーゲンに粘着したフォンウィルブランド因子（von Willebrand factor：vWF）が血小板膜糖蛋白（glycoprotein：GP）であるGPIb/IX/V複合体と反応する（可逆性の粘着，tetheringとよばれている）．この刺激でGPIa/IIaが活性化してコラーゲンと反応する（血小板粘着）．粘着後活性化する血小板の膜上には活性型GPIIb/IIIaが発現して（血小板活性化），動脈血栓形成過程の後期である，活性化血小板とフィブリノゲンやvWFの結合，あるいは活性化した血小板から放出される強力な血小板惹起物質であるトロンボキサンA_2をはじめとする種々の因子を血小板外へ放出し正のフィードバック機構により血小板同士が強固に凝集（血小板凝集）して安定した動脈血栓をもたらす．このように動脈血栓形成過程に重要なはたらきをもつ膜受容体は近年，抗血小板療法のターゲットとして注目されている．そして，これら膜受容体の多型には脳卒中に関連するものが報告されている。

A．GPIIb/IIIa複合体（インテグリンαIIbβ3）

血小板フィブリノゲン受容体でGPIIb/IIIaは血小板シグナル伝達の最終共通経路である．WeissらはGPIIIa^{33}Leu/Pro多型とCADとの関連を初めて報告した[8)]．しかしこの関連を否定する報告もある．多くの結果を総合すると，GPIIIa^{33}Leu/Pro多型は少なくとも健常人やlow risk groupでは危険因子とならないと思われる．こ

の多型は日本人ではまれであるため，わが国における血栓症の発症にこの遺伝子多型が深くかかわっている可能性は低い．

B. GPIb/IX/V複合体

GPIb/IX/V複合体は内皮下組織に存在するvWFを認識する膜受容体である．GPIbのα鎖に存在する三つの多型はよく研究されている．われわれは[145]Thr/Metおよび#399－411の13アミノ酸反復多型とCADとの関連について検討し，Met-allele，4-repeat alleleがCADの有病率，重症度と関連があることを報告した[9]．[145]Thr/Metはまた虚血性脳血管障害にも関係することが最近の研究で明らかになっている[10]．すなわち虚血性脳血管障害200例について，年齢，性がマッチしたコントロールと遺伝子頻度の比較をすると脳血管障害群で[145]Met-alleleの出現頻度が有意に（p＝0.0005）高く，とくに対象を女性，若年者，非喫煙者，高血圧を有さないなど後天的リスクファクターが少ない個体にかぎって解析するとこの傾向は一層顕著であり，たとえば60歳未満の非喫煙女性では，そのオッズ比は10.6であった．

C. GPIa/IIa複合体（インテグリンα2β1）

コラーゲン受容体の一つで血小板のコラーゲンに対する粘着をつかさどる．この受容体は主要な血小板膜受容体のなかでは血小板膜上の発現量が少なく，しかも正常人での発現量の個体差が大きい．Reinerらはα2の多型と虚血性脳血管障害の関連を報告している[11]．

4 全ゲノムアプローチ

一方，全ゲノムアプローチによる血栓症の責任遺伝子の同定についても注目すべき報告がみられる．とくに虚血性心疾患に関する研究は特筆に値する．Harrapら[12]はマイクロサテライトマーカーを用いて，急性冠動脈症候群の患者のなかで同胞発症のある，なし，を比較し，2番染色体にその責任座位を同定している．一方，Broeckelら[13]は心筋梗塞513家系でlinkage analysisをおこない，14番染色体上に心筋梗塞遺伝子があるとした．わが国からは92,788ヵ所のSNPを解析したデータがOzakiらにより報告された．それによれば6番染色体（6p21）に心筋梗塞感受性遺伝子があるとされる．この部位に存在するlymphotoxin alpha遺伝子の[26]Thr/Asn多型が心筋梗塞と強く関連し，また実験的にもこの多型がVCAM-1などの接着分子の発現に影響するという[14]．

虚血性脳血管障害についても，以下に述べるように全ゲノムアプローチによる原因遺伝子同定が試みられている．

● 1．アイスランドにおけるゲノムワイド関連研究

deCODE Genetics of IcelandのGretarsdottirらは最近，アイスランド住民を対象とした全ゲノムアプローチにより脳卒中遺伝子を同定している[15]．総計179家系において，476人の脳卒中患者およびこれら患者の親戚438人が調べられた．ゲノム上の約1,000のマイクロサテライトマーカーについて遺伝子タイピングをおこなった結果，5番染色体（5q12）に脳卒中と最も関連が強い領域が発見された，という研究である．はじめLOD scoreが1を超える領域が3ヵ所に見出されたが（図❷）[15]，このなかで最もscoreの高かった5番染色体について，さらに45の多型マーカーを調べていったところ，5q12に絞りこみが成功した．彼らはこの領域をそれまで脳卒中との関連の報告がみられない，新しい領域として*STRK1*と名づけた．その後の詳細なmappingの結果，5q12に存在するphosphodiesterase 4D（PDE4D）遺伝子が脳卒中と最も強い関連があることを発見しこれを彼らは『*Nature Genetics*』に報告した[16]．この報告によればPDE4D遺伝子は三つのハプロタイプに分類可能で，それぞれ「wild-type」「at-risk」「protective」とされている．さらに，これは*in vitro*の研究で，遺伝子型による機能の相違が報告されている．

● 2．SWISS Protocol

The Siblings With Ischemic Stroke Study（SWISS）protocolは，同胞対を対象としたlinkage analysisによる，虚血性脳血管障害の新しい遺伝的危険因子の同定を目的としている[17]．この研究では画像診断で確認された脳卒中患者のなかで，少なくとも1人の脳卒中の既往があり生存している同胞が存在する患者を対象としている（図❸）[17]．同胞に対しては質問表などによりconcordant，discordantの判断がなされ，対象者の血液からはEB virusによって不死化されたリンパ球のcell lineが作製される．ゲノムを対象に237のマイクロサテライトマーカーがタイピングされる予定である．その結果は今後報告されると思われるが，上述のゲノムワイド研究の結果と