

No Association of Factor XIII Val34Leu Polymorphism with Primary Intracerebral Hemorrhage and Healthy Controls in Korean Population

The polymorphism in the factor XIII A-subunit gene (FXIII Val34Leu) has been recognized as a risk factor for primary intracerebral hemorrhage (PICH). In addition, FXIII Val34Leu has a significant ethnic heterogeneity. FXIII Val34Leu was detected in 41.7-54.8% of the Westerners, but in 2.5% of the Asians. We aimed to evaluate the prevalence of FXIII Val34Leu in patients with PICH and in healthy controls among Koreans. We recruited 58 in-patients with PICH, defined by brain computed tomography or magnetic resonance imaging, and 48 controls matched for age, sex, and risk factors for cerebrovascular diseases. Genomic DNA was extracted from blood. A 183-bp fragment of exon 2/intron B of the factor XIII A-subunit gene was amplified by polymerase chain reaction (PCR). The factor XIII genotype was determined through a single-stranded conformational polymorphism. Fifty-eight patients and 48 controls showed the same band patterns on SSCP. In addition, we directly sequenced six random-selected DNA segments using DNA auto-sequencer. In conclusion, the results of this study suggest that FXIII Val34Leu be absent or rare both in patients with PICH and in healthy controls among Koreans.

Key Words : Factor XIII; Point Mutation; Polymorphism (Genetics); Cerebral Hemorrhage; Polymerase Chain Reaction; Polymorphism, Single-Stranded Conformational

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INTRODUCTION

Blood coagulation factor XIII (FXIII, also called fibrin-stabilizing factor) is a transglutaminase that plays an important role in clot stabilization by crosslinking fibrin chains (1). FXIII exists in a tetrameric form consisting of two catalytic A-subunits (FXIIIA) and two carrier protein B-subunits (FXIIIB) that are held together by non-covalent bondings and weigh approximately 320 kDa (2, 3). While FXIIIA clearly have the catalytic activity, FXIIIBs are thought to protect FXIIAs from proteolysis and stabilize their structure (4). FXIII has a pivotal role in the coagulation process. Cross-linked stable fibrin contributes to the resistance to chemical, mechanical, and proteolytic insults.

The gene coding for FXIIIA maps to chromosome 6p24-25, encodes a mature protein of 731 amino acids (5, 6), and spans altogether over 160 kb. It contains 15 exons (6) that code for a 4-kb mRNA (7). FXIIIB is comprised of 641 amino acids with 10 tandem repeats of 60 amino acids each and its coding region has been assigned to chromosome 1q31-32 (8). Only three mutations have been reported in the gene of FXIIIA (9-11), and two mutations in the gene of FXIIIB (12). Both genes exhibit common protein polymorphisms among various population groups (13, 14).

There is an increasing interest in the role of FXIII in cardiovascular and cerebrovascular diseases. It has been previ-

ously reported that a common G→T point mutation in exon 2, codon 34 of the FXIIIA gene, which elicits a valine-to-leucine change (FXIII Val34Leu), is protective against thrombotic disease, but seems to increase the risk of intracerebral bleeding.

Surprisingly, the less frequent allele (Leu34) does not increase the risk of thrombosis, but has been described as being a protective factor against myocardial infarction (15, 16), brain infarction (17), and deep vein thrombosis (18, 19). On the other hand, there seems to be an increased risk for intracerebral hemorrhage (15, 20). Regarding the prevalence, FXIII Val34Leu was shown to be highly prevalent in Caucasians, but a very low prevalence in Japanese (21).

To our knowledge, there has been no report on the prevalence of FXIII Val34Leu in Koreans. In this study, we investigated the distribution of FXIII Val34Leu, and then evaluated the relationship between the polymorphism and primary intracerebral hemorrhage in Korean population using PCR-SSCP method and gene sequencing.

MATERIALS AND METHODS

Subjects

Fifty-eight patients with primary intracerebral hemorrhage

(PICH) admitted to the Department of Neurology at Chonnam National University Hospital were prospectively recruited between April 1999 and April 2000. Diagnosis of PICH was verified by computed tomography (CT) or magnetic resonance imaging (MRI) in each patient on admission, within 24 hr from the onset of symptoms. Exclusion criteria were 1) subarachnoid hemorrhage, 2) bleeding tendency disorders, 3) history of previous cerebrovascular accidents or recent trauma, 4) history of other neurological disorders and 5) intracerebral hemorrhage (ICH) caused by congenital vascular malformation. During the same period, we selected 48 healthy control subjects from those visiting to the health screening center. They had no history of vascular, thromboembolic, or hemorrhagic disease, or ongoing antithrombotic therapy. They were also matched for age, sex, and selected risk factors for arterial thromboembolic disease (smoking history, hypertension, cholesterol level and diabetes) with the respective patient. All subjects gave informed consent.

Analysis of ICH

Hemorrhage volumes were measured by a simplified formula for the volume of an ellipsoid, $ABC/2$ (22). For the bedside ABC/2 method, the CT slice with the largest area of hemorrhage was identified. The largest diameter (A) of the ICH was measured on this slice. Then the largest diameter 90° to A was measured next (B) on the same slice. Finally, the approximate number of 1 cm slices containing the ICH was calculated (C). C was calculated by comparison of each CT slice with hemorrhage to the CT slice with the largest hemorrhage on that scan.

Classification of each hematoma was based on the location of the epicenter of the hematoma as basal ganglia, lobar (frontal, parietal, temporal, or occipital), thalamus, cerebellum, brain stem, or other (combined with ventricle).

Extraction of DNA from venous blood

Peripheral blood (3 mL) was anticoagulated with 1.6 mg/mL EDTA. Genomic DNA was extracted from 100 μ L venous blood using the Dr. GenTLE™ kit (TaKaRa Shuzo Co., Ltd, Japan).

DNA amplification by polymerase chain reaction (PCR)

A 183-bp fragment of exon 2/intron B of the factor XIII gene was amplified by the polymerase chain reaction (PCR), with 5'-ACCCAGAGTGGTGGGGAAG as a 5' primer and 5'-GACCTTGTAAGTCAAAAATGTC as a 3' primer. The DNA extract was amplified by PCR in 50 μ L of reaction mixture containing 2 μ L (200 ng) of DNA extracts, 5 μ L of 10 \times reaction buffer, 6 μ L of 1.5 mM MgCl₂, 0.5 μ L of *Taq* DNA polymerase (TaKaRa), 4 μ L of 2.5 μ M dNTP, 1 μ L (50 pmol) of each primer, and 30.5 μ L of sterilized dis-

tilled water. The reaction samples were preheated at 94°C for 5 min; then, they were subjected to cycling of denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and elongation for 2 min at 72°C for 35 cycles; this was followed by post-elongation reaction at 72°C for 2 min in a GeneAmp PCR system 9600 (Perkin Elmer Co., Norwalk, CT, U.S.A.).

After the reaction, electrophoresis of the mixture containing 5 μ L of post-PCR solution and 2 μ L of loading buffer (0.25% bromophenol blue/0.25% xylene cyanol/50% glycerol) was performed on 1.8% polyacrylamide gel that contained 1 μ g/mL of ethidium bromide. DNA bands were identified through ultraviolet transilluminator.

Single-stranded conformational polymorphism (SSCP) analysis and identification of the Factor XIII Val34Leu genotype

To detect the substitution of thymine to guanine (Val34Leu), we performed the single-stranded conformational polymorphism (SSCP) analysis on the PCR products. Five microliters of the PCR product was mixed with 1 μ L of alkaline denaturing solution containing 0.2 M NaOH and 20 mM EDTA, 4 μ L of sterilized distilled water, and SSCP loading buffer. The samples were heated at 95°C for 6 min, cooled briefly on ice, and loaded onto a 20% TBE Gel (5:1 TBE/bisacrylamide ratio; NOVEX™). Electrophoresis was run for 9 hr at room temperature (14–18°C) at 8 W constant power in 0.5 \times TBE running buffer. During the electrophoresis, performed in a renaturing condition, the DNA fragments undergo conformational changes according to their base sequence. The migration in the electrical field depends on the size and conformation of the DNA fragment, so that an aberrant migration is usually detected both in deletions/insertions and single-base substitutions. The gel was subjected to SYBR® Green II staining for 20 to 40 min. The bands were identified through FLARE 3000 (image pro).

The PCR product was further purified by a QIAquick gel extraction kit (Qiagen, Germany), and was inserted into a PGEM T easy vector and directly sequenced using dye-labeled terminators (ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kits version 2.0, Perkin-Elmer) on an ABI Prism 377 DNA Sequencer. We confirmed the genotypes of 183 bp-fragments by sequencing randomly selected 6 samples (3 patients with PICH and 3 healthy controls).

RESULTS

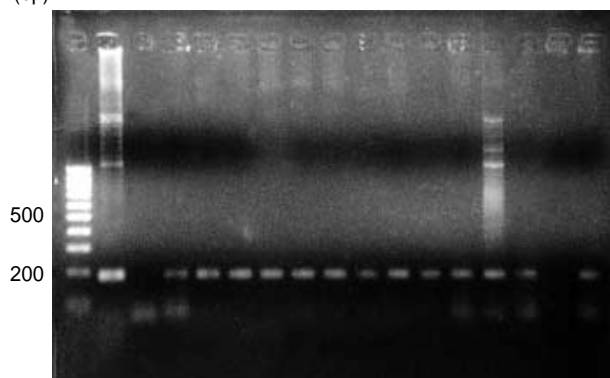
Characteristics of the study population

Table 1 summarizes the relevant general characteristics of the patients. Detailed information concerning the presence of major risk factors for cerebrovascular disease (hypertension, dyslipidemia, diabetes, obesity, and smoking) was available

Table 1. The characteristics of patients with primary intracerebral hemorrhage (PICH) and healthy controls

Groups Age (yr)	Patients with PICH			Healthy controls		
	Male (n)	Female (n)	Total (n)	Male (n)	Female (n)	Total (n)
30-39	1	0	1	1	0	1
40-49	5	4	9	4	3	7
50-59	7	6	13	6	6	12
60-69	14	10	24	13	8	21
70-79	3	5	8	2	3	5
>80	2	1	3	1	1	2
Total	32	26	58	27	21	48
Mean±SD (yr)	58.8±9.8	63.3±12.1	60.9±11.1	59.2±8.7	62.9±11.8	60.8±10.6

(bp) M P1 P2 P3 P4 P5 P6 P7 P8 C1 C2 C3 C4 C5 C6 C7 C8

**Fig. 1.** Identification of 183 bp segment of Factor XIII A-chain cDNA. The electrophoresed PCR products were visualized by ethidium bromide staining. Lane M, DNA molecular size markers for 100 bp; Lanes P1-8, DNA from patients with ICH; Lanes C1-8, DNA from healthy controls.

for patients and controls. No significant differences were found in the prevalence of selected risk factors for cardiovascular disease between patients and controls. Of the 58 patients evaluated, the mean volume (cm³) of hematoma was 20.07 (range: 0.13-81.39). The most common site of ICH was basal ganglia (29.3%). Genomic DNA from venous blood was available in all cases (n=58) and controls (n=48).

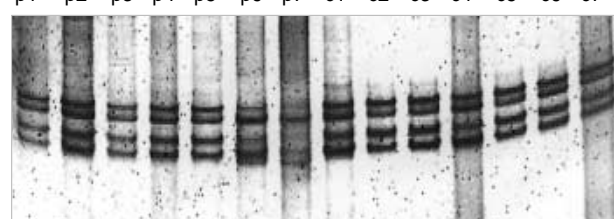
Identification of FXIII 183-bp fragments on electrophoresis after PCR

After PCR amplification of 183-bp fragments, DNA bands were identified through ultraviolet transilluminator, and photographed (Fig. 1). The PCR products of the patients were indistinguishable from those of the healthy controls on the agarose gel, suggesting that no major deletion or insertion in that exon area was responsible for the disease.

Screening for mutations in the XIII A gene by PCR-SSCP and DNA sequencing

To screen mutations of the A-subunit gene of FXIII, the

p1 p2 p3 p4 p5 p6 p7 c1 c2 c3 c4 c5 c6 c7

**Fig. 2.** Analysis of the Val34Leu point mutation in exon2/intron B of the factor XIII A subunit gene by single-stranded conformational polymorphisms (SSCP). The figure shows the genotype pattern obtained by SSCP. SSCP of 183 bp segment of the factor XIII A subunit gene reveals no variant mobility shifts. Lanes p1-7, DNA from patients with ICH; lanes c1-7, DNA from healthy controls.

genomic DNA from patients and healthy controls were analyzed by PCR-SSCP. The conditions for SSCP analysis were optimized by limiting the size of the PCR products and by varying the buffer and glycerol concentrations for electrophoresis. No mobility difference was detected between the patients and healthy controls on SSCP (Fig. 2).

To confirm the presence of a point mutation, the PCR products were subjected to direct sequencing analysis, which revealed no mutations in FXIII exon2/intronB in patients with ICH and in control. This demonstrates the absence of a common G→T point mutation in exon 2, codon 34 of the FXIII-A gene.

DISCUSSION

Genetic polymorphism of FXIII subunits was first reported by Board (23). Following studies demonstrated the existence of several allelic variants of FXIII-A with a wide distribution among racial groups. Data from gene, cDNA, and protein sequencing revealed several amino acid substitutions in apparently normal subjects. One of the common polymorphisms is a G→T point mutation in codon 34, exon 2 of the A-subunit gene, which codes for a Valine→Leucine change (FXIII Val34Leu) only three amino acids apart from the

thrombin activation site (15, 24). Because of this proximity, this polymorphism might well influence the process of proteolytic activation and is therefore a candidate for a role in the pathogenesis of thrombotic disorders.

The role of common polymorphism of FXIII Val34Leu has been recently recognized as a protective genetic factor against arterial and venous thrombosis. In addition, the less frequent Leu34 allele has been described as a risk factor for ICH. The FXIII Val34Leu has been recently reported to confer protection against arterial and venous thrombosis (15-19, 25, 26) and predisposition to PICH (20, 27), although not all studies report its protective role against ischemic diseases (20, 28, 29). How FXIII Val34Leu protects against thrombosis and predisposes to hemorrhage are not completely understood, but it seems that owing to an early activation, a premature depletion of the circulating FXIII Leu34 is a protective effect against thrombosis (30, 31). FXIII Val34Leu may be responsible for the formation of weaker fibrin structures through a common mechanism for protection against myocardial ischemia or cerebral infarction and predisposition to PICH.

Genetic polymorphisms underlie the diversity of any ethnic group. Most of such inherited changes in DNA structure are neutral, but others could affect the function of proteins and, with varying degrees of severity, the efficiency of a whole physiological system, thus modifying susceptibility to a particular disease.

The FXIII Val34Leu polymorphism was originally described in a Finnish population composed of 600 controls with a allele frequency of 23% (24), similar to that in other study population (15) and in small groups of Finnish, Russian, German, and Japanese subjects (32). Renner *et al.* (26) found a prevalence of 26.2% heterozygous and 7.8% homozygous carriers of FXIII Val34Leu among controls in Austria. McCormack *et al.* (33) reported that Asians, Caucasians, and Pima Indian groups had different leucine allele frequencies (0.13, 0.28, and 0.40, respectively). The Leu allele seems to be less common in Japanese than in the three Caucasian populations (32).

Reported allele frequencies of FXIII Val34Leu range from 0.25 to 0.30, with 32% to 43% heterozygous, and 4% to 10% homozygous carriers (16, 18, 19, 34). Recently, FXIII Val34Leu was detected in 44.3% of Caucasians, 28.9% of Blacks, 2.5% of Asians, and 51.2% of Amerindians (21). However, limited data are available about the prevalence of the FXIII Val34Leu among Asians. To our knowledge, there has been no information on the exact prevalence of FXIII Val34Leu polymorphism in Koreans until now.

Our results are in line with those of previous studies that showed FXIII Val34Leu was absent or had a very low prevalence among Japanese (35), and then demonstrate that FXIII Val34Leu is also rare in Korean population. The high prevalence of FXIII Val34Leu in Caucasians and a very low prevalence of that in Koreans and Japanese, imply a heterogeneous distribution of the polymorphism. Taken together, our data

showed that FXIII Val34Leu exhibits a significant ethnic heterogeneity, a finding that is relevant to studies relating this polymorphism with thrombotic and bleeding disorders.

On the other hand, three points should be borne in mind when interpreting our results. First, the number of our subjects was too small. Second, our study might have false negative results because we could not carry out the direct sequence of 183 bp segment of Factor XIII A-chain cDNA in all subjects. Third, our study was performed in a limited area (Chonnam Province).

However, these results should facilitate future investigations to identify genetic variations in clotting factor genes that play a role in the risk for cerebrovascular diseases. We suggest that further studies with larger numbers of participants should be performed.

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