

A Mutation Unique in Serine Protease Inhibitors (Serpins) Identified in a Family with Type II Hereditary Angioneurotic Edema

Javier G. Ocejo-Vinyals, Francisco Leyva-Cobián,
and José L. Fernández-Luna

Servicio de Inmunología, Hospital Universitario Marqués de Valdecilla,
Santander, Spain

ABSTRACT

Background: Hereditary angioneurotic edema (HANE) is an autosomal dominant disease due to genetic alterations at the C1 inhibitor gene. Mutations within the C1 inhibitor gene are responsible for the molecular defect in type II HANE. Most of the dysfunctional proteins result from mutations involving the Arg-444 (the P-1 site of the reactive center) or amino acids NH₂-terminal to the reactive center.

Materials and Methods: We have studied a Spanish family with type II HANE by using polymerase chain reaction (PCR) to amplify the exon eight of the C1 inhibitor gene. The purified 338-bp PCR product was subcloned and transformed into competent cells. After overnight cultures, we extracted the cloning vector from the positive colonies and sequenced both strands of the PCR

product from each patient and healthy members of the family.

Results: We show that affected individuals in this family have a missense mutation, changing an adenine to cytosine in the codon 445. This substitution changes threonine at the P-1' site of the reactive center to a proline. This mutation generates a new restriction site, recognized by Bsi YI.

Conclusions: To our knowledge, this is the first molecular defect characterized in a Spanish family with type II HANE, and to date, this is the first reported mutation at the P-1' site of the reactive center in individuals with type II HANE. This new mutation located at the reactive center emphasizes once more time the enormous heterogeneity of this gene.

INTRODUCTION

Hereditary angioneurotic edema (HANE) is characterized by potentially life-threatening episodes of painless, nonpruritic, nonpitting edema, usually involving the face, upper airway, gastrointestinal tract and extremities. There are two variants of HANE (1,2): type I is characterized by low antigenic and functional levels of a normal C1 inhibitor protein. In type II, there are normal or increased antigenic levels of a dysfunctional mutant C1 inhibitor protein together with a reduced concentration of the functional protein.

The C1 inhibitor is a member of the serine protease inhibitors (SERPINS) (3) which shows

activity against the C1r and C1s subcomponents of the first component of complement (4–8). Moreover, it also inhibits kallikrein (9), activated Hageman factor (factor XIIa) and plasma thromboplastin antecedent (factor XIa or PTA) (10), plasmin (9), and tissue plasminogen activator (11,12).

The C1 inhibitor shows structural and functional homologies with other human SERPINS such as α -1-antitrypsin, antithrombin III, α -1-antichymotrypsin, α -2-antiplasmin, and heparin cofactor II (13–16). The reactive center of SERPINS mimics the substrate of the protease. In the C1 inhibitor protein, this reactive center is an Arg-Thr sequence at residues 444–445 (16). Binding of the protease cleaves the Arg-444 (P-1)-Thr-445 (P-1') peptide bond and releases a small polypeptide molecule from the carboxy-

Address correspondence and reprint requests to: Javier G. Ocejo-Vinyals, Servicio de Inmunología, Hospital Universitario Marqués de Valdecilla, 39008-Santander, Spain.

terminal end of the C1 inhibitor. The sequences and lengths of SERPINS-reactive center are different (14,16–19), and the P-1 site seems to be involved in determining the target protease specificity of individual SERPINS (16–19).

The complete nucleotide sequence of the human C1 inhibitor gene, which contains eight exons, has been recently determined (14,21). A significant feature of the intronic sequences is the increased frequency of *Alu 1* repeats. Another feature is that among normal individuals a restriction fragment length polymorphism can be observed after digestion of genomic DNA with HgiAI (14), which is sometimes useful for following the inheritance of normal and mutant alleles in HANE kindreds (22). In type I HANE the *Alu 1* repeats predispose to intragenic breakpoints and unequal crossing-over (23), which together with partial deletions (24,25), nonsense mutations (26), and other point mutations (13,27) are responsible for this disease.

In type II HANE, the dysfunctional C1 inhibitor molecule usually has an amino acid change due to point mutations. Most often, at codon 444 (CGC) in the P-1 site of the reactive center, point mutations changes Arg to His or Cys (13,27). CG dinucleotides are the major methylated sequences in vertebrates and they are hotspots for mutations by deamination of 5-methylcytosine (22) and other mechanisms (28,29,35). Other point mutations leading to dysfunctional C1 inhibitor molecules include Ala-434, Ala-436, and Val-432, among others (13,20,27,30). In addition, a deletion of Lys-251 that results in acquisition of a new N-glycosylation site (31), a nucleotide insertion (32), and a cluster of mutations within exon 5 (33) has also been described.

Our study represents the first report of a single base mutation at codon 445 (P-1' site of the reactive center) that changes Thr to a Pro. This substitution generates also a new restriction site recognized by Bsi YI.

MATERIALS AND METHODS

Patients

We studied five patients and two unaffected members of one family (ages from 18 to 72 years) with type II HANE (Fig. 1A). Consent informed was given to begin the study. The diagnosis of type II HANE was based on typical clinical symptoms and signs, normal antigenic C1 inhibitor plasma levels, low C4 levels as mea-

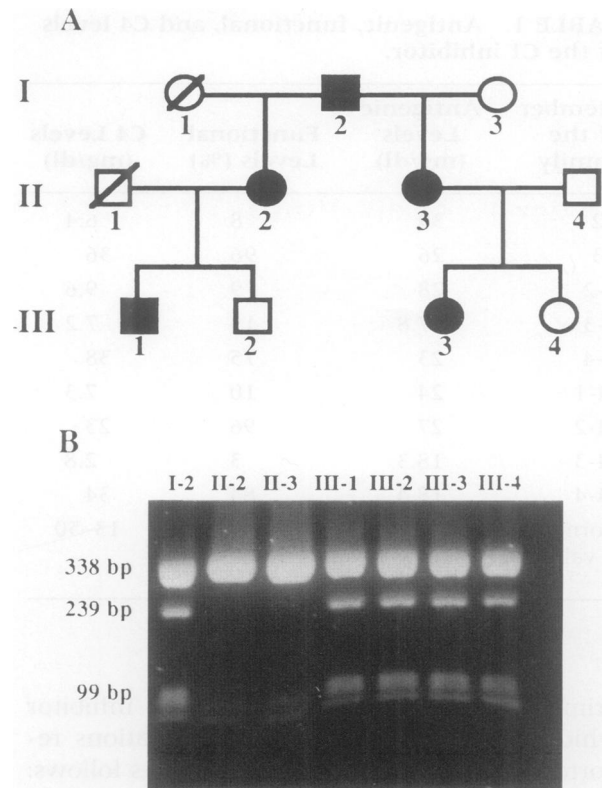


FIG. 1. Pedigree of the family with type II HANE and segregation of the BsiHKAI polymorphism

(A) The entire pedigree for the type II HANE family. Solid circles (females) and squares (males) represent affected individuals. Note the two marriages of the grandfather who had one affected daughter from each marriage. (B) Restriction fragment length polymorphism after digestion of the amplified exon eight with BsiHKAI (an isoschizomer of HgiAI). The normal PCR product is 338 bp and the BsiHKAI restriction fragments of the mutant allele are 239 bp and 99 bp in size. Lanes are numbered as in Panel A.

sured by a nephelometric technique (Behring Nephelometer-Analyzer), and low or absent functional C1 inhibitor activity as measured by a chromogenic assay (Immunochrom; Immuno, Vienna, Austria). The analytical values of all the subjects investigated in this study are shown in Table 1.

PCR Amplification of Genomic DNA

DNA was extracted from peripheral mononuclear cells of affected and healthy members of the family using standard procedures (34). The primers for the PCR analysis were designed from the published sequence (21). We selected intronic

TABLE 1. Antigenic, functional, and C4 levels of the C1 inhibitor.

Member of the Family	Antigenic Levels (mg/dl)	Functional Levels (%)	C4 Levels (mg/dl)
I-2	39	8	6.4
I-3	26	96	36
II-2	28	9	9.6
II-3	17.8	15	7.2
II-4	23	75	38
III-1	24	10	7.3
III-2	27	96	23
III-3	18.3	3	2.8
III-4	18.4	84	34
Normal values	15-35	>40	13-50

primers flanking exon eight of the C1 inhibitor which concentrates most of the mutations reported so far. The primer pair used is as follows: sense primer (5'-GCGAATTCTCATGCTGGCTTC TGA CTCTGT-3'), and antisense primer (5'-GCGA ATTCTCGCCCTAACCTGATCCTGCAG-3'). The specific primer pair resulted in a 338-bp fragment after PCR amplification. Each PCR reaction contained 1 μ g genomic DNA, 30 pmol of each primer, 200 μ M dNTPs (Pharmacia, Uppsala, Sweden), 1.5 mM MgCl₂, 10 mM Tris-HCl pH 8.3, 50 mM KCl, and 2.5 U Taq DNA polymerase (Promega, Madison, WI, U.S.A.) in a final volume of 50 μ l. Reactions were carried out in a GeneAmp PCR System 9600 (Perkin Elmer, Norwalk, CT, U.S.A.) and the PCR conditions were as follows: 95°C for 2 min as initial denaturation and 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 30 cycles. Amplified products were run on 1% agarose gel and visualized by ethidium bromide staining.

Cloning and Sequencing

The 338-bp PCR product encompassing exon 8, was fractionated on a 1% low-melting point agarose gel and extracted using the Wizard PCR preps system (Promega Corp., Madison, WI). The purified product was ligated with the pCRIITA-cloning vector (Invitrogen Corp., San Diego, CA, U.S.A.) and transformed into competent DH5 α cells (Life Technologies, Gaithersburg, MD, U.S.A.). DNA minipreps of 2-ml overnight cul-

tures were prepared with the Wizard minipreps DNA purification system (Promega) and sequenced using the Sequenase version 2.0 kit (United States Biochemical Corp., Cleveland, OH, U.S.A.) in accordance with the manufacturer's instructions. Both strands were sequenced for each PCR product from at least three independent clones.

RFLP Analysis

The purified PCR products (0.5-2 μ g) were digested overnight in a total reaction volume of 30 μ l with 10 U of either BsiHKAI (an isoschizomer of HgiAI) (New England Biolabs GmbH, Schwalbach, Taunus, Germany), or BsiYI (Boehringer-Mannheim GmbH, Mannheim, Germany), using the manufacturer's buffer. Digestion products were fractionated on a 2% agarose gel and stained with ethidium bromide.

RESULTS

RFLP Analysis of Alleles Coding for Dysfunctional C1 Inhibitor Protein

The HgiAI restriction fragment length polymorphism has been previously used to follow inheritance of individual C1 inhibitor alleles in HANE kindreds and to identify those carrying abnormal genes (22). In order to carry out this family study, exon 8 was amplified by PCR using intronic primers flanking the exon. The amplified product (338 bp) was then digested to completion with BsiHKAI (an isoschizomer of HgiAI) and the resultant fragments (239 and 99 bp) resolved by agarose gel electrophoresis. As shown in Fig. 1B, the BsiHKAI RFLP is present in both patients (I-2, III-1, and III-3 are heterozygous, and II-2 and II-3 are homozygous for this polymorphism) and unaffected members (III-2, III-4) of the family, which makes this polymorphism to be not informative in this family.

Nucleotide Sequence Analysis of the PCR Amplified Exon 8

Most of the mutations affecting the C1 inhibitor gene are located within exon 8, which encodes the reactive center of the protein. Exon 8 was amplified by PCR using primers flanking the entire exon, subcloned into a pCRIITA vector and sequenced from both ends.

The screening detected a missense mutation

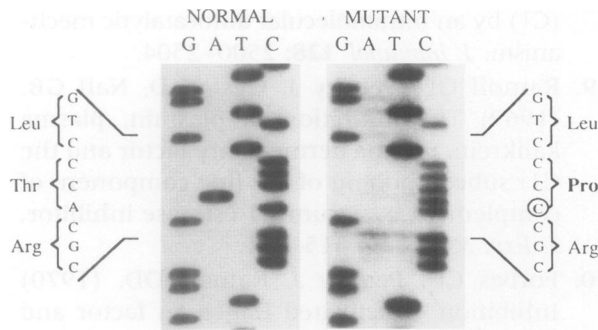


FIG. 2. DNA sequence of the PCR-amplified exon 8

On the left, partial sequence from a normal control. On the right, an A to C change (marked with a circle) in a sample of an individual with type II HANE (I-2) which is identical to those seen in the other affected members of the family.

that substitutes an A to C at nucleotide 16791. The substitution was detected in all family members affected with type II HANE disease but not in any unaffected members (Fig. 2). This mutation changes Thr to a Pro at codon 445. The substitution of an A to C creates a new BsiYI restriction site.

Digestion of Amplified Exon 8 with BsiYI

Digestion of the purified PCR product (338 bp) with BsiYI gave rise to two fragments with the expected sizes (200 and 138 bp), and we used this analysis to demonstrate the segregation of this mutation in the five affected members of the studied family (Fig. 3).

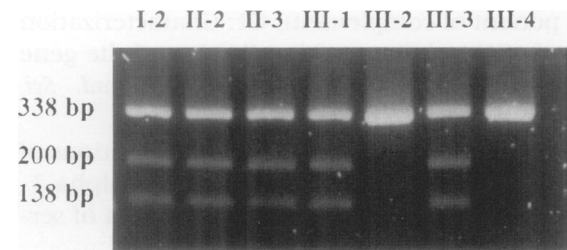


FIG. 3. BsiYI digestion analysis of PCR products

Digestion of the amplified exon 8 (338 bp) with BsiYI give rise to two fragments of 200 and 138 bp in the affected individuals. Lanes are as in Fig. 1.

DISCUSSION

Type II HANE represents almost 15% from total HANE and is characterized by normal to elevated levels of C1 inhibitor protein in plasma, but with reduced functional activity (1,2).

To date, mutations resulting in amino acid changes at the P1 site of the reactive center are the most frequent cause of type II HANE. Other substitutions located 5' to the reactive center are also responsible for the defect. These substitutions seem to generate conformational changes affecting interactions of the C1 inhibitor with its targets.

The reactive center mutations occur in a CG dinucleotide, which has been demonstrated to be a hotspot for mutations, probably resulting from deamination of 5-methylcytosine to thymidine (22,35).

Target protease specificity must be influenced by non-reactive-center residues, as many investigators have reported. Mutations that generate amino acids substitutions in the hinge region near the reactive center and in other regions of the protein also result in a dysfunctional C1 inhibitor molecule (13,20,27-33). Substitutions of Arg-444 by His, Cys, Leu, and Ser at the reactive center of the C1 inhibitor molecule are the most frequent defects responsible for the dysfunctional protein. Similarly, replacement of Ala434 (P-12) by Glu, Val432 (P-14) by Glu, and Ala436 (P-10) by Thr also causes the dysfunction. This is probably due to conformational changes in the loop near the reactive center. All SERPINS have a P-12 Ala residue that seems to be critical for the conformational change that occurs during successful interactions between SERPINS and serine proteinases, and this codon is conserved among SERPIN genes (13).

The reactive center of serpins mimics the substrate of the protease. Recognition of this reactive-center peptide bond results in complex formation between the amino acid residue amino-terminal to this bond (the P1 residue) and the active-site of the protease. Proteases inactivated by the C1 inhibitor recognize the Arg444 (P1)-Thr445 (P-1') dipeptide.

Here we report the first mutation involving the codon for Thr445, at the P-1' site of the reactive center, which is substituted by a Pro. This mutation leads to a new restriction site recognized by BsiYI. This new RFLP has never been observed among normal subjects and cosegregates with HANE in this family. The natural HgiAI polymorphism detected among normal in-

dividuals has not been useful for following the inheritance pattern of the molecular defect in this family because there were healthy members with the HgiAI polymorphism.

The substitution of a Thr by a Pro may interrupt the secondary structure of the protein, originating conformational changes that disturb the interaction of the C1 inhibitor molecule with its substrates. Finally, it is worthwhile to emphasize the enormous heterogeneity of this gene, as other authors have reported (13,27).

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