MUTATION IN BRIEF

Detection of C1 Inhibitor (SERPING1/C1NH) Mutations in Exon 8 In Patients With Hereditary Angioedema: Evidence For 10 Novel Mutations

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Hereditary angioedema (HAE) is caused by mutations in the C1 inhibitor gene (SERPING1, C1NH) and the result is C1 inhibitor deficiency, either in levels or function. We have searched exon 8 for mutations by direct sequencing and analyzed the rest of the exons by SSCP in 87 Spanish families affected by HAE. Out of 87 screened families, we have detected exon 8 mutations in 26. Among these, 17 different mutations were identified: 14 point mutations and 3 frameshift. Seven of the point mutations and the three frameshift were not previously reported. Mutations were: S438P; R444P; V451G; W460X; V468D; G471E; X479R; S417fsX427; I440fsX450; E429fsX450. The rest of the families presented previously reported mutations, 5 missense and two nonsense. In none of the 26 families was an additional change identified in the rest of the exons by SSCP, and, in 20 out of the 22 families with point mutation, we verified that the mutation did not affect a healthy relative. Seven of these families had no history of the disease, and in five of them we were able to verify that the progenitors did not have the mutation. Therefore, they were *de novo* mutations. © 2002 Wiley-Liss, Inc.

KEY WORDS: C1 inhibitor; C1NH; C1 inh; SERPING1; hereditary angioedema; HAE; complement

INTRODUCTION

Hereditary angioedema (HAE, MIM# 106100) is an autosomal dominant disease due to alterations of the C1-inhibitor gene (C1 inh or C1NH; approved symbol, SERPING1; MIM# 606860) (Rosen et al., 1971; Tosi, 1998). Individuals affected by HAE have recurrent episodes of subcutaneous tissue swelling, particularly of the gastrointestinal mucosa, and the upper airways, that can be life threatening (Agostoni and Cicardi, 1992; Späth and Wuthrich, 1998).

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The C1 inh gene (GenBank accession number X54486) maps to chromosome 11q12-q13.1 and consists of 8 exons distributed over a DNA stretch of 17 Kb, with most introns particularly rich in repetitive Alu sequences(Carter et al., 1991).

There are two HAE phenotypes: Type I and II. Patients with type I HAE (approximately 85%) have low antigenic and functional C1 inhibitor levels. Patients with type II HAE (approximately 15%) have normal or elevated C1 inhibitor concentrations, with low C1 inhibitor functional activity caused by the secretion of a dysfunctional C1 inhibitor protein (Rosen et al 1965, 1971).

Patients with type I and type II HAE have different patterns of C1 inhibitor mutation. The structural abnormalities in the C1 inh gene in patients with Type I HAE are very heterogeneous and include deletions, insertions and point mutations that lead to an apparent failure to synthesize or secrete the inhibitor protein. In affected members of families with Type II HAE, the abnormal allele usually presents a point mutation that produces a single amino acid change in the primary structure of the molecule. Almost all type II patients have missense mutations within the reactive loop of the C1 inhibitor. Mutations at Arg444 (the P1 residue) are estimated to be present in up to 70% of type II HAE (Parad et al., 1990). Sequencing of exon 8, which contains the critical hinge region and reactive center of the C1 inhibitor molecule, identified multiple mutations within exon 8 (Davis et al., 1993; Verpy et al., 1995; Bowen et al., 2001)

Identification of additional C1 inhibitor mutations causing HAE could help to elucidate structure-function relationships.

MATERIALS AND METHODS

Patients

Eighty-seven independent unrelated families were studied. They were previously diagnosed with hereditary angioedema due to C1 inhibitor deficit and followed up at different centers around the country. All human samples were obtained with informed consent. The Ethical Committee of the Hospital Universitario "La Paz" granted ethical approval. White blood cells, serum and plasma were obtained from at least one member of each family and in most cases samples were also obtained from one healthy relative.

Complement studies

The laboratory diagnosis of HAE was made through demonstration of decreased C1 inhibitor antigenic or functional levels. C3, C4, C1 inhibitor levels were measured by nephelometry and, in patients without family history, C1q was measured to rule out acquired angioedema. C1 inhibitor function was measured by an assay of the ability of the C1 inhibitor to bind C1s as previously described (De Smet et al., 1993).

Screening for point mutations at exon 8

Eighty-seven patients and 60 healthy controls were screened for mutation by automatic sequencing. Genomic DNA was extracted from peripheral blood leukocytes with a Puregene kit (Gentra Systems. Minneapolis, MN) according to the manufacturer's instructions. Genomic DNA (1 µg) was subjected to 30 cycles of PCR amplification in a final volume of 50 µl using an AmpliTaq DNA polymerase kit (Applied Biosystems, Foster City, CA), 0.2 mM dNTPs, 1.5 mM $MgCl_2$ and 17 pmols of the primers that amplify exon 8 and exon-intron boundaries (Verpy et al., 1996) (forward: 16474 GAGGATCCCACGAACTGCCAG; reverse: 17128 GTGAACTTGAACTAGAGAAAGC) in the presence of 2.5 U of Taq polymerase. The reaction was performed using a Perkin Elmer Cetus 9600 (Applied Biosystems) thermocycler according to the following conditions: 1 min each at 95 ^{o}C, 59 ^{o}C, and 72 ^{o}C. Amplified product was purified from the 2% low melting agarose gel by using a QIAquick gel extraction kit (Qiagen Inc., Valencia, CA). The sequence reactions were performed by using a DNA sequencing kit (Big Dye terminator cycle sequencing. Applied Biosystems). Each purified product was sequenced with three different primers, the two primers used in the PCR reaction and a third inner primer (Bissler et al., 1997) in order to sequence the entire active center: 16609 TTCTGACTCTGTTTTTCTCTGGTTTTGCCC. The sequencing products were precipitated with ethanol/sodium acetate and analyzed on an automated ABI-PRISM 310 (Applied Biosystems). Nucleotides were numbered according to Carter et al. (1991). All the mutations were verified with a second independent PCR product.

SSCP analysis

Exons 1 to 7 and their flanking intronic regions were amplified by PCR using 500 to 1000 ng of template DNA and specific primers for each exon (Table 1), except exon 3 that was amplified in 3 overlapping segments.

The PCR products were mixed with a loading buffer (95% formamide, 10 mM EDTA, 50 mM NaOH, 0.05% xylene cyanol, 0.05% bromophenol blue), heat denatured and then resolved on polyacrylamide gels under two different conditions: a) percentage of acrylamide optimized for each exon, acrylamide/bisacrylamide ratio 29:1, run at 4 ^{o}C and constant 140-180 V current, for 15-18 hours. Except exon 4 which was run at room temperature for 8 hours and exon 3c which contained 7.5% glycerol; b) 10% acrylamide, acrylamide/bisacrylamide ratio 37.5:1, run at room temperature, constant 250-350 V current, for 3-6 hours.

Table 1. C1 Inhibitor Primers Used for SSCP Analysis (Exons 1 to 7)

Exon	Forward primer	Reverse primer	Ta	Fragment size (bp)
1*	ATTTGTAACTGGGCAGTGTCCCGG	AAGTCCCAGGTGGAAGCAAGCCTA	58	271
2*	AGGGAGGAGGAGGGAATTCGCTAA	GCCTGAAGGGTTAATCCTCAGCCA	61	294
3a	GGACTGTGCCTCGTAGTAAGA	GGTTGTTGAGTTGGTTGTCGG	55	217
3b	AAGGGAAGGTCGCAACAACAGT	GCCTCTGTTGAATGACTCTCC	55	295
3c	CTCCTACCCAGCCCACTACT	TCTGGATTGGTGACTCTTATGG	55	280
4+	GCAAGTATCTTTCATCTCTGCCCTTTGTTG	CTCTGCAGACACTGCCCATTCCTGGGCACT	60	201
5+	GACTCATGCCTCCCTTTCTCAACATACCCC	AATGGGAAGACTAACTGGCTAAGGGCACCC	60	271
6+	CATTAGAGCAACCCTCCCACCTCTTCCCTC	TTCAAACAGGAGAAGGAAGGTTAAGAACT	60	206
7+	GAAGACTGTTAAGGTGCATCTCTTATTTTC	GCCTGGGAGTAACCCTAAGCTGCCAGAGCT	55	286

* According to Zuraw et al.

+ According to Bissler et al.

RESULTS

We have sequenced exon 8 of the C1 inhibitor gene in 87 HAE families (10 type II and 77 type I, according to C1 inhibitor levels and function) and in 60 healthy controls in order to rule out polymorphic variations. We have identified 17 different mutations within the C1 inhibitor exon 8 coding region (Tables 2 and 3) affecting 26 unrelated families (29,88 %). Out of the 17 mutations, 14 were point mutations (3 nonsense and 11 missense) affecting 22 families, and 3 were frameshift mutations affecting 4 families.

All the mutations were studied at both strands and verified with a second independent PCR product. We did not find any additional change in the rest of the exons by SSCP. Ten of these mutations were previously unreported. Seven of these families did not have family history, and, in five of them, both recognized parents were available for DNA and plasma studies; C1 inhibitor levels and function were normal in both parents and C4 was within the normal range. In these cases, the progenitors did not present the mutations of the affected descendant; thus, we were able to demonstrate that they were *de novo* mutations (6 %) (Patients: BW, AW, BS, DK and AP).

In 20 of the 22 families with point mutations we were able to demonstrate that exon 8 of the C1 inhibitor gene was not mutated in at least one healthy relative, since for two patients (AV and DI) no healthy relatives were available for study.

Table 2. Point Mutations in Exon 8

Patient Code	Angioedema Type	Nucleotide Change	Predicted effect on C1 inhibitor protein	Family history
BW	I	g.16720A>G	p.H421R	No^{+}
I	II	g.16759C>A	p.A434E	No
DI	**II**	**g.16770T>C**	**p.S438P**	**No**
AA	II	g.16788C>T	p.R444C	Yes
AW	II	g.16788C>T	p.R444C	No^{+}
BR	II	g.16788C>T	p.R444C	Yes
DT	II	g.16788C>T	p.R444C	Yes
BS	II	g.16789G>A	p.R444H	No^{+}
DK	II	g.16789G>A	p.R444H	No^{+}
AQ	I-II*	g.16789G>T	p.R444L	Yes
BD	**II**	**g.16789G>C**	**p.R444P**	**Yes**
LL	**I**	**g.16810T>G**	**p.V451G**	**Yes**
DS	**I**	**g.16838C>T**	**p.W460X**	**Yes**
Z	I	g.16842C>T	p.Q462X	Yes
BX	**I**	**g.16861T>A**	**p.V468D**	**Yes**
AV	**I**	**g.16870G>A**	**p.G471E**	**Yes**
AP	I	g.16872C>T	p.R472X	No^{+}
AR	I	g.16872C>T	p.R472X	Yes
BY	I	g.16872C>T	p.R472X	Yes
DR	I	g.16872C>T	p.R472X	Yes
Q	I	g.16872C>T	p.R472X	Yes
X	**I**	**g.16893T>A**	**p.X479R**	**Yes**

* Intermediate phenotype.

$^{+}$Verified *de novo* mutations.

New mutations are in **boldface** type.

Table 3. Exon 8 Frameshift Mutations in Type I Angioedema Patients*

Patient Code	Mutation	Predicted Change	Family history
AC	g.16707delT	p.S417fsX427	Yes
AÑ	g.16775-16776insC	p.I440fsX450	Yes
R	g.16742-16743insA	p.E429fsX450	Yes
Y	g.16742-16743insA	p.E429fsX450	Yes

*None of these frameshift mutations were previously reported.

DISCUSSION

We have performed mutational studies at exon 8 of the C1 inhibitor gene in a group of 87 unrelated families affected by HAE. Of these, 10 were Type II angioedema families and the rest type I. In all, we found 14 different point mutations affecting 22 families and 3 frameshift mutations affecting four.

We detected three different nonsense mutations affecting seven families. A previously unreported one affected the DS family. This mutation corresponded to a C to T transition at position 16838 and it originated the appearance of a stop codon after Trp460. The second nonsense mutation involved a C to T transition at position 16842 (patient Z). The result of this mutation was translation termination at C1 inhibitor aminoacid 461. Other authors (Siddique et al 1993) found this mutation. Five families (AP, AR, BY, DR and Q) presented a C to T transition at position 16872 that was responsible for the Arg472stop change. This mutation produced the synthesis of a truncated protein seven residues shorter than normal, which was rapidly degraded intracellularly (Verpy et al., 1995). Several authors (Verpy et al., 1996; Bissler et al., 1997; Zuraw et al., 2000) previously found this change.

We found 11 different missense mutations affecting 15 families. The observed changes in these families were absent in a sample of 60 healthy controls. Six of these were previously unreported and they affect patients: DI, BD, LL, BX, AV and X. Furthermore, in four of them (BD, LL, BX and X) the mutation is absent in a healthy relative.

Among the eleven missense mutations six are type II HAE mutations, four directly affecting the active center, and two outside the active center.

The four type II missense mutations at the active center concern Arg444. In our series, the Arg444Cys mutation was found in four patients (AA, AW, BR and DT) and the Arg444His mutation in two (BS and DK). These are the most frequent active center mutations and they have been widely reported (Skriver et al., 1989; Donalson and Bissler, 1992; Bissler et al., 1997; Pappalardo et al., 2000; Zuraw and Herschbach, 2000). Both mutations are caused by the deamination of the dinucleotide CpG that is considered to be a "hotspot" for mutation in vertebrate genomes. The third mutation at the active center introduced the aminoacid Leu instead of Arg and was previously found in Type II (Frangi et al 1992; Bissler et al 1997; Pappalardo et al 2000; Zuraw et al 2000). Taking into account C1 inhibitor levels, patient AQ is type I, but the mutation found corresponds to Type II. Recently, Pappalardo et al 2000 described that this mutation resulted in a biochemical phenotype that is intermediate between type I and Type II. This patient also presents this phenotype (C1 inhibitor levels: 70%; C1 inhibitor function: 9%). The unreported mutation at the active center corresponds to a G to C change at position 16789 with a change of Arg to Pro (patient BD).

There were two type II patients who had a mutation outside the active center (Patient I and DI). Patient I presented a change C to A at position 16759 with a change of Ala434Glu. This mutation was found in type II patients by other authors (Skriver et al., 1991). Patient DI presented a substitution of T by C at position 16770, with a change of Ser for Pro at codon 438 close to the active center. Several mutations have been described around this site, mostly in Type II patients (Späth and Wuthrich, 1998).

The other five missense mutations were found in type I patients. One was previously described (His421Arg) and four were first reported in this paper (Val451Gly, Val468Asp, Gly471Glu, Stop479Arg). Three out of these new four mutations: Val451Gly; Val468Asp and Gly471Glu are located at a hydrophobic pocket, which is highly conserved among the serpins (Eldering et al., 1995). Val451 is located, according to the antitrypsin model, in the β sheet C strand 1. This residue is important for the folding of C1 inh into its native conformation (Huber and Carrell, 1989). The other two residues (Val468 and Gly471) are located, according to the antitrypsin model, in strand 5 of the β sheet B, which is one of the most conserved features of serpins. Eldering et al. (1995) have suggested that mutations in this region, could induce a conformational change in the protein that could explain the lost of inhibitory activity. The most unusual missense mutation corresponds to a transversion of T to A (TGA→AGA) at position 16893 (patient X). This change implies the conversion of the stop codon to Arg. The resultant protein could be as long as 563 aminoacids instead of 478 (normal protein). Western blot analysis using monoclonal antibodies and 7.5% polyacrylamide gels failed to identify a band corresponding to the longer than normal 478 amino acid protein, suggesting that it is not synthesized, secreted or, alternatively, is rapidly degraded in plasma. Mutations at the stop codon have not been previously described in HAE patients but they have been found in other genes as the α2-globin (HBA2), causing hemoglobin Constant Spring, an abnormal haemoglobin that occurs frequently in South-East Asia (Clegg et al., 1971; Milner et al., 1971). The associated α–globin chain is 172 aminoacids in length, rather longer than the normal 141 amino acids, as a result of a TAA→CAA transition in the termination codon. In these patients, translation extends into the 3' non-coding region of the α2-globin (HBA2) mRNA. The Constant Spring HBA2 mRNA is highly unstable, resulting in low haemoglobin production in the red blood cells of heterozygous carriers (Hunt et al., 1982).

Eleven of the 14 missense mutations were clustered at the active center or downstream from the reactive site, in the COOH-terminal region of the C1 inh. Mutations in this region have been considered to prevent proteins folding properly (Verpy et al., 1995).

In four patients we found three frameshift mutations, one produced by the deletion of a T and two by the insertion of a single base (Table 3). These mutations were previously undescribed. One of these mutations, an

insertion of A after position 16742, affected two unrelated patients (R and Y). Two of these mutations (16775-16776insC, 16742-16743insA) were located in an imperfect repeat, or quasipalindrome (nucleotides 16740-16787). This molecular region might be considered as a sequence that facilitates frameshift mutations (Bissler et al., 1998).

In conclusion, in a total of 87 families we found that 26 presented mutations at exon 8, as the only alteration. The identification of particular mutations in HAE families, where the mutation is specific for each affected family, is crucial for the correct diagnosis of relatives showing minor or inexistent clinical symptoms and only slightly reduced complement levels.

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REFERENCES

Agostoni A, Cicardi M. 1992. Hereditary and acquired C1-INH deficiency: biological and clinical characteristics in 235 patients. Medicine 71: 206-215.

Bissler JJ, Aulak KS, Donaldson VH, Rosen FS, Cicardi M, Harrison RA, Davis III AE. 1997. Molecular defects in hereditary angioneurotic edema. Proc Assoc of Am Physicians. 109: 164-173.

Bissler JJ, Meng Q-S, Emery T. 1998. C1 inhibitor gene sequence facilitates frameshift mutations. Molecular Medicine 4: 795-806.

Bowen B, Hawk JJ, Sibunka S, Hovick S, Weiler JM. 2001. A review of the reported defects in the human C1 esterase inhibitor gene producing hereditary angioedema including four new mutations. Clin Immunol 98: 157-163.

Carter P, Duponchel C, Tosi M, Fothergill JE. 1991. Complete nucleotide sequence of the gene for human C1 inhibitor with an unusually high density of Alu elements. Eur J Biochem 197: 301-308.

Clegg JB, Weatherall DJ, Milner PG. 1971 Haemoglobin Constant Spring - a chain termination mutant?. Nature 234: 337-340.

Davis AE III, Bissler JJ, Aulak KS. 1993. Genetic defects in the C1 inhibitor gene. Complement Today 1: 133-150.

De Smet BJ, De Boer JP, Agterberg J, Rigter G, Bleker WK, Hack CE. 1993. Clearance of human native, proteinase-complexed, and proteolytically inactivated C1-inhibitor in rats. Blood 81: 56-61.

Donaldson VH, Bissler JJ. 1992. C1-inhibitors and their genes: an update. J Lab Clin Med 119: 330-333.

Eldering E, Verpy E, Roem D, Meo T, Tosi M. 1995. COOH-terminal substitutions in the serpin C1 inhibitor that cause loop overinsertion and subsequent multimerization. J Biol Chem 270: 2579-2587.

Frangi D, Aulak KS, Cicardi M, Harrison RA, Davis III AE. 1992. A dysfunctional C1 inhibitor protein with a new reactive center mutation (Arg444-Leu). FEBS Lett 301: 34-36.

Huber R, Carrell RW. 1989. Implications of the three-dimensional structure of α1 -antitrypsin for striker and functions of serpins. Biochemistry 28: 8951-8966.

Hunt DM, Higgs DR, Winichagoon P, Clegg JB, Weatherall DJ. 1982. Haemoglobin Constant Spring has un unstable a chain messanger RNA. Br J Haematol 51:405-413.

Milner PF, Clegg JB, Wetherall DJ. 1971. Haemoglobin H disease due to a unique haemoglobin variant with an elongated a chain. Lancet i: 729-732.

Pappalardo E, Cicardi M, Duponchel Ch, Carugati A, Choquet S, Agostoni A, Tosi M. 2000. Frequent de novo mutations and exon deletions in the C1 inhibitor gene of patients with angioedema. J Allergy Clin Immunol 106: 1147-1154.

Parad RB, Kramer J, Strunk RC, Rosen FS, Davis III AE. 1990. Dysfunctional C1 inhibitor Ta: deletion of Lys-251 results in acquisition of an N-glycosylation site. Proc Natl Acad Sci USA 87: 6786-6790.

Rosen FS, Alper CA, Pensky J, Klemperer MR, Donaldson VH. 1971. Genetically determined heterogeneity of the C1 esterase inhibitor in patients with hereditary angioneurotic edema. J Clin Invest 50: 2143-2149.

Rosen FS, Charache P, Pensky J, Donaldson VH. 1965. Hereditary angioneurotic edema: two genetic variants. Science 148: 957-958.

Siddique Z, McPhaden AR, Fothergill JE, Whaley K. 1993. A point mutation in the C1-inhibitor gene causes type I hereditary angioedema. Hum Hered 43: 155-158.

Siddique Z, McPhaden AR, Whaley K. 1992. Type II hereditary angio-edema associated with two mutations in one allele of the C1-inhibitor gene around the reactive-site coding region. Hum Hered 42: 298-301.

Skriver K, Radziejewska E, Silbermann JA, Donaldson VH, Bock SC. 1989. CpG mutations in the reactive site of human C1 inhibitor. J Biol Chem 264: 3066-3071.

Skriver K, Wikoff WR, Patston PA, Tausk R, Schapira M, Kaplan AP, Bock SC. 1991. Substrate properties of C1 inhibitor Ma (Alanine 434-Glutamic acid). Genetic and structural evidence suggesting that the P12-region contains critical determinants of serin protease inhibitor/substrate status. J Biol Chem 266: 9216-9221.

Späth PJ, Wüthrich B. 1998. Inherited and acquired deficiencies of C1 esterase inhibitor in humans. In: Rother K, Till GO and Hänsch GM editors. The Complement system. 2nd ed. Berlin Heidelberg: Springer-Verlag p. 353-410.

Tosi M. 1998. Molecular genetics of C1 inhibitor. Immunobiology 199: 358-365.

Verpy E, Biasotto M, Brai M, Misiano G, Meo T, Tosi M. 1996. Exhaustive mutation scanning by fluorescence-assisted mismatch analysis discloses new genotype-phenotype correlations in angioedema. Am J Hum Genet 59: 308-319.

Verpy E, Couture-Tosi E, Eldering E, López-Trascasa M, Späth P, Meo T, Tosi M. 1995. Crucial residues in the carboxi-terminal end of C1 inhibitor revealed by pathogenic mutants impaired in secretion or function. J Clin Invest 95: 350-359.

Zuraw BL, Herschbach J. 2000. Detection of C1 inhibitor mutations in patients with hereditary angioedema. J Allergy Clin Immunol 105: 541-54.