

Letter to the Editor

De novo homozygous mutation of the C1 inhibitor gene in a patient with hereditary angioedema*To the Editor:*

Hereditary angioedema (HAE; OMIM #106100) is a rare autosomal-dominant disease resulting from congenital deficiency of C1 esterase inhibitor protein (C1-INH) that controls complement, contact-kinins, coagulation, and fibrinolytic cascades.¹ Symptoms relate to acute edema of subcutaneous tissues, viscera, and the upper airways (submucosal tissue). Edema of the larynx is the most fearsome feature of this disorder and is often life-threatening.²

HAE due to C1-INH deficiency occurs as type I (85%), characterized by functional and antigenic C1-INH deficiency, or type II (15%), characterized by normal C1-INH antigenic levels and reduced functional activity.^{1,2} Individuals affected by type I and II HAE carry a mutation in the C1-INH gene (*C1NH*, *SERPING1*; OMIM #606860).^{3,4} The *C1NH* gene maps onto chromosome 11q12-q13.1 and is organized into 8 exons and 7 introns, particularly rich in repetitive *Alu* sequences.⁵ More than 300 deficiency-causing mutations have been identified, and approximately 25% of them occur *de novo*.⁶ Patients with HAE are generally heterozygous for the *C1NH* gene mutation; only recently, 3 subjects from 2 different families with a homozygous genetic defect have been identified.^{7,8}

We describe here the molecular genetic analysis of a 21-year-old woman who showed clinical and laboratory findings typical of HAE; we also provide evidence for a *de novo* homozygous mutation in the *C1NH* locus. The proband presented with recurrent peripheral and abdominal attacks of angioedema, starting at the age of 16 years. Both parents and 1 sibling did not manifest a history of angioedema attacks. The patient experienced about 8 to 10 attacks per year, with about 50% of them as abdominal attacks and 50% at cutaneous or facial sites. There was no history of laryngeal attack. On-demand treatment of acute attacks with icatibant led to prompt resolution of symptoms in the majority of cases.

The laboratory assessment showed very low antigenic and nondetectable functional levels of C1 INH and reduced C4, with normal levels of C3 and C1q. Neither the parents nor the sibling showed alterations in complement parameters (Table I).

Western blot analysis of plasma protein with a specific rabbit antihuman C1 INH antibody revealed the presence of the 2 bands (105 and 96 kD, native and cleaved form of C1 INH protein, respectively) both in parents and in the sibling, while no significant bands were evident in the proband plasma (Fig 1, A).

Mutational screening of *C1NH* in the proband allowed us to identify a novel genetic defect, the insertion/deletion insTCAGTGTCTGdelA at position 646 in exon 4, p.Lys216-Serfs*4 (Fig 1, B). Interestingly, the proband was homozygous for the mutation. This genetic defect has been not previously reported and was absent in 100 healthy and unrelated subjects used as control. The complete sequencing of the *C1NH* revealed 2 additional single nucleotide polymorphisms (SNPs), that is, Val458Met in exon 8 and IVS7-20A>G, that were previously reported not to affect C1 INH activity. Genetic analysis of *C1NH* of both parents showed that they did not carry the mutation of the affected daughter; thus, the genetic defect of the proband was a

TABLE I. Clinical and laboratory features of the proband and relatives

Parameter	Proband	Father	Mother	Sibling
Clinical HAE	Yes	No	No	No
C1 INH antigenic (NV 21–32 mg/dL)	4	28	30	32
C1 INH functional (NV 68%-132%)	ND	108	102	104
C1q (NV 70%-130%)	83	105	93	110
C4 (NV 10-40 mg/dL)	6	18	21	19

ND, Not detectable; NV, normal values.

de novo mutation. To rule out that a small deletion of exon 4 could simulate a homozygosity in the patient, we used different procedures. First, we performed a long-range PCR by amplifying a segment of *C1NH* that encompasses the repetitive *Alu* sequences 1 to 8 surrounding exon 4. As shown in Fig 1, C, only a fragment of 3.9 kb was observed in the proband and her parents, as expected by amplifying the wild-type allele with no deletion. Second, we performed *RsaI* restriction analysis of genomic DNA by amplifying a 2.9-kb fragment encompassing exon 4 (Fig 1, D). PCR products from the proband were completely digested into subfragments of 1301, 1053, and 528 bp, consistent with the absence of a small deletion. Finally, quantitative real-time PCR analysis was performed by amplifying a 202-bp fragment carrying the homozygous mutation. The signal for the *C1NH* product was normalized to that of the transferrin receptor gene, as reported in the Methods section (see the Online Repository at www.jacionline.org). We found that the proband showed a $\Delta\Delta C_t$ ratio of 0.99; her father and mother had a ratio of 1.05 and 1.21, respectively, in agreement with the $\Delta\Delta C_t$ ratio measured in 10 healthy subjects (1.16 ± 0.1). This result suggests a normal amount of the exon 4 PCR product in the proband and her parents.

Having excluded the deletion of exon 4, we investigated by means of intragenic polymorphisms whether uniparental isodisomy or gene conversion could have occurred. A panel of 9 SNPs was evaluated in the family. Among these SNPs, 8 were fully informative and showed typical Mendelian inheritance with paternal and maternal alleles that were detected in the proband (see Fig E1 in this article's Online Repository at www.jacionline.org). However, because the 2 informative SNPs closer to the mutation are located 251 bp upstream and 1070 bp downstream of the homozygous ins/del, a small gene conversion within this region cannot be completely excluded, possibly mediated by *Alu* sequences 1 to 8. If the ins/del mutation had occurred postzygotically, one could expect a mosaic pattern in the DNA of the patient. Then, we performed genetic analysis on DNA isolated from buccal cells, which have ectodermal origin, a convenient alternative for collecting genetic material. The inspection of the sequence chromatogram confirmed the presence of the mutation in the proband (Fig 1, B).

Blanch et al⁷ reported the first 2 siblings with the I440S missense mutation occurring in homozygosity. Analysis of the family demonstrated consanguinity and the presence of multiple heterozygous relatives who were free of symptoms. In 2010, López-Lera et al⁸ described a new case of homozygous deficiency in a patient carrying the R378C substitution. Five asymptomatic relatives of the proband were heterozygous for the mutation.

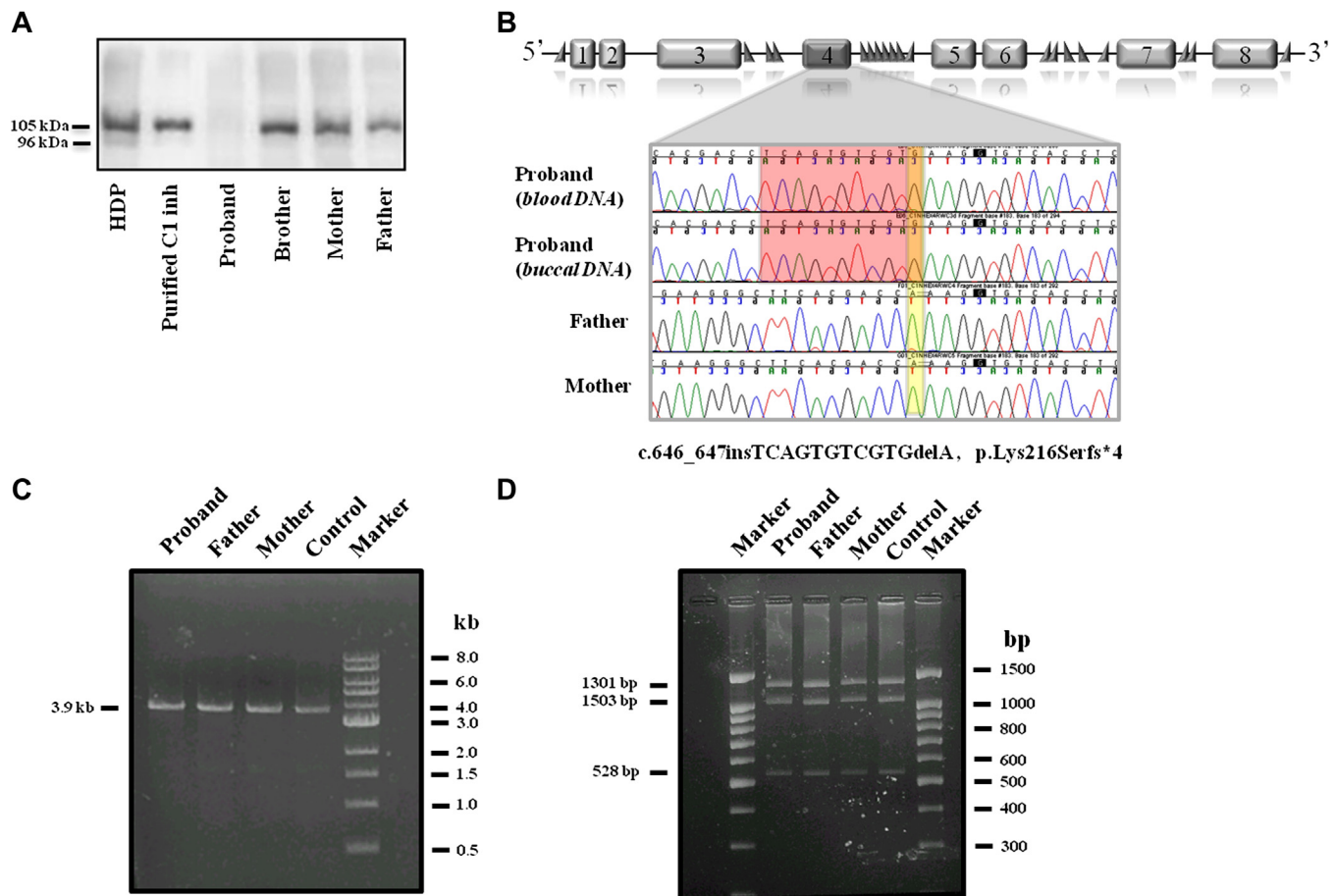


FIG 1. Biochemical and genetic studies on the ins/del mutant. **A**, Western blot of plasma samples of the family members showing the native (105 kd) and cleaved (96 kd) forms of C1-INH. **B**, Sequence chromatograms showing the presence of the mutation in the DNA of the proband and her parents. **C** and **D**, Long-range PCR and *RsaI* restriction analysis in the proband and family members. *HDP*, Healthy donor plasma.

This is the fourth HAE patient with a homozygous defect of the *C1NH* gene. The specific genetic alteration was not reported before, even in heterozygous form. The patient had a disease resembling phenotypically to the heterozygous form of HAE. The intensity, frequency, and localization of attacks did not show peculiar features, and did not delineate a more severe disease. In the previous reported cases, the disease course was severe in 1 and milder in 2.^{7,8} Moreover, while the other 3 cases showed undetectable circulating C1q levels (a feature of acquired angioedema), our patient had normal levels of C1q, as normally observed in heterozygous HAE patients.⁹

Our patient showed very low antigenic levels of C1 INH by standard nephelometry; however, western blot analysis did not show any evident 105 or 96 kDa band related to the C1 INH protein, and the functional assay of C1 INH showed undetectable activity. A different sensitivity or discriminating capacity at low concentration of the clinical tests used could account for this discrepancy.

Genetic analysis showed that the proband carried the c.646_647 insTCAGTGTCTGdelA located in exon 4 that introduced a premature stop codon, 3 residues after the first affected amino acid at position 216 of the mature protein. The ins/del mutation had not previously been described, but it could be considered pathogenic because it segregated with the C1 INH

deficiency in the family, was the only change in the *C1NH*, and was not present in a healthy control population. Unlike the other homozygous cases reported, the proband is the first example of a true null mutation in which there is no protein secretion, as confirmed by western blot.

In summary, this is the first report of a patient with HAE due to a homozygous *de novo* null mutation of the *C1NH* gene. In contrast with the previously reported cases, the proband had a clinical phenotype resembling the typical heterozygous form of HAE, as expected in a classical Mendelian-dominant disorder. Further investigations are required to demonstrate the specific mechanism underlying the mutation.

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Disclosure of potential conflict of interest: V. Montinaro has consultant arrangements with Shire HGT and Viropharma; has grants/grants pending with CSL Behring; has received payment for lectures, including service on speakers' bureaus, from CSL Behring and Shire HGT; has received payment for development of educational presentations from Shire HGT; and has received travel/accommodations/meeting expenses from Shire HGT, CSL Behring, and SOBI. The rest of the authors declare that they have no relevant conflicts of interest.

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METHODS

Study subjects

We report the clinical and laboratory features of a 21-year-old Caucasian woman who showed recurrent attacks of angioedema. Both parents and 1 sibling of the proband were also investigated for circulating levels of C1 INH, while the genetic analysis was performed in the proband and her parents. The study was carried out in accordance with the Principles of the Declaration of Helsinki, and a written informed consent was obtained from the proband and her parents.

Laboratory diagnosis

Serum and plasma EDTA samples were obtained from all the family members. An aliquot of serum was directly used to measure the complement levels. The remaining serum and plasma samples were frozen at -80°C until used. C1-INH and C4 antigenic levels were assessed by using nephelometry (Siemens, Milano, Italy), while C1 INH functional activity was evaluated by using an EIA test (MicroVue C1 inhibitor Enzyme Immune Assay, Quidel Co, San Diego, Calif). C1q levels were measured by using the Human Complement C1q Binding Radial Immunodiffusion Kit (The Binding Site Group Ltd, Birmingham, United Kingdom).

Western blot analysis of C1-INH

Plasma samples from the proband and relatives were run on 7.5% SDS-PAGE under nonreducing conditions; 0.25 to 2.0 μL of plasma was analyzed in different experiments. Fifty nanograms of purified C1-INH (Berinert; CSL Behring, Marburg, Germany) were run in each experiment. After the electrophoretic run, proteins were transferred onto a polyvinylidene fluoride membrane (Millipore, Bedford, Mass) at 250 mA and 4°C overnight. The filter was blocked with 5% nonfat dry milk powder in PBS that contained 0.1% tween-20 (TBS) and incubated with a rabbit anti-human C1-INH antibody (provided by Prof. M. Daha, University of Leiden) 1:1000 dilution in TBS for 2 hours at room temperature. The membrane was washed twice in TBS and incubated with horseradish peroxidase-conjugated sheep anti-rabbit IgG (Santa Cruz Biotechnology; Santa Cruz, Calif) 1:10,000 dilution in TBS. Immune complexes were detected by using the ECL enhanced chemiluminescence system (Amersham, GE Healthcare, Milano, Italy), as recommended by the manufacturer. Bands were quantified by using the Image J 1.34 Software.

Molecular diagnosis

Genomic DNA was isolated from peripheral blood leukocytes according to standard protocols.^{E1} Genetic analysis was performed by direct DNA sequencing. Proband DNA was also isolated from buccal mucosa cells by using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA eluates were kept at 4°C while undergoing genotyping assays and kept at -80°C for long-term storage.

We have standardized the PCR conditions by using primers designed with Primer 3 software (www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) on the basis of known sequences of *C1NH* as reported in ENSEMBL database (Wellcome Trust Sanger Institute, Cambridge, United Kingdom): *SERPING1* ENSG00000149131.

Briefly, PCR was carried out on 50- μL samples in a Bio-Rad thermal cycler (Bio-Rad Laboratories, Inc, Hercules, Calif). Each sample contained 0.15 μg of genomic DNA, 0.3 μM of each primer, 200 μM of deoxyribonucleotide triphosphates, 1X PCR buffer (with 1.5 mM MgCl_2), and 1.5 U of AmpliTaq gold polymerase with appropriate 1X buffer (Applied Biosystems, Inc, Foster City, Calif). PCR products were purified and subjected to direct-cycle sequence analysis by using the Taq dye-deoxy terminator method and an ABI Prism 3100 genetic analyzer (Applied Biosystems) according to the manufacturer's instructions. Primers and PCR conditions for DNA analysis are reported in Table E1. We used samples from 100 unrelated healthy subjects as the control group to distinguish novel mutations from rare polymorphisms.

Deletion tests

To investigate the presence of a deletion of exon 4 in *C1NH* gene, we used different techniques reported below.

• Long-Range PCR

Long-range PCR was performed as reported by Roche et al.^{E2} The selected primers amplified a segment of *C1NH* encompassing the repetitive *Alu* sequences 1 to 8 that surround exon 4. The expected 3.9-kb fragment will be barely detected in the patients with deletion because the PCR preferentially will amplify the shorter bands corresponding to the alleles carrying the mutation.

The PCR was performed in a final volume of 50 μL , using 1.5 U of TaKaRa La Taq DNA polymerase (Takara Bio, Inc, Otsu, Shiga, Japan), 15 pmol of each primer, 200 μM of deoxyribonucleotide triphosphate, and 0.15 μg of genomic DNA. The cycling conditions were as follows: 30 cycles at 98°C for 30 seconds, 56°C for 30 seconds, and 72°C for 5 minutes. The final extension was 72°C for 5 minutes. PCR products were separated in a 0.9% agarose-ethidium bromide gel. To confirm accuracy of the results, long-range PCR products were also sequenced by conventional DNA sequencing (data not shown).

• PCR-RFLP

PCR-RFLP was conducted in a 15- μL reaction volume containing *Rsa I* (New England Biolabs, Beverly, Mass) restriction enzyme and genomic DNA amplified by PCR by using the following primers: int3.3fw 5'-TTGTCCAGCTACTTGGGAGG3' and int4.2rw 5'-GCTCATTTTTGGGTCAAAGG3'. The restriction enzyme cleaved the PCR product of 2.9 kb in 3 fragments of 1301, 1053, and 528 bp, respectively. Upon digestion performed at 37°C overnight, the resulting DNA fragments were visualized in 4% agarose gel.

• Quantitative real-time PCR

The quantitative real-time PCR assay to measure gene dosage was performed by using the 7500 Fast Real Time PCR system (PE, Applied Biosystems) according to the manufacturer's protocol. Primers to amplify the exon 4 of *C1NH* were RTex4fw: 5'-TCTTTCATCTCTGCCCTTGTG-3' and RTex4rw: 5'-AGTGTCTGCAGAGGAGGGTCC-3'. As a reference gene, we used the transferrin receptor (*TFRC*): TFRC forward primer is 5'-ACGGAATATGAAGATGATCTCAGCAAGG-3'; TFRC reverse primer is 5'-GCGCAGATCACGAGATGGA-3'.^{E3} PCR was performed in a total volume of 20 μL in each well, containing 10 μL of SYBR Green PCR Master mix (PE, Applied Biosystems), 20 ng of genomic DNA, and 15 pmol of *C1NH* and *TFRC* primers. Each sample was run in triplicate. To make sure that the amplification efficiencies for the 2 segments of the target and reference gene were similar, standard curves were generated with serial dilutions of DNA from a normal individual. The relative gene copy numbers were calculated by using the comparative C_t method, using the ratio formula ($\text{ratio} = 2^{-\Delta\Delta C_t}$). This ratio was expected to be about 1 in normal control and 0.5 in heterozygous patient.^{E4}

• SNP genotyping

Nine polymorphisms (1 in 5'UTR, 5 in intron 3, 1 in intron 4, 1 in intron 7, and 1 in exon 8) throughout *C1NH* were used for haplotyping of the mutant allele, that is, c.-21C>T (rs28362944), c.550+748A>G (rs2432809), c.550+794C>A (rs1812005), c.551-500C>G (rs28362947), c.551-495A>C (rs28362948), c.551-155A >G (rs2936694), c.685+1100C>T (rs78364821), c.1030-20A>G (rs2511988) and c.1438G>A (rs4926). Genetic analysis was performed by direct DNA sequencing. Primers and PCR conditions for the analysis of SNPs are reported in Table E1.

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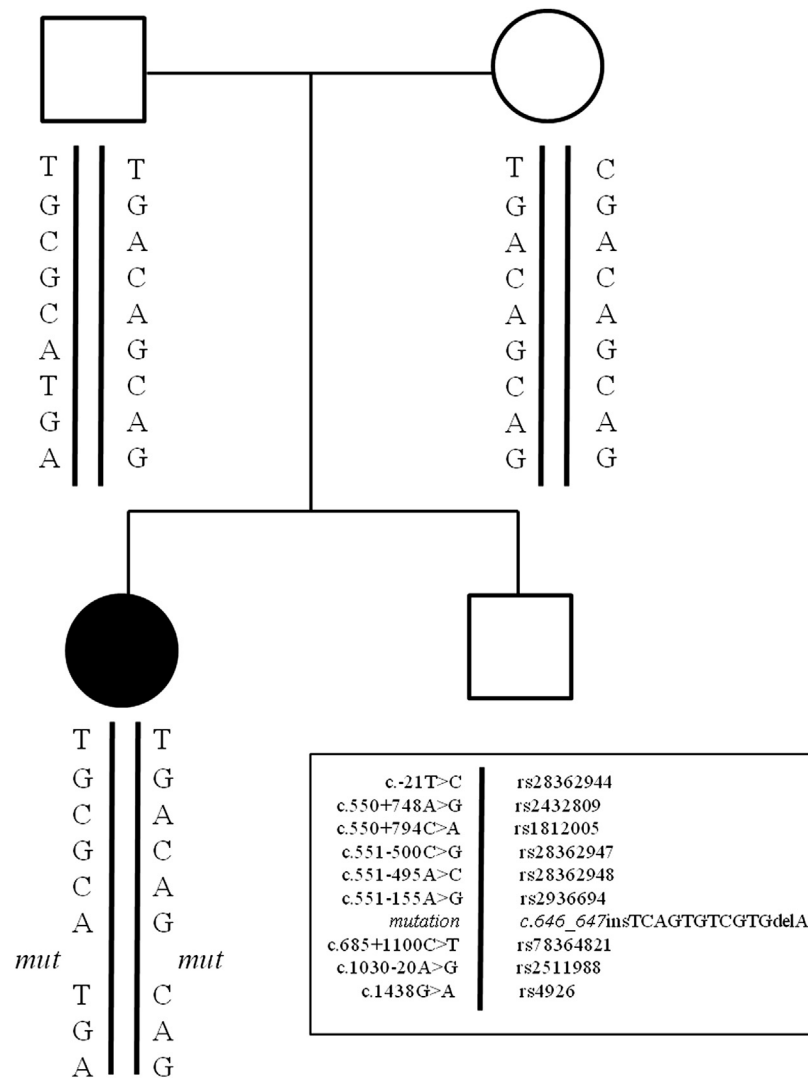


FIG E1. Pedigree of the family. Results of the *C1NH* mutation analysis and the haplotypes using 8 intragenic different SNPs are presented.

TABLE E1. Primers and PCR conditions for the genetic analysis of *C1NH*

Exons	Primer forward	Primer reverse	T annealing (°C)
1	5'AGCTGAGAACTGCACCCAAG 3'	5'CGGGTCTCTGTCCATCTGTT 3'	57
2	5' CTCTGGCCTCATTGTTTGGT 3'	5' CGGAGCCTGAAGGGTTAAT3'	56
3	5' CCACACCTTCTCTTCCTGCT 3'	5' CCAGAGGCATGGCTTTGTAA 3'	54
4	5'AACCCTCATTCCCAAGGAAG 3'	5' TTCCCTCTGTCCTTTCTTTCC 3'	56
5-6	5' GCATGCTCACTCTCAAATCG 3'	5' CAAAATGATGGGACTACAGCA 3'	54
7	5' ACTGCAGGACAGCATTGTGA 3'	5' CTAGAGAATGCTAACTACATCTTAGGG 3'	56
8	5' GGACAAAGGTCTCCATCAGC 3'	5' TAGCCTGGGTGACAGATTGA3'	62
Int3.1	5'GCCTCTGGGAAGAAGCTGTA 3'	5'CTGCCTCAGCCTCCTGAGTA 3'	59
Int3.2	5' TGAACACGGGAGGCAGAG 3'	5' CAGGTGAAGTCCTTGGGGTA 3'	58
Int4	5' ATGGTGGTGTGCACCTGTA 3'	5' GCTCATTTTGGGTCAAAGG 3'	57