

## Original article

## Normal C1 inhibitor mRNA expression level in type I hereditary angioedema patients: newly found C1 inhibitor gene mutations

**Background:** C1 esterase inhibitor (C1INH) plays a key role in the classical pathway of the complement cascade. Mutations in this gene cause a decreased level of antigenic (type I hereditary angioedema, HAE) or functional (type II HAE) C1INH.

**Objective:** To find novel mutations in C1INH and evaluate the expression of C1INH gene in HAE patients.

**Methods:** Direct sequencing mutation analysis was performed for genomic DNA from three unrelated families (14 HAE patients and 18 family members). Genomic DNA from one family was also analyzed for larger genomic rearrangements, using Southern blotting analysis. We used real-time quantitative polymerase chain reaction (PCR) to evaluate C1INH mRNA expression level.

**Results:** Four mutations in exons (2311 T → C, 14 034 G → A, 16 830 G → A, and 16 979–16 980 G insertion) and four in introns (738 G → A, 8531 A → G, 14 254 A → G, and 14 337–14 378 TT deletion) were found. Interestingly, all of the nine patients in one family share the same mutation of Gly345Arg (14 034 G → A) in the seventh exon. In another family, a single base mutation near the splice site (14 254 A → G) was found in all of the three patients. In the last family, although a significant mutation was not found by direct sequencing, patients showed an abnormal 16 kb fragment in addition to the normal allele (21 kb Bcl I fragment). The C1INH mRNA expression of HAE patients in two families was not significantly different compared with that of normal controls.

**Conclusion:** The two novel exonal mutations (G → A and A → G) and one large gene deletion were associated with the clinical phenotypes of HAE. Considering the normal C1INH mRNA levels but below normal protein levels in two families, their phenotypes would be associated with the post-translational defect.

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Hereditary angioedema (HAE) is an autosomal dominant disease that afflicts one in 10 000–50 000 persons (1), characterized by quantitative or qualitative deficiency of a plasma protein called C1 esterase inhibitor (C1INH), the main control element of enzymatic activity of the first component of complement cascade. Historically, two types of HAE without sex predominance had been described until a variant, possibly X-linked, type 3 HAE has recently been described (2). Type I HAE, accounting for 85% of HAE patients, is characterized by decreased levels of C1INH, which is 5–30% of normal value. In contrast, type II HAE patients show normal or raised C1INH levels in spite of diminished enzymatic functional activity. Types I and II HAE are suggested to be a result of mutations within the C1INH gene (chromosome 11q12-q13.1). Up to the present, little is known

about the molecular nature of the gene defect causing low serum C1INH level in type I HAE.

Since this clinical entity was described for the first time by Osler (3), many western researchers have reported HAE-related genetic mutations. In Asian populations, there have been a few case reports and no investigation into the genetic mutations involved. We have investigated the genetic findings of three unrelated Korean HAE families, including 14 patients and their 18 healthy family members and measured the levels of C1INH mRNA.

## Materials and methods

## Subjects

We screened 32 individuals representing three distinct families (A: 23, B: 4, C: 5). A careful personal history of the subjects was obtained by physicians. All subjects, or their parents, gave informed consent to the study for seeking mutations on C1INH gene. Family trees are depicted on Fig. 1.

**Abbreviations:** C1INH, C1 esterase inhibitor; HAE, hereditary angioedema; PCR, polymerase chain reaction.

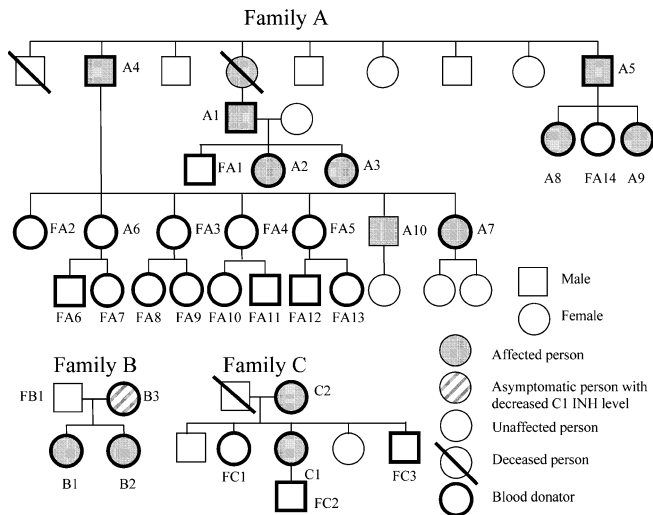


Figure 1. Family trees of three Korean families with hereditary angioedema.

#### Complement assay

Plasma concentrations of C1INH, C4, and C1q antigens were measured by radial immunodiffusion (RN019.3, RN025.3, and RN020.3; Bindarid™, Birmingham, UK).

#### C1INH gene sequencing

DNA was extracted from 10 ml of whole blood in ACD tubes using Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). DNA samples were dissolved in 10 mM Tris/1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0 and stored at  $-20^{\circ}\text{C}$  until polymerase chain reaction (PCR) amplification. Eight exons and intervening intron 5 of C1INH were amplified by PCR from genomic DNA of 32 subjects. The primers of all eight exons were designed according to the published sequence (GenBank accession number X54486). We used OLIGO PRIMER ANALYSIS Software Version 5 (Molecular Biology Insights Inc., Cascade, CO, USA) to design PCR primer pairs. The PCR fragments were analyzed on 2% agarose/1X tris-acetate EDTA (TAE) gels. The ingredients of 50  $\mu\text{l}$  PCR mix consisted of 1.25 U of *Taq* Polymerase (Boehringer Mannheim, Mannheim, Germany), 200  $\mu\text{M}$  of each dNTP (Boehringer Mannheim), 5  $\mu\text{l}$  10X PCR buffer (Boehringer Mannheim), 300 ng of genomic DNA and 10 pM of each primer. Reactions were carried out using MJ Research PTC-200 thermal cycler (Watertown, MA, USA). Direct sequencing was performed with a Big Dye Terminator Kit (Applied Biosystems, Foster City, CA, USA) on ABI PRISM 3100 DNA Sequencer (Applied Biosystems).

#### C1INH gene mRNA

Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of patients and healthy donors, respectively, by centrifugation in Ficoll-Paque gradient (Pharmacia, Uppsala, Sweden). Resultant PBMCs ( $1 \times 10^6$  cells) were treated with lysis buffer containing guanidium isothiocyanate. Then, total RNA was isolated from the lysates. After ethanol precipitation, the pellet was dissolved in RNase-free  $\text{H}_2\text{O}$ . The yield and purity of the RNA

were determined by optical density reading at 260 and 280 nm. cDNA was synthesized by using 1  $\mu\text{g}$  of total RNA and 45 U of avian myeloblast virus (AMV) reverse transcriptase (Promega), 40 U RNasin and 10 mM dNTPs in a final volume of 49  $\mu\text{l}$ . To quantify the amount of C1INH mRNA, we performed real-time PCR using TaqMan probe. The ABI PRISM 7700 Sequence Detection System (PE Biosystems, Foster City, CA, USA) was used. C1INH mRNA was detected using the following primers and dual-labeled TaqMan probe. Forward: 5'-CTC CGA TAC CCG CCT TGT C-3'; reverse: 5'-GCA CTT TTA TAA CTG AGT TTT TGA AGT GA-3'; probe: 5'-TCA ATG CTA TCT ACC TGA GTG CCA AGT GGA A-3'.

The real-time TaqMan PCR analysis was carried in 25  $\mu\text{l}$  reaction volumes containing 2  $\mu\text{l}$  DNA, 9  $\mu\text{M}$  of each amplification primer, and 2.5  $\mu\text{M}$  of the dual-labeled TaqMan probe and the necessary components provided in the TaqMan PCR Core Reaction Kit (Perkin Elmer, Foster City, CA, USA). The program consisted of stage 1,  $50^{\circ}\text{C}$  for 2 min; stage 2,  $95^{\circ}\text{C}$  for 10 min; and stage 3, 45 cycles of  $95^{\circ}\text{C}$  for 15 s and  $60^{\circ}\text{C}$  for 1 min. All samples were analyzed in duplicate and scored in a blinded manner.  $\beta$ -Actin was used as endogenous controls to normalize for differences in the amount of total RNA added to each reactions.

#### Southern blotting

Southern blotting of Bcl I-digested genomic DNA (10 mg) were performed by using the whole cDNA of the C1INH gene with radioactive ( $\alpha^{32}\text{P}$ -dCTP) labeling followed by autoradiography. The full-length cDNA probe (1.6 kb) was generated from human lung cDNA library by direct PCR amplification using the following primer pairs – sense: 5'-GGT GAC CAG AAG TTT GGA GTC-3'; antisense: 5'-AAC TGA GAG CTG AGG CTG GA-3'.

#### Statistics

The significance of differences between patients and healthy relatives were assessed by the nonparametric independent *t*-test. The correlation between C1INH mRNA expression and serum C1INH level was assessed by Spearman's correlation test. Variables were analyzed using the spss 11.0 for Windows. Values of  $P < 0.05$  were considered significant.

## Results

#### Clinical phenotypes

Three index patients (A1, B1, and C1) of each family had a typical history of HAE and were diagnosed as a type I HAE based on the decreased serum level of C1INH and C4.

In family A, a 44-year-old male (A1) had suffered multiple episodes of self-limiting localized cutaneous swelling and abdominal pain for more than 10 years. His 9-year-old monozygotic twin daughters (A2, A3) who were asymptomatic also showed low C1INH and C4 levels. They became symptomatic at the age of 13. His deceased mother died of sudden suffocation. Two uncles (A4, A5) and their daughters (A6, A7, A8, and A9) and son (A10) were also diagnosed as HAE. In family B, a 17-year-old girl (B1) and her younger sister (B2) suffered

frequent painless edema on their extremities and were diagnosed as HAE. Their 42-year-old mother (B3) was asymptomatic but her serum C1INH and C4 levels were below normal. In family C, a 46-year-old female (C1) and her 78-year-old mother (C2) were symptomatic and had low levels of serum C1INH and C4 at the time of diagnosis.

Mutations in C1INH gene

In family A, six separate mutations were found (Table 1). Interestingly, all nine HAE patients have a unique missense mutation present in the seventh exon of C1INH; at nucleotide 14 034 (amino acid 345), a G to A mutation transforms Gly to Arg, which has not been previously reported. All three patients of family B share the same mutation on their C1INH gene: a single base change A to G at 14 254 in intron 7 near the splice site (14 252). Common mutation was not detected in patients C1 and C2 by direct sequencing method but both patients were confirmed to have an abnormal 16 kb fragment besides the normal allele of 21 kb Bcl I fragment (Fig. 2).

RNA expression and serum protein level of C1INH gene

Basal C1INH mRNA expression levels of HAE patients in families A and C were not lower than those of healthy members (mean  $\pm$  SD:  $2.30 \pm 0.79$  vs  $1.99 \pm 1.06$ ) while their serum levels were significantly reduced below the normal level (Fig. 3). Patients from family B showed relatively low C1INH mRNA level. In healthy controls, a negative correlation was observed between serum C1INH and C1INH mRNA expression levels (Spearman's correlation coefficient,  $\gamma = -0.697$ ,  $P = 0.017$ ; Fig. 4), but not in HAE patient group (Spearman's correlation coefficient,  $\gamma = 0.508$ ,  $P = 0.092$ ).

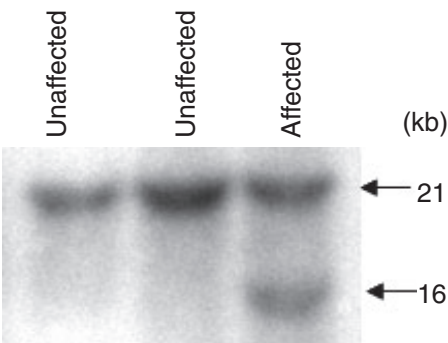


Figure 2. Southern blot analysis demonstrating C1 esterase inhibitor (C1INH) gene rearrangement. Distinct restriction patterns were found in patients C1 compared with unaffected controls. Patient C2 showed the same pattern (data not shown.)

Effect of Danazol treatment

In response to Danazol, there appeared to be a correspondence between serum C1INH and C4 but not with the mRNA level. In some Danazol-treated patients, their serum levels were still below the normal range (15–35 mg/dl) despite enormous increase of mRNA expression (Fig. 3). We followed mRNA expression level of one patient. Her mRNA expression increased remarkably with Danazol 400 mg/day and went down with lowering the dosage to 200 mg/day (Fig. 5).

Discussion

The main C1INH gene mutations were missense mutations (36%) and frame shifts (23.8%). In addition, nonsense mutations (8.8%), splice-site mutations (8.8%), small insertions or deletions (4.8%), and changes in the promoter (1.4%) or other regulatory elements

Table 1. Mutations found in the C1INH gene\*

| Subjects       | Intron 2<br>(G738A) | Exon 3<br>(GTT → GCT, Val 56 Ala) | Introns 5 and 6<br>(A8531G) | Exon 7<br>(GGG → AGG, Gly 345 Arg) | Intron 7     |                 | Exon 8                 |           |
|----------------|---------------------|-----------------------------------|-----------------------------|------------------------------------|--------------|-----------------|------------------------|-----------|
|                |                     |                                   |                             |                                    | 14 254 A → G | 14 337–8 del TT | GTG → ATG, Val 480 Met | 16,979–80 |
| <b>A1, 3–9</b> | GG                  | TT                                | AA                          | <b>GA</b>                          | AA           | –               | GG                     | G ins     |
| <b>A2</b>      | GG                  | TC                                | AA                          | <b>GA</b>                          | AA           | TT del          | GA                     | G ins     |
| FA1            | GG                  | TC                                | AA                          |                                    | AA           | TT del          | GG                     | G ins     |
| FA2–14         | GG                  | TT                                | AA                          | GG                                 | AA           | –               | GG                     | G ins     |
| <b>B1–2</b>    | GG                  | TT                                | AG                          | GG                                 | <b>AG</b>    | –               | GG                     | G ins     |
| <b>B3</b>      | GG                  | TT                                | AA                          | GG                                 | <b>AG</b>    | TT del          | GA                     | G ins     |
| FB1            | GG                  | TT                                | AG                          | GG                                 | AA           |                 | GG                     | G ins     |
| <b>C1</b>      | GG                  | TT                                | AA                          | GG                                 | AA           | –               | GG                     | G ins     |
| <b>C2</b>      | GG                  | TT                                | AA                          | GG                                 | AA           | –               | GA                     | G ins     |
| FC1, 3         | GG                  | TT                                | AA                          | GG                                 | AA           | –               | GA                     | G ins     |
| FC2            | GG                  | TC                                | AA                          | GG                                 | AA           | TT del          | GA                     | G ins     |

\*Subjects are detailed in Fig. 1.

HAE patients (written in bold stroke) of A and B family share a common mutation (in italics) with other patients among their relatives. C1INH, C1 esterase inhibitor; HAE, hereditary angioedema.

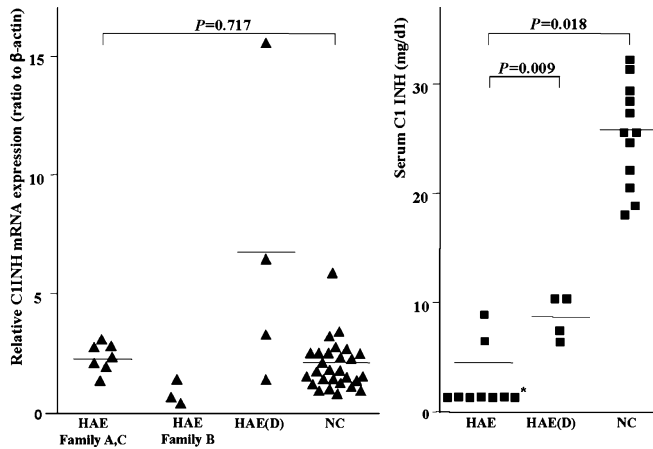


**Figure 3.** Comparison of C1 esterase inhibitor (C1INH) mRNA expression and serum C1INH levels of hereditary angioedema (HAE) patients and normal controls. HAE patients in families A and C showed normal C1INH mRNA expression levels in spite of significantly low serum C1INH protein levels. \*Seven serum C1INH levels below the lower detectable level ( $< 3.8$  mg/dl) were depicted as 3.8 mg/dl. HAE(D), HAE patient in family A with Danazol medication; NC, normal control.

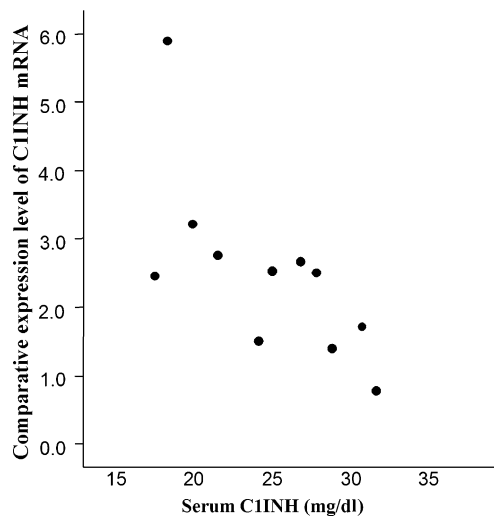


**Figure 4.** Inverse correlation between C1 esterase inhibitor (C1INH) mRNA expression levels and serum C1INH levels in healthy controls. Spearman's correlation coefficient,  $\gamma = -0.697$ ,  $P = 0.017$ .

(1.4%) have been reported (4, 5). Until now, more than 150 mutations have been reported and many of them are close to splice junctions (6, 7). Large gene rearrangements account for approximately 15% of C1INH defects and are thought to be due to unequal recombination between Alu repeats present within introns (4). Among all the mutations reported (1, 7–10), nothing was identical to mutations that we found. Nonetheless, for the huge number of mutations reported, only a few cases have been investigated by functional studies (11, 12). We found that

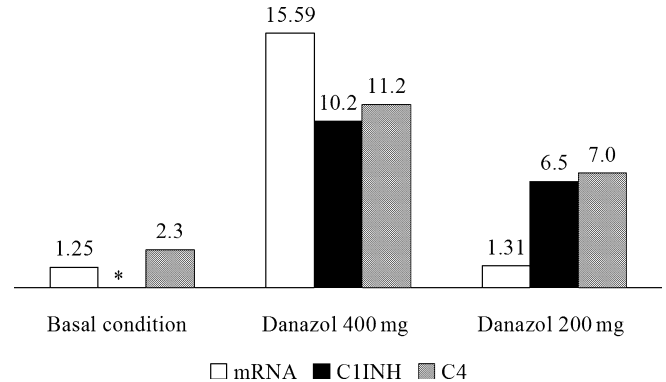


**Figure 5.** Serial follow up of C1 esterase inhibitor (C1INH) mRNA expression, serum C1INH and C4 in patient A6. Unrelated increase of serum protein level and mRNA expression after Danazol medication. \*Below the lower detectable level.

all nine patients in family A had the mutation  $G \rightarrow A$  in the seventh exon that changes the 345th amino acid Gly to Arg. Considering the fact that none of the 14 healthy members of the same family did not possess this mutation, it is more likely to be an important marker of HAE than a family trait. The single base change found in patients of family B,  $A \rightarrow G$ , is located near the splice site. Splice site mutations have not been uncommonly reported to account for 7–10% of the molecular defects of type I HAE. In family C, patients had a large genomic deletion that was unable to be detected by direct sequencing.

The C1INH mRNA expression level of HAE patients has been reported to be reduced by various investigators (13–17). Patients of family B had relatively lower mRNA expression level compared with normal controls, like previous reports. However, patients of families A and C had normal C1INH mRNA expression levels corresponding to that of the controls. This is a very important finding as this is the first report of type I HAE patients with normal mRNA expression. Considering normal mRNA expression, the reduced serum C1INH level may be the result of errors in post-translational processes. Although post-translational defect has never been proved in HAE, it has been raised as a cause of other various rare genetic disorders, such as hereditary inclusion body myopathies, osteogenesis imperfecta, and multiple sulfatase deficiency.

In the present investigation, C1INH mRNA expression was negatively correlated with the serum C1INH level in the healthy control group (Spearman's correlation coefficient,  $\gamma = -0.697$ ,  $P = 0.017$ ). This suggests that they have a negative feedback mechanism that enables delicate control of serum levels. Considering the fact that this correlation was not observed in HAE patient group (Spearman's correlation coefficient,  $\gamma = 0.508$ ,  $P = 0.092$ ), there is a possibility of impaired perception of negative feedback in those HAE patients.

Androgens reverse the clinical symptoms and the biochemical defect of HAE successfully since Gelfand et al. reported their effect in 1976 (18). In spite of widespread use of Danazol for HAE, the exact mechanism has not been elucidated yet. Previously, a transgenic mouse model with entire cDNA failed to show any increase of C1INH mRNA or protein synthesis upon hormonal treatment with Danazol (5). Pappalardo et al. reported that C1INH mRNA expressions and not C1INH plasma levels were significantly increased in patients treated with minimal dose of Danazol compared with those of untreated patients (19). We followed C1INH mRNA expression before and after Danazol treatment in one patient (A6; Fig. 5). The fact that C1INH mRNA expression rose with Danazol administration (400 mg/day) suggests that the therapeutic effect of Danazol be mediated by increasing the level of mRNA expression. With a lower Danazol dose, serum protein level was still higher than untreated level whereas mRNA expression returned to basal level. This discrepancy between mRNA expression and protein level suggests that Danazol may take part in mRNA synthesis but also may be involved in the process of post-transla-

tional modification. We also analyzed four patients of family A who were medicated with Danazol. Although their mRNA expressions were variable from normal to extremely high levels, their serum protein levels did not reach the normal range. This implies that mRNA increased by Danazol did not result in equivalent protein production in those HAE patients. These findings are compatible with our hypothesis that the mutation found in family A may give rise to post-translational defect.

So far, few studies on Asian HAE patients exist, and we report three mutations of C1INH in type I HAE. Neither of these mutations has previously been reported. The diversity of mutations suggests that HAE is a heterogeneous disease with the same clinical manifestations. Functional genetic studies would be helpful to make mechanisms clear.

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