ONLINE MUTATION REPORT

The effect of sequence variations within the coding region of the C1 inhibitor gene on disease expression and protein function in families with hereditary angio-oedema

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Hereditary angio-oedema (HAE; OMIM#106100) affects 1 in 50 000 of the population[1] and results from deficiency of the plasma protein C1 inhibitor. It is characterised clinically by recurrent episodes of subcutaneous, intestinal, and laryngeal oedema, which vary in severity between affected individuals. In some cases the laryngeal oedema may be so severe as to occlude the upper airway and threaten life. Two distinct categories of HAE are now recognised. Type I affects approximately 85% of all patients with HAE, and is characterised by low antigenic and functional levels of C1 inhibitor. Type II is found in approximately 15% of patients with HAE, and is defined by normal or elevated levels of C1 inhibitor with low functional activity caused by the secretion of a dysfunctional protein.[2] Family studies of HAE suggest that the disease has an autosomal dominant inheritance with incomplete penetrance, so that loss of one C1 inhibitor allele may be sufficient to cause disease expression. The C1 inhibitor gene (*C1-inh*) maps to chromosome 11q12-q13.1 and comprises eight exons. Rare mutations causing deficiency or dysfunction of C1 inhibitor have been identified throughout the entire length of the gene, as well as common sequence variations of unknown significance.[3–11] Moreover, the C1 inhibitor locus has clusters of intragenic Alu repeats that predispose to deleterious gene rearrangements. Such gross alterations in *C1-inh* have been reported in up to 20% of individuals with type I HAE.[1] Despite our understanding of the mutations that underlie the deficiency of C1 inhibitor, there is a poor correlation between the mutation, the plasma level of C1 inhibitor, and the disease phenotype. The majority of studies on mutations contributing to HAE have been cross-sectional. We report here a family based study designed to determine the relationship between genotype and clinical phenotype in patients with C1 inhibitor deficiency and hereditary angio-oedema. This allows segregation analysis of sequence variations. In particular, all affected individuals (and related family members) have been screened for both small mutations and gross deletions in the coding region of *C1-inh*, and for polymorphisms that may modify the clinical phenotype. We were therefore able to address whether common sequence variants found within the gene affect disease expression and, where appropriate, protein function.

METHODS

The kindreds and control populations

Sixteen families containing 27 individuals with clinically evident HAE were identified and characterised. All individuals were assessed by clinical examination and were scored for clinical phenotype. Patients were unlikely to have an acquired form of the disease, as plasma levels of C1q were normal. The severity of HAE was graded by location of the disease and the frequency of attacks (table 1). Severe, moderate, and mild disease were defined as disease affecting

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Key points

- The segregation of sequence variations within the coding region and splice sites of the C1-inhibitor gene (*C1-inh*), and its effect on disease expression was studied in 16 families with hereditary angio-oedema.

- We describe two new mutations in *C1-inh* associated with hereditary angio-oedema: Thr285Ala, a coding variation at a highly conserved residue in the serpin super family; and a 4 bp deletion at position 2458–2461 that will cause premature protein termination.

- Four out of 16 families have a relatively common sequence variation at the exon 2 splice site. Alone, this variation is not associated with symptoms of the disease but in affected families the sequence variation was present in more severely affected individuals, suggesting it may be a modifier allele for disease expression.

- The Val458Met polymorphism was shown to have no effect on protein stability or activity with respect to inhibition of the enzyme C1s, despite changing a conserved amino acid.

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the larynx, intestine, and skin respectively. High, medium, and low reflect the frequency of the attacks. These data are summarised in table 2.

All individuals had blood drawn to measure the plasma level of C1q, C3, C4, and C1 inhibitor. A deficiency of C1 inhibitor leads to the consumption of C4, whereas consumption of C3 suggests that the defect lies elsewhere in the complement pathway. Therefore, plasma levels can be used as indicators for disease. Immunoreactive protein levels of C1 inhibitor, C1q, C3, and C4 were measured using a Dade-Behring nephelometer II (Dade-Behring, Marburg, Germany). Anti-C1 inhibitor, anti-C3 and anti-C4 antibodies were purchased from Dade-Behring. C1 inhibitor activity was determined using a proprietary method (Technoclone, Surrey, UK) that assessed its ability to inhibit the enzyme C1s. Purified C1 inhibitor or plasma was incubated with an excess of C1s in 50 mmol/l Tris, 300 mmol/l NaCl, pH 8.5 at 37°C for 5 min. Residual C1s activity was assessed by the addition of the synthetic chromogenic substrate C_2H_5CO-Lys(ϵ-Cbo)-Gly-Arg-pNA and monitoring the colour change at 405 nm. Where possible, the plasma level and functional

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Abbreviations: HAE, hereditary angioedema; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecylsulphate

activity of C1 inhibitor, and the clinical phenotype were determined prior to treatment with danazol or C1 inhibitor replacement therapy. The control samples were obtained from 102 apparently healthy blood donors from the same local population as the patients.

Characterising mutations and deletions in the C1 inhibitor gene

Genomic DNA was extracted from whole blood using a Wizard Genomic DNA purification kit (Promega, Madison, WI, USA). PCR amplification of the coding exons of C1 inhibitor was carried out using the primers and reaction conditions described by Zuraw & Herschbach.[11] PCR products were purified using a QIAquick PCR purification kit (Qiagen, Crawley, UK), sequenced using the ABI Prism Big Dye terminator cycle sequencing ready reaction kit and analysed on an ABI Prism 310 genetic analyser (Warrington, Cheshire, UK). All exons and splice junctions were sequenced in both directions, and chromatograms were compared directly to a control sequence. This method considerably reduces ambiguity in base calling.[12]

Deletions within *C1-inh* were determined by quantitative fluorescent multiplex PCR using the primers described by Duponchel *et al*[13] Essentially, the amplification of the *C1-inh* exons are compared quantitatively to a control exon (*BRCA1* exon V) under conditions where template concentration is rate limiting. Despite extensive optimisation of reaction conditions, it was impossible to multiplex five exons with an internal control (exon V of *BRCA1*) and obtain an output sensitive enough to detect exon deletions. Consequently, exons IV and V of *C1-inh* and exon V of *BRCA1* were run in triplex, and exon VII of *C1-inh* and exon V of *BRCA1* were run as duplex assays. Amplification efficiency for exons VIII and III of *C1-inh* were compared to the *BRCA1* control exon in singleton assays. PCR conditions were optimised accordingly. Based on the distribution of Alu repeats, only five of the seven exons were screened for the detection of Alu mediated rearrangements because exon VI is separated from exon V by only 193 bp with no Alu sites, and no Alu sequence is present in intron 1 or intron 2. The optimised assays were validated by establishing that they could accurately detect a 50% reduction or increase in template concentration with control genomic DNA, as ratios of peak areas were critically dependent on template concentration in some of the assays. Each patient sample was also validated in a similar manner using mean peak areas from a minimum of three independent PCR reactions.

Characterisation of polymorphisms within the C1 inhibitor protein

C1 inhibitor was purified from 300 ml of plasma obtained from two healthy volunteers homozygous for Val458 and Met458 respectively. Serum C1 immunoreactive protein and activities were within the reference range. Fractionation with 40% ammonium sulphate was followed by anion exchange and then affinity chromatography, using Jacalin agarose as described by Pilatte *et al*[14] The purified protein was stored in PBS/0.04% (w/v) azide, 0.5 mol/l NaCl, 10 mmol/l EDTA, pH 7.0 at 4℃ (short term) and −20℃ (long term), and characterised by 7.5–15% (w/v) sodium dodecylsulphate (SDS) and 4.5% (w/v) non-denaturing polyacrylamide gel electrophoresis (PAGE). Conformational stability was determined by 0–8 mol/l urea transverse urea gradient gel electrophoresis.[15] Thermal stability was assessed by heating C1 inhibitor at 0.4 mg/ml and a range of temperatures between 50 and 65℃ for up to 60 minutes in 0.5 mol/l NaCl, 10 mmol/l EDTA, pH 7.0.[16] Complex formation was assessed by incubating dilutions of C1 inhibitor with an excess of C1s at 37℃ for 5 min. The ability of C1 inhibitor to form stable complexes was determined by 7.5–15% w/v SDS-PAGE. C1 inhibitor activity was measured using an Immunochrom assay kit (Technoclone) as described above.

Table 1 Assessment of the clinical phenotype in individuals with HAE

Phenotype	Frequency	Score
Fatal		10
Severe	High	9
	Medium	8
	Low	7
Moderate	High	6
	Medium	5
	Low	4
Mild	High	3
	Medium	2
	Low	1
Low levels of C1 inhibitor	No symptoms	0.5
Normal levels of C1 inhibitor	Unaffected	0

Severe, moderate, and mild disease were defined as disease affecting the larynx, intestine, and skin respectively.
High, medium, and low reflect the frequency of the attacks.
Score indicates level of clinical severity: 0 is unaffected and 10 is fatal.

Statistical analyses

Differences among normally distributed sets of data were evaluated by one-way analysis of variance, *t* tests, χ^2 tests and the Shapiro-Wilk test for normality.

RESULTS

Mutation detection in the C1 inhibitor gene in individuals with C1 inhibitor deficiency and hereditary angio-oedema

Sixteen families containing 27 individuals with HAE were identified and characterised (table 2). Fifteen families had one or more members with type I HAE and one family had two members with type II HAE based on plasma immunoassay criteria. Exonic sequence was obtained by sequencing the coding exons as seven different PCR products amplified from genomic DNA extracted from all 27 individuals. As Alu site recombination is a common feature of C1 inhibitor deficiency, all patients with HAE were screened for exon deletions and duplications by quantitative PCR. A total of 28 point variations and four partial deletions of *C1-inh* were detected (table 3).

New mutations

A mutation in exon VI at position 8755 of the nucleotide sequence was identified in two families. The a→g transition converted codon Thr285→Ala. This mutation was confirmed by sequence analysis and by restriction digestion using the enzyme *Cvi*JI. The Thr285 residue is strongly conserved within the serpin super family.[17] Ala285 was not found in normal blood donors (table 4).

Deletions

Of the two partial deletions found, the first removed exons VII and VIII, and affected two families. The second removed exons IV, V and VI, and was identified in both affected members of a single family (fig 1). For each of these deletions, the boundaries could not be confidently mapped within the gene due to the large number of Alu sites, particularly 3′ of exon VIII.

Previously described mutations

The previously described sequence variants t566c, a14011g, Val458Met, g14030a were also detected in these families (table 2). Allele frequencies are described in table 4 where appropriate.

Table 2 Summary of patient and family member details including results of clinical evaluation, measurement of plasma levels of C1 inhibitor, C4 and C3, symptoms, and score indicating level of clinical severity: 0 is unaffected and 10 is fatal.

| Patient | Age | Clinical details | | | Family history | C3 levels | C4 levels | Score | Variant |
		LO	IO	CO					
L-1	54	+++	+	+	No	Normal	Low	9	t566c. Exons VII & VIII deleted
L-2	24	−	−	+	Yes	Normal	Low	1	Met458. Exons VII & VIII deleted
L-3	22	Asymptomatic			Yes	Normal	Low	0.5	Exons VII & VIII deleted
S-1	59	−	++	+	Yes	Normal	Low	6	t566c
S-2	34	−	−	+	Yes	Normal	Low	1	
S-3	19	−	+	+	Yes	Normal	Very low	5	
S-4		Asymptomatic			Yes	Normal	Low	0.5	
B-1	45	−	+	+	Yes	Normal	Low	4	Exons IV, V & VI deleted
B-2	13	Asymptomatic			Yes	Normal	Low	0.5	Exons IV, V & VI deleted
P-1	73	+	++	++	NK	Normal	Low	7	g14030a
P-2	43	−	+	++	Yes	Normal	Low	4	g14030a
D-1	32	+	++	−	Yes	Normal	Low	7	
V-1	39	−	+	++	Yes	Normal	Low	4	Met458. 4bp deletion (2458-2461)
V-2	28	−	++	++	Yes	Normal	Low	5	Val458Met. 4bp deletion (2458-2461)
V-3	36	−	+++	++	Yes	Normal	Low	6	Val458Met. 4bp deletion (2458-2461)
V-4	16	Asymptomatic			Yes	Normal	Normal	0	Val458Met
V-5	60	−	+	++	Yes	Normal	Low	2	Val458Met. 4bp deletion (2458-2461)
Dr-1	56	+	+	++	NK	Normal	Low	7	
C-1	32	−	+	++	No	Normal	Low	6	Exons VII & VIII deleted
Cl-1	34	−	++	+	Yes	Normal	Low	4	
W-1	56	Asymptomatic			No	Normal	Low	0.5	Thr285Ala, a14011g. Val458Met
W-2	30	−	++	++	No	Normal	Low	6	Thr285Ala, a14011g Val458Met, t566c
G-2	21	−	+	++	No	Normal	Normal	4	t566c
R-1	35		+	++	Yes	Normal	Low	4	Arg444His
R-2	62	+	+	++	No	Normal	Low	7	Arg444His
N-1	48	−	+	+	No	Normal	Low	3	Thr285Ala, g14011. Val458Met
By-1	17	+	+	++	No	Normal	Low	7	
By-2	35	Unaffected			No	Normal	Normal	0	
J-1	21	−	−	++	Yes	Normal	Low	4	
Sh-1	21	−	−	++	Yes	Normal	Low	2	

LO, laryngeal oedema; IO, intestinal oedema; CO, cutaneous (peripheral) oedema.
All individuals with a clinical score ≥0.5 had a C1 inhibitor level below the reference range except R-1 and R-2, who had type II HAE.
NK indicates the results were unavailable.

Type II hereditary angio-oedema family

In family R with type II HAE (abnormal serum protein activity with normal plasma concentration), sequence analysis revealed a single base change at nucleotide 16789 in two patients (father and daughter, clinical severity index for each individual was 7 and 4 respectively). This G→A mutation converted the P1 reactive centre residue Arg444 (cgc) to His (cac). C1 inhibitor levels were within the reference range, but the functional activity of the protein was significantly less. This variant has greatly diminished activity against the target proteinase C1s.[18] The pre-treatment level of C1 inhibitor in the index case was 20.7 mg/dl (reference range 10.5–23.0 mg/dl) and the functional activity was <5 U/ml (reference range 70–130 U/ml). The antigenic levels of C1 inhibitor in the daughter were 18.1 mg/dl, but functional levels were also low (7 U/ml).

Type I HAE families: single sequence variants

In five of the families with type I HAE (abnormal plasma protein concentration) (P, B, C, S, and G), heterozygous mutations were detected in *C1-inh* of affected individuals. Unlike the type II patients, the plasma C1 inhibitor concentration in these individuals was below the reference range (10.5–23.0 mg/dl).

A potential splice site mutation at the intron VI/exon VII junction (g14030a)[10] was identified in both the mother and son in family P (clinical severity index 7 and 4 respectively). It was likely to contribute to the clinical phenotype of both patients as it segregated with disease.

A deletion comprising exons IV, V, and VI was found in both affected individuals analysed in pedigree B. An extensive pedigree was obtained indicating nine affected members over four generations. The mother of the index case died from laryngeal oedema (clinical severity index 10) and all the members of that generation were severely affected by the disease (clinical severity index ranging from 7 to 10). The daughter of the index case is young and it is known that the disease often presents with the onset of puberty. Therefore, the eventual outcome for this individual remains to be determined.

Family C is a large family but the index case was the only affected member. His clinical score index was 6 and his immunoreactive protein level was 2.2–5.9 mg/dl. A deletion of exons VII and VIII was detected in this individual. His mother and his three siblings were all unaffected, and did not have a deletion of exons VII and VIII. Unfortunately, the

Table 3 Summary of mutations identified in type I HAE patients

No. of families	Location	Mutation	Reference
4	Exon II	t566c	10
12	Intron VI	a14011g polymorphism	10
8	Exon VIII	Val458Met polymorphism	20
1	Exon VIII	Arg444His	18
1	Intron VI/exon VII junction	g14030a splice site	10
1	Exon III	4bp deletion caac (2458–2461bp)	New
2	Exon VI	a8755g (Thr285Ala)	New
1	Exons IV, V, VI	Del IV,V,VI	
2	Exons VII, VIII	Del VII,VIII	

Previously described mutations are included and referenced.
The boundaries of the exon deletions could not be confidently mapped due to the large number of Alu repeats.

Table 4 Allele frequency of sequence variants in normal controls and in patients with HAE

Sequence variation	Allele frequency (donors, n = 102)	Allele frequency (patients, n = 25)
Val458Met	0.69/0.31	0.56/0.44
t566c	0.96/0.04	0.92/0.08
a14011g	0.28/0.72	0.32/0.68
Thr285Ala	1.0/0.0*	0.93/0.07

The frequency of the Val458Met and Thr285Ala amino acid substitutions was determined by NlaIII and CviJI digestion respectively.
The t566c nucleotide substitution was determined by AvaII digests. The validity of the digests was confirmed by sequence analysis.
The allele frequency of a14011g in a control population is taken from.[10]
*Values from n = 40 normal controls.

father was unavailable for genotyping. This individual may represent a de novo mutation, which has been frequently described in HAE pedigrees.[19]

The relatively common t566c sequence variation was detected in families G (fig 2) and S (fig 3). The mutation is detected in affected individuals, but also in unaffected siblings. t566c was also found with a gene frequency of 4% (table 4) in unaffected blood donors. Unfortunately, we have been unable to find an individual homozygous for this mutation.

Type I HAE families: compound sequence variants

The 4 bp nucleotide deletion was identified in family V (caac deleted in exon III at position 2458–2461) as well as the Val458Met polymorphism (fig 4). The deletion segregates with disease and is likely to explain the disease inheritance in this family as the mutation produces a truncated protein. This is unlikely to be secreted. Alternatively, it may not be recognised by the C1 inhibitor antibodies used in the plasma immunoassay. It is unlikely to be active. The common Met458 allele is the *cis* form with this mutation, suggesting that the deletion occurred on this allele. We found this polymorphism at a gene frequency of 31% in the blood donor controls, similar to values described in the literature.[20] Affected individual V-1 has two copies of the Met458 allele, but has no worse a CSI than her affected heterozygous siblings.

Family N contains the previously undescribed Thr285Ala point mutation as well as the relatively common Val458Met and the a14011g sequence variations. Unfortunately, we were
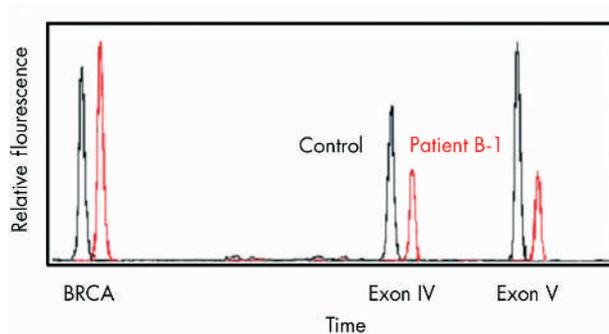

Figure 1 Quantitative fluorescence multiplex PCR results for exons IV and V from *C1-inh*, and the control exon V from *BRCA1*. Amplified products were separated using capillary electrophoresis on an ABI 310 instrument using gene scan software. The patient sample profile (red) is displayed offset from a control sample (black) for ease of viewing. Deletions of exons IV and V are clearly visible.
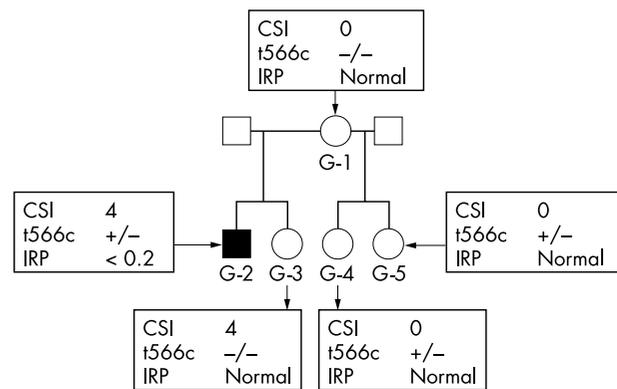

Figure 2 Haplotype of family G. Black symbols represent severely affected individuals. Presence of a heterozygous mutations is denoted by +/− and absence by −/−. IRP, immunoreactive protein levels (normal range 10.5–23.0 mg/dl). CSI, clinical severity index: 0 is unaffected, 10 is fatal.

only able to genotype one affected parent and her as yet unaffected daughter. The affected individual carries Thr285Ala and Val458Met but the phase cannot be ascertained. This patient is also homozygous for the a14011g polymorphism. The unaffected child is an obligate carrier of a14011g but carries neither of the other sequence variants. The Thr285 residue is strongly conserved within the serpin superfamily.[17] The Ala285 variant was not found in normal blood donors. The clinical severity index score of the index case was 3, and immunoreactive protein level of C1 inhibitor was 5.0–9.2 mg/dl. One son also had low C1 inhibitor levels but no phenotype. This is most likely due to his age (4 years old). The unaffected daughter had normal levels of C1 inhibitor. We were unable to obtain sufficient plasma from the index case to purify this protein, but suspect that this mutation contributes to disease in this family.

Family W

Family W (fig 5) also carry the Thr285Ala mutation as well as the more common a14011g, t566c, and Val458Met sequence variants. We cannot exclude that family N and W are related, but neither family thought this likely on questioning. In this small family the affected offspring (W-2), a 30 year old male who suffered frequent attacks of cutaneous and intestinal oedema, is heterozygous for three variations (a14011g, t566c,Val458Met) and the Thr285Ala mutation. His unaffected mother W-1 was heterozygous for the same mutations as W-2 with the notable exception of the t566c polymorphism. t566c and Thr285Ala are in the *trans* form in this family. The father, W-3, also unaffected, was heterozygous for t566c but not Thr285Ala. This suggests that *trans*-t566c may affect disease expression in this family. W-2 is more severely affected than either his asymptomatic mother or the mildly affected Thr285Ala carrier from family N. From these two families, it would appear that heterozygosity for Thr285Ala alone is not associated with severe HAE.

Family L

Family L (fig 6) also carry the t566c and Val458Met variations plus a deletion of exons 7 and exon 8. Individual L-1 carries t556c and the deletion *in trans*. The patient is severely affected by the disease and has frequently suffered from laryngeal oedema. L-2 and L-3 do not carry t566c but have the exon deletions. The former, who also carries Met458, suffers from mild episodes of cutaneous oedema. The latter has low levels of C1 inhibitor but is asymptomatic.
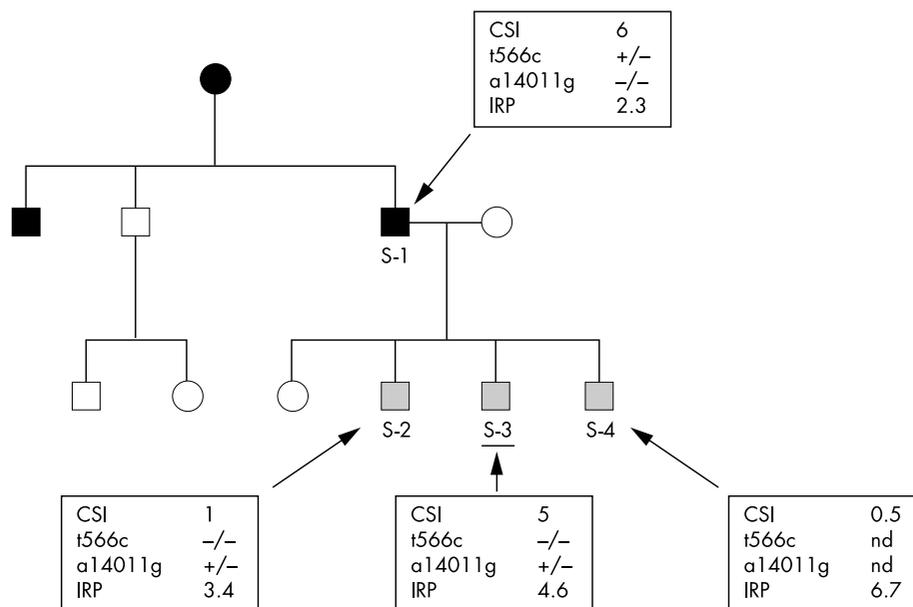
Figure 3 Haplotype of family S. Black symbols represent severely affected individuals and grey symbols represent individuals with milder forms of the disease. Presence of a heterozygous mutations is denoted by +/− and absence by −/−. IRP, immunoreactive protein levels (normal range 10.5–23.0 mg/dl). CSI, clinical severity index: 0 is unaffected, 10 is fatal.

This family also suggest that t566c may affect expression of the disease in a codominant manner.

Type I HAE families: no sequence variants detected

No mutations or exon deletions were detected in the remaining six families who had an index case with low levels of C1 inhibitor and symptoms of HAE. Their clinical severity index score ranged from 2 to 7 but this could not be explained by mutations in the coding region or Alu recombination leading to deleterious rearrangements of the gene. We did not exclude the presence of mutations in the promoter, intronic or downstream elements in *C1-inh*.
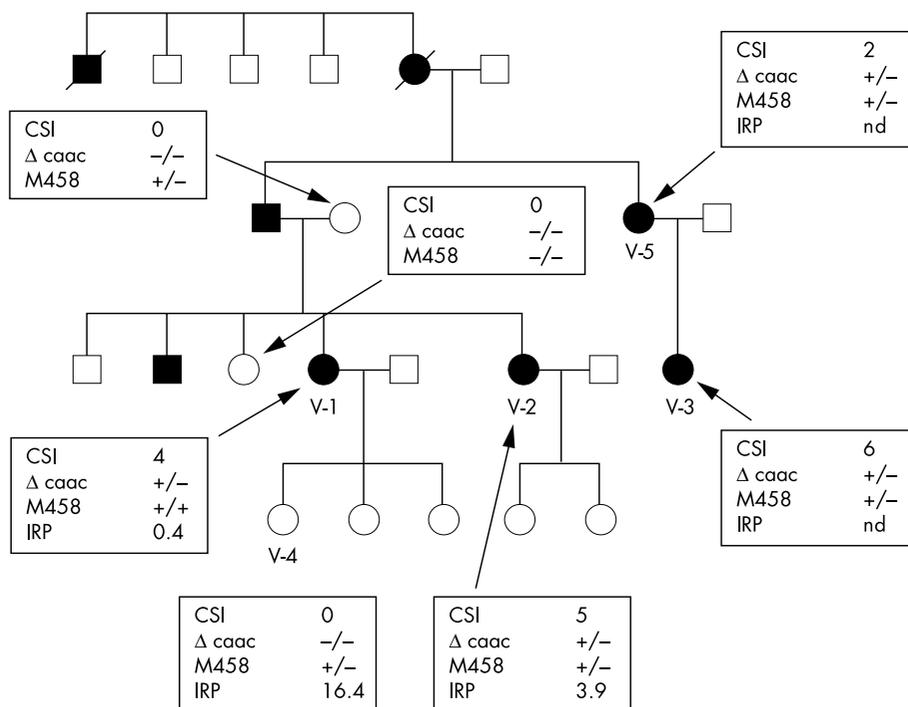
Figure 4 Haplotype of family V which has type I HAE associated with a single mutated copy of *C1-inh*. Segregation analysis of the 4 bp deletion in exon III (Δ$^{+/-}$) is illustrated. Black symbols represent affected individuals. M458$^{+/+}$ represents homozygous alleles for the 458 polymorphism in exon VIII with M458$^{+/-}$ showing heterozygous alleles for this polymorphism. IRP, immunoreactive protein levels (normal range 10.5–23.0 mg/dl). CSI, clinical severity index: 0 is unaffected, 10 is fatal. V-1 and V-2 represent index cases.
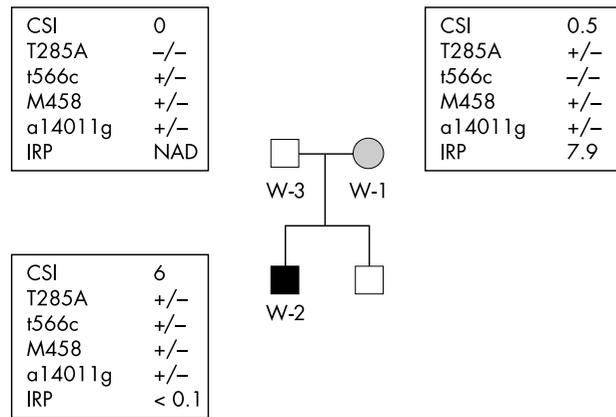
Figure 5 Haplotype of family W. Black symbols represent severely affected individuals and the grey symbols represent individuals with more mild forms of the disease. Presence of a heterozygous mutation are denoted by +/− and absence is represented by −/−. M458 indicates the polymorphic sequence in exon VIII. IRP, immunoreactive protein levels (normal range 10.5–23.0 mg/dl). CSI, clinical severity index: 0 is unaffected, 10 is fatal.

Characterisation of the effect of common sequence variations on plasma levels and function of C1 inhibitor

Three sequence variations (a14011g, t566c, Val458Met) were present with a frequency greater than 3%. Of these variations, only Val458Met affects the primary protein structure of C1-Inh. The allele frequencies of g16830a (Val458Met) are shown in table 4 and were in agreement with previously published data for a healthy control population.[10] This variant was not over-represented in the patient group (χ^2 test p = 0.1, table 4). However, the polymorphism results in the substitution of a conserved Val for Met in the hydrophobic core of the C1 inhibitor.[17] The effect of this amino acid change on plasma levels and/or function of C1 inhibitor was assessed by measuring the plasma level of C1 inhibitor in 102 healthy individuals (table 5). The data showed that the polymorphism had no effect on the circulating level of C1 inhibitor *in vivo*. This population had sufficient power to detect a difference of more than 13% in plasma level of C1 inhibitor. While having no effect on plasma levels of C1 inhibitor, the polymorphism may affect functional activity. As plasma activity can be variable, accurate detection of the specific activities of these isoforms was assessed by purifying C1 inhibitor from the plasma of individuals who were homozygous for Met458 C1 inhibitor or Val458 C1 inhibitor. The specific activities of Val458 and Met458 were not significantly different. The mean (2SD) specific activities for Val458 and Met458 C1 inhibitor were 3.4 (0.7) U/mg and 3.4 (0.6) U/mg respectively, where 1 U of C1 inhibitor corresponds to the average C1 esterase inhibitor activity present in 1 ml of fresh citrated normal plasma (p>0.1; *t*-test). In addition, the Met458 mutation had no effect on C1 inhibitor activity as assessed by complex formation with C1s, on protein stability in urea (data not shown), thermal denaturation (melting temperature 56±2℃ for Met458 and 57±3℃ for Val458; p>0.1, *t* test) or polymer formation. A difference in protein structure or function of the Val458Met mutation was not detected using these methods, suggesting that this polymorphism is unlikely to cause or contribute to the phenotype seen in patients with HAE, although an effect on another function of C1 inhibitor, apart from the inhibition of C1, cannot be excluded.

While the a14011g and t566c common sequence variants do not affect protein structure, they have the potential to
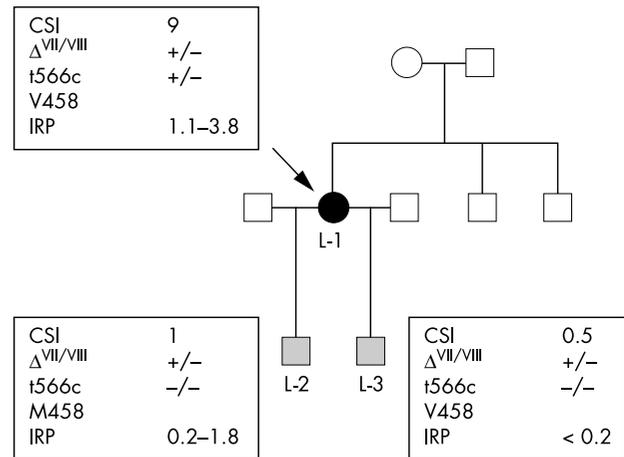

Figure 6 Haplotype of family L. Black symbols represent severely affected individuals and the grey symbols represent individuals with more mild forms of the disease. Presence of a heterozygous mutation are denoted by +/− and absence is represented by −/−. V458 indicates the wildtype sequence in exon VIII and M458 represents the polymorphism. $\Delta^{VII/VIII}$ indicates that exons VII and VIII are deleted. IRP, immunoreactive protein levels (normal range 10.5–23.0 mg/l). CSI, clinical severity index: 0 is unaffected, 10 is fatal.

Table 5 Distribution of serum C1 inhibitor protein concentration in a population of 102 healthy individuals based on the Val458Met or t566c polymorphism

Allele	Val458 homozygote	Val/Met458 heterozygote	Met458 homozygote
Number	45	48	9
Serum C1 inhibitor protein concentration/gL^{-1} Geometric mean (95% CI)	0.193 (0.182–0.206)	0.188 (0.179–0.198)	0.175 (0.153–0.201)

Allele	t566 homozygote	t566c heterozygote	c566 homozygote
Number	94	8	0
Serum C1 inhibitor protein concentration/gL^{-1} Geometric mean (95% CI)	0.190 (0.182–0.199)	0.180 (0.159–0.204)	n/a

Protein concentration was log normally distributed.

affect disease penetrance in HAE families by effects on message transcription.

The first, a14011g, has a frequency of 32%, is intronic and is present at the same frequency in affected patients and healthy blood donors (χ^2 test p = 0.74). As the polymorphism was common and has no effect on protein structure, it was thought unlikely to play any role in disease expression and was not investigated further.

The t566c variation is the second nucleotide of exon II and is part of the canonical acceptor splice site.[10] This variant may affect message splicing, but will not affect the protein structure. This nucleotide substitution has an allele frequency of 4% in the healthy blood donors (table 4). This is consistent with the allele frequencies reported by Freiberger *et al* (2002).[21] In affected patients, the c allele frequency is roughly double that for blood donor controls, although this did not reach significance because of the small number of patients (χ^2 test p = 0.22). The segregation analysis suggests that this

variant may be associated with increased penetrance of HAE in affected individuals, but plasma concentrations of C1 inhibitor did not vary between healthy donors who were t566c heterozygous and non-carriers. Unfortunately, material was not available to study the effect of this mutation at the level of mRNA.

DISCUSSION

HAE presents with a wide spectrum of clinical severity, even within families thought to carry the same mutations.[22] Plasma levels of C1 inhibitor are also a poor predictor of clinical phenotype (fig 7). This variable presentation is thought to be caused by either genetic or non-genetic elements distinct from the defect in the C1-inh. We present data here to suggest that a relatively common mutation within C1-inh (t566c) may be a modifier of disease penetrance. This variation has a gene frequency of around 4%. Although this mutation alone is not sufficient to cause symptoms of the disease, in four families presented here this variation is associated with a more severe disease expression. While the t566c is within the canonical acceptor splice site for exon II, and is therefore likely to affect message splicing or stability, a direct effect of this variation awaits studies on message expression. Alternatively, this variation may be in linkage disequilibrium with another sequence variation within C1-inh. The presence of a relatively common modifier allele within C1-inh may alter the interpretation of family studies where subsequent family members of an index case are only screened at a single genetic locus.

It is of particular interest that in patients whose disease can be described by segregation of a mutation within a single allele of C1-inh, plasma protein concentrations are between 5 and 30% of normal instead of the expected 50% if the unaffected allele were fully expressed. This discrepancy between the expected and observed levels of C1 inhibitor in HAE has been explained by an increased catabolic rate of C1 inhibitor and a decreased rate of synthesis.[23 24] However, other data have suggested a transinhibition of wild-type C1 inhibitor translation by mutant mRNA and/or protein.[25] In healthy blood donors, the t566c sequence variation does not greatly affect C1 inhibitor protein levels. However, if catabolism also determines steady state levels of C1 inhibitor, this effect of t566c may only be unmasked if protein levels are low because of a coexisting mutation on the opposing allele. As for the segregation studies described above, protein clearance studies must also be interpreted with caution if potentially common unidentified modifier alleles exist.

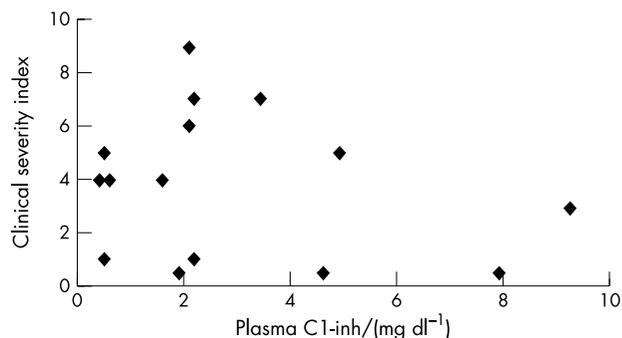A second common sequence variation within C1-inh (Val458Met) has no detectable effect on protein stability, plasma levels or HAE disease expression. The function of Met458 as an inhibitor of C1 was studied and shown to be unaffected. This cannot exclude a consequence of this polymorphism on other functions of C1 inhibitor as a modulator of the coagulation and kinin release pathways[26–28]

Mutations were not detected in 6/16 families affected with symptoms of HAE using this strategy. This is different to the conclusions of Verpy et al,[10] who were able to detect mutations in 34/36 cases using a more comprehensive chemical cleavage method. These authors questioned the validity of the diagnosis in the patients where no sequence abnormality was found. The strategy presented here would not have detected four of the mutations detected by Verpy et al.[10] Duponchel et al[13] also report one patient in whom the chemical cleavage strategy and a screen for exon deletions has failed to detect a mutation in the C1-inh. This emphasises the fact that in many clinical syndromes, even extensive sequencing strategies, despite high diagnostic specificity for the detection of heritable disease, may have less than optimum sensitivity.

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Figure 7 Clinical severity index and plasma level of C1 inhibitor in patients with type I HAE. Plasma levels are a poor indicator of clinical severity.

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