

Haploinsufficiency due to deletion within the 3'-UTR of C1-INH-gene associated with hereditary angioedema

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Purpose: Sequences within the non-coding 3'UTR (untranslated region) of genes were reported to be involved in the regulation of gene expression by modifying pathways of (co)transcription, post-transcriptional processing and RNA transport. However, direct biological evidence (i.e., knock-out models) is sparse. This report intends to correlate the first reported alteration within the 3'UTR of the C1 inhibitor (C1-INH) gene with clinical presentation of hereditary angioedema (HAE). **Methods and Results:** Direct sequencing of genomic DNA revealed in all affected members of a family suffering from HAE a heterozygous 155 bp deletion 100 bp downstream of the physiological stop-codon in exon 8. A substantial reduction of both mRNA as well as C1-INH protein expression was revealed by RT-PCR and nephelometry, respectively. **Conclusion:** We suppose that the mutation within the 3'UTR interferes with integral pathways of gene expression leading to pathogenic haploinsufficiency in this family. *Genet Med* 2006;8(4):249–254.

Key Words: 3' UTR, C1-inhibitor, mRNA, hereditary angioedema, deletion

The M(r) approximately 105,000 C1-inhibitor (C1-INH) glycoprotein is a plasma proteinase inhibitor of the serpin (serin protease inhibitor) superfamily that controls initiation of the (classical, alternative and lectin) complement system, the contact system of kinin generation and the intrinsic coagulation pathway, especially factors XI and XII.^{1–3} Decreasing the ultimate generation of pro-inflammatory peptides, competitively interacting with selectin molecules on endothelial cells or preventing endotoxin shock via a direct interaction with lipopolysaccharide, C1-INH thereby represents a key regulator protein to balance inflammatory response and activity.^{2,4,5}

Autosomal-dominant inherited heterozygosity for a transcriptionally-, translationally-, and secretory-deficient, serum level-reduced (hereditary angioedema type 1) or dysfunctional, activity-reduced but anti-genetically and quantitatively normal (type 2) C1-INH leads to the clinical entity of hereditary angioedema (HAE) which is characterized by recurrent, self-limited attacks/episodes of localized increased vascular permeability restricted to three specific sites, i.e., subcutaneous

space, submucosal gut and upper airway. The occasional involvement of intestinal or laryngeal mucosae causes abdominal pain and potentially life-threatening airway obstruction. Besides these and other hereditary forms,^{6,7} variants of acquired angioedema due to auto-antibodies or associated lymphoproliferative diseases, causing accelerated catabolism of C1-INH, exist.

Bradykinin, a vasoactive cascade-intermediate overwhelmingly generated as a result of insufficient suppression of contact system activation, has been proven to be the primary pathogenic mediator in HAE by a variety of clinical, in vitro and animal experiments.^{2,8} The consequences of concurrent dysregulations at different interfaces in HAE, however, can be diverse and far-reaching. Through either insufficient complement-mediated signaling affecting B lymphocyte maturation and activation or, alternatively, insufficient deposition of C2 and C4 on immune complexes due to an impaired C1-INH mediated repression of C1r and C1s initial proteases activated via classical complement pathway, HAE is supposed to mediate its frequent association with autoimmune diseases like systemic lupus erythematoses.¹

Established alterations in exon-coding C1-INH gene segments underlying inherited forms of HAE comprise missense mutations, frameshifts, stop codon mutations, promoter variants, splice site mutations and insertions, deletions or duplication variants, all causing subsequent insufficient gene and protein expression.^{1,9–11} Whereas mutations in type I HAE are highly heterogeneous, almost all patients with type II HAE have point mutations within the reactive center encoded by exon 8 of C1-INH. The presence of the CpG dinucleotide within the CGC codon for the reactive center arginine (Arg)

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renders this codon susceptible to single base substitutions which result in the replacement of the reactive center Arg residue reducing the specificity toward normal target proteases. The large number of C1-INH mutations identified to date (more than 100), together with the observed variability in angioedema severity among affected kindreds, suggests that, besides exogenous and epigenetic modifiers (trauma, drugs, infections, emotional stress, etc.), different mutations may have differential effects on C1-INH phenotype.

Herein, we report on a family with hereditary angioedema displaying an unique heterozygote 155 bp deletion within the 3'UTR (untranslated region) of the C1 inhibitor gene that highlights its specific (pathogenetic) relevance to sequences of transcription, posttranscriptional processing and RNA transport.

PATIENTS, MATERIALS AND METHODS

Clinical features

We investigated three members of a family with history of angioedema. The mother (50 a) complained about recurrent episodes of cutaneous and mucosal swellings associated with abdominal pain and dyspnea in the past. Although taking substitutive danazol medication (800 mg Danokrin™ per day, Sanofi-Synthelabo, Fawdon, UK) has been accompanied by a marked clinical improvement, she still suffers from occasional genital swellings. Both of her children (20 a and 30 a) have been on supportive C1-INH therapy for life, associated with generally milder clinical manifestations mainly restricted to the early childhood. Although the unaffected father and other family members unfortunately were inaccessible for exploration, the maternal grandmother as well as the aunt anamnestically reported typical clinical symptoms of HAE (pedigree is shown in Fig. 1).

Polymerase chain reaction (PCR)

Genomic DNA was isolated from peripheral blood of our patients as recommended by the manufacturer's protocol (Puregene™ DNA Isolation Kit, Gentra, Minneapolis). Primers published in Bowen et al.¹² for amplification of exons 2-8 of the C1-INH gene were acquired from MWG-Biotech (Ebersberg, Germany). For amplification, 100 ng of genomic DNA was used as a template in a volume of 30 μ l containing 1x enzyme buffer, 1,5 mM MgCl₂, 6% DMSO, 0,2 mM dNTPs, 10 pmol of each primer and 1,25 U polymerase (BioThermRed™, GeneCRAFT, Muenster, Germany). Amplification conditions were: initial denaturation at 94°C for 5 minutes, 40 cycles of denaturation (94°C for 15 seconds), annealing (52°C [exon 7], 58°C [exon 3, 5, 6, 8], 62°C [exon 4] and 64°C [exon 2] for 45 seconds) and extension (72°C for 30 seconds), followed by a final extension step at 72°C for 5 minutes. PCR products were analyzed by agarose gel electrophoresis (1% agarose) along with 250 ng of a molecular weight marker (100 bp DNA Ladder, New England Biolabs, Beverly, MA).

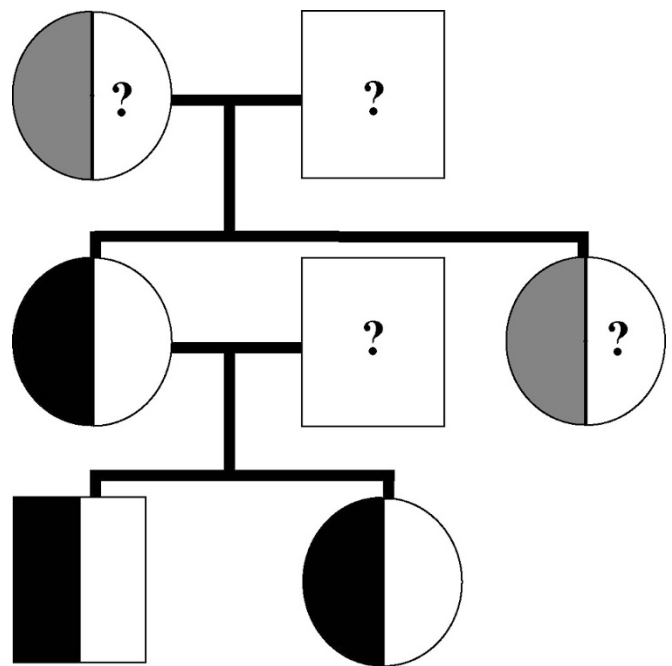


Fig. 1. Pedigree of the index family. While mother, son and daughter herein have been molecularly characterized, other family members were inaccessible for genetic analyses (indicated by question marks). However, the maternal grandmother and aunt are also reported to suffer from clinical manifestations typical of HAE (gray colored).

Sequencing

PCR products were sequenced at MWG-Biotech (Ebersberg, Germany) with a concentration of 20 ng DNA/100 bases and 10 pmol of the respective primers.

RNA Isolation

Total RNA was extracted from the patient's blood using an RNA extraction kit (RNeasy™ Mini Kit, Quiagen, Hilden, Germany) according to the manufacturer's recommendations. RNA was visualized by formaldehyde agarose gel electrophoresis (1,2% agarose).

cDNA synthesis

cDNA (first strand) was synthesized by M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) with oligo(dT)primers corresponding to the manufacturer's instructions using 1 μ g total RNA isolated from the patient's blood.

Quantification of C1-INH mRNA

Amplification conditions of C1-INH cDNA (first strand) were: 94°C denaturation for 5 minutes, 40 cycles of denaturation (94°C for 15 seconds), annealing (60°C for 30 seconds) and extension (72°C for 45 seconds) followed by a final extension step at 72°C for 5 minutes. Primers for amplification of C1-INH were: 5'-gtg ggg cag ctg ctc t-3' (sense, nucleotide position 15213-15231 referring to NCBI sequence viewer for human C1-INH gene at <http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=X54486>) hybridizing to exon 7 and 5'-tca ggc cct ggg gtc ata tac tc-3' (antisense, nucleotide position 18065-18077) binding to exon 8 (5' of the 155bp deletion). Amplifi-

cation of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using primers 5'-aat ccc atc acc atc ttc ca-3' (sense) and 5'-cct gct tca cca cct tct tg-3' (antisense) served as internal reference for equivalent loading.

Cycling conditions of Real Time (RT)-PCR (iCycler, BioRad, Hercules, USA) using the primers mentioned above were: 95°C denaturation for 3 minutes, 55 cycles of denaturation (95°C for 30 seconds), annealing (60°C for 30 seconds) and extension (72°C for 30 seconds), followed by one cycle of denaturation (95°C for 1 minute) and 35 cycles with increase of setpoint temperature (60°C) after cycle 2 by 1°C for 8 seconds, enabling melt curve data collection and analysis. Calculated threshold for data analysis using the maximum curvature approach was 317,5. Per-well baseline cycles have been determined automatically. Data analysis window was set at 95% and centered at end of the cycle. Weighted mean digital filtering was applied, global filtering was off. To account for possible replicate variations, RT-PCR was done in triplicate with corresponding data.

For mutational analyses at cDNA level, a reverse primer located 3' of the deletion was synthesized (5'-ttt ttt ttt atg gtc tgt cag g-3'; antisense, nucleotide position 18329-18341). Combined with primer 5'-gtg ggg cag ctg cag ctc t-3' (sense, nucleotide position 15213-15231) hybridizing to exon 7, C1-INH cDNA (first strand) was amplified under the following conditions: 94°C denaturation for 5 minutes, 50 cycles of denaturation (94°C for 30 seconds), annealing (57°C for 30 seconds) and extension (72°C for 45 seconds), followed by a final extension step at 72°C for 5 minutes. PCR products were concentrated with a Microcon YM-100 Centrifugal Filter (Millipore, Bedford, MA).

Immunodetection of C1-INH and C4 in serum

C1-INH was functionally determined in patient's serum by Berichrom C1-Inhibitor test (Dade-Behring, Marburg, Germany) using a Beckmann 640 spectrophotometer. Complement factor C4 was quantified by nephelometric plasma protein analysis (Dade-Behring BN™ 100).

RESULTS

PCR-amplification by using DNA isolated from peripheral blood mononuclear cells and subsequent sequencing of the C1-INH gene revealed no alterations in the coding sequence. However, a unique alteration identical in all three patients, i.e., a heterozygote 155 bp deletion 100 bp downstream of the stop-codon of exon 8 in the 3'UTR was determined (Fig. 2). When calibrating with housekeeping gene GAPDH, C1-INH cDNA-expression analyses using primers 5'-gtg ggg cag ctg cag ctc t-3' (sense, nucleotide position 15213-15231) and 5'-tca ggc cct ggg gtc ata tac tc-3' (antisense, nucleotide position 18065-18077) revealed a significant reduction of amplification in comparison to a control sample (Fig. 3A,B). Semi-quantification by real-time PCR confirmed a 25-fold reduction of C1-INH mRNA expression in the patient vs. control (Fig. 3C). The 155bp deletion was verified as a very faint 586bp PCR band at cDNA level using a reverse primer located 3' of the deletion

(5'-ttt ttt ttt atg gtc tgt cag g-3'; antisense, nucleotide position 18329-18341) (Fig. 4). Cloning experiments of the 586bp PCR product harboring the 155bp deletion failed because of quantitatively insufficient extraction.

A corresponding decrease of C1-INH at protein level was immunologically detected in serum of all three family members (2–20% [mother] and 5–25% [children], varying on danazol intake; reference range: 70–130%), accompanied by significant reduction of C4 (0.02–0.07 g/l [mother] and 0.05 g/l [children]; reference range: 0.1–0.4 g/l). The molecular alteration of our patients was not reproducible in genomic DNA of 55 probands of a control group.

DISCUSSION

The pathogenicity of the, to our knowledge, first large deletion within the 3'-flanking region of C1-INH is speculative. However, given the well-established notion that precise processes dependent on specific inhibitory and/or stimulatory interactions of cis-acting sequence determinants and a complex transacting protein machinery of cooperative binding factors within the 3'UTR account for regulation of mRNA steady state levels and gene expression, respectively,³ it is plausible to assume a pathogenetic relevance of the mutated non-coding region to the phenotype in this family. Sequence motifs in the 3'UTR are implicated in the determination of mRNA polyadenylation (affecting nuclear export and stabilization),^{13–15} transcript-selective translatability (ribosome binding, scanning, initiation, elongation)¹⁶ and nucleolytic degradation (mRNA decay).^{17,18} Investigative reports have repeatedly underscored this pathophysiological relevance. For example, a single point mutation in 3'UTR of the prothrombin gene (G20210A) causing familial thrombophilia was reported to be associated with elevated prothrombin levels probably due to increased efficiency of polyadenylation of prothrombin mRNA transcripts.^{14,15} In two related individuals with beta thalassemia, Bilenoglu et al.¹⁹ further described a 13 nt (nucleotide) deletion in the 3'UTR of beta-globin gene leading to a sixfold reduction in the relative level of mutant mRNA. It was speculated that the mutation may act to decrease beta-globin mRNA levels by inhibiting the efficiency of nuclear processing events. Accordingly, a relative excess of fully stable but unprocessed betaDelta13 mRNA in erythroid progenitors from beta-Delta13 heterozygotes was detected.

Notably, the 3'UTR also harbors a remarkable high number of repetitive DNA-Alu elements which are known to favor rearrangements of sequences such as duplications or large deletions seen in our patients secondary to unequal homologous recombination/unequal cross-overs.^{20,21} Moreover, the C1-INH gene has an unusually high density of Alu repeats,²² and one of these is in close vicinity to the site of our deletion (Fig. 2a).

This refined knowledge about structure and function of 3' UTR motifs, enlightening their major significance, argues for the specific correlation between its disruption in this family and the distinct clinical phenotype. In line with these regula-

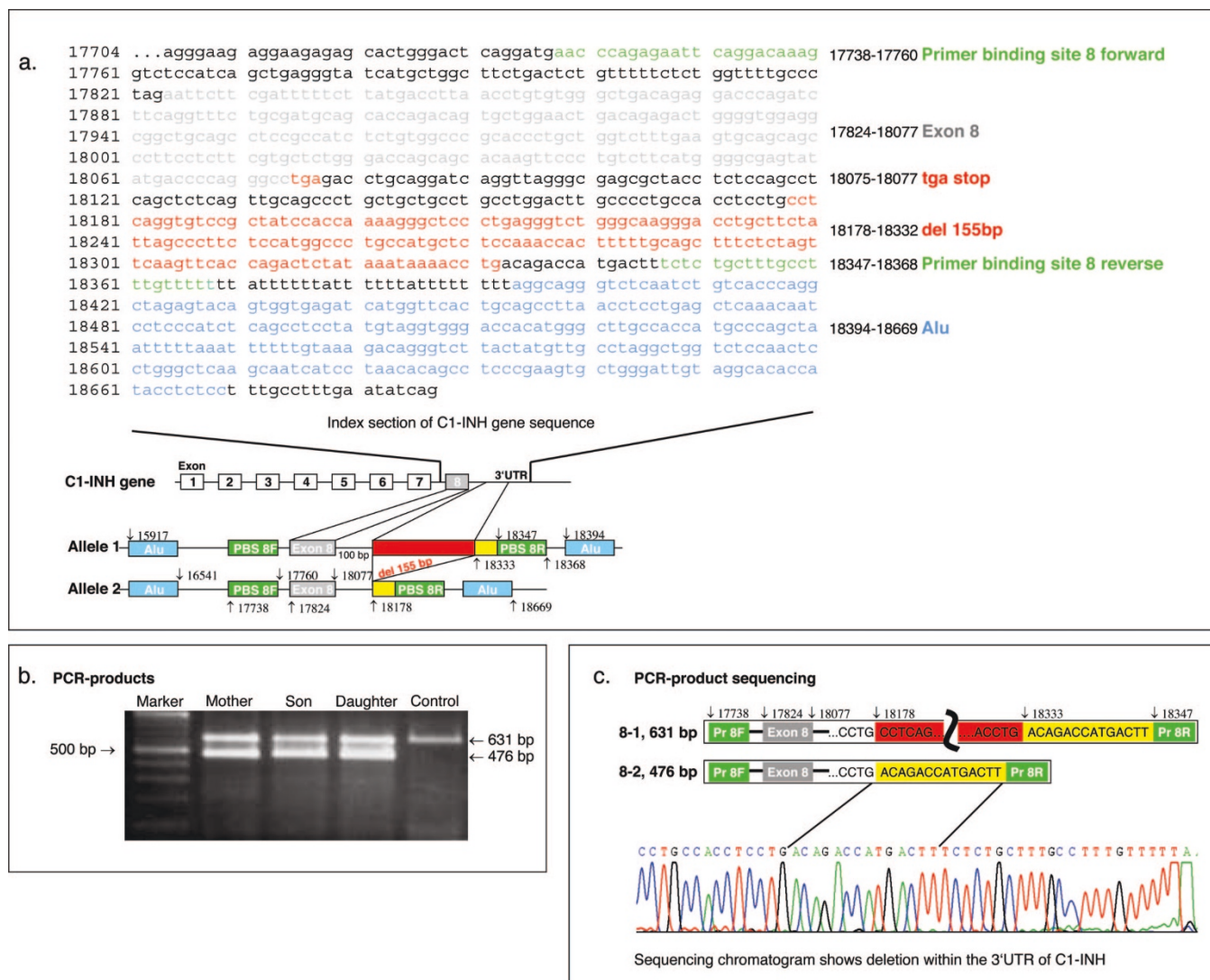


Fig. 2. A) Schematic overview of the sequence of molecular alterations and their effects implicated to be pathogenic in this case. Nucleotide position refers to NCBI sequence viewer for human C1-INH gene at <http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=X54486> (PBS = primer binding site, Alu = alu sequence) B) PCR amplification of exon 8 and neighboring sequences of the C1-INH gene shows a double band due to a 155 bp deletion within the 3'UTR in clinically affected members of the investigated family. (Marker = 100bp marker) C) Sequencing data show 155bp deletion within the 3'UTR of the second C1-INH allele. (Pr 8F = Primer 8 forward, Pr 8R = Primer 8 reverse).

tory functions we detected a substantial reduction of C1-INH mRNA associated with a significant decrease in C1-INH protein in serum. This reduction could be caused by degradation/inactivation of the mutation carrying allele by means that prevent (co-) transcription or abolish mRNA expression post-transcriptionally. It further highlights the importance of dosage for maintaining the biological function and overall level of enzymatic activity of C1-INH at specific sites. The individual who is heterozygous for the gene mutation (or hemizygous at a particular locus) is clinically affected because a single copy of the normal gene is incapable of providing sufficient protein production (levels) as to assure normal phenotype.²³ This so-called haploinsufficiency has been described in a diverse variety of pathologies.^{24–27} For instance, the reduction of the transcription factor MyoD protein-level to an amount below a threshold required to activate differentiation specific genes during muscle

regeneration is pathogenetically implicated in Myotonic dystrophy (DM) associated with the 3'UTR CTG repeat expansion mutation DM1 in the dystrophia myotonica protein kinase (DMPK) gene.²⁸

In accordance, we propose that the deletion within the presented family leads to a similar/analog dosage-dependent loss of C1-INH function induced by haploinsufficiency. Interestingly, although in type I HAE only one allele is functioning, plasma values of C1-INH are usually 5–30% of normal rather than the 50% value that might be expected. A decreased inhibitor level, resulting in increased activation of some or all of the proteases regulated by C1-INH and the consecutive higher catabolism of remaining C1-INH, a decreased production or deficient transport and segregation of the protein have been implicated to account for this phenomenon.^{7,29,30} Our RT-PCR data show a C1-INH reduction of much more than 50% al-

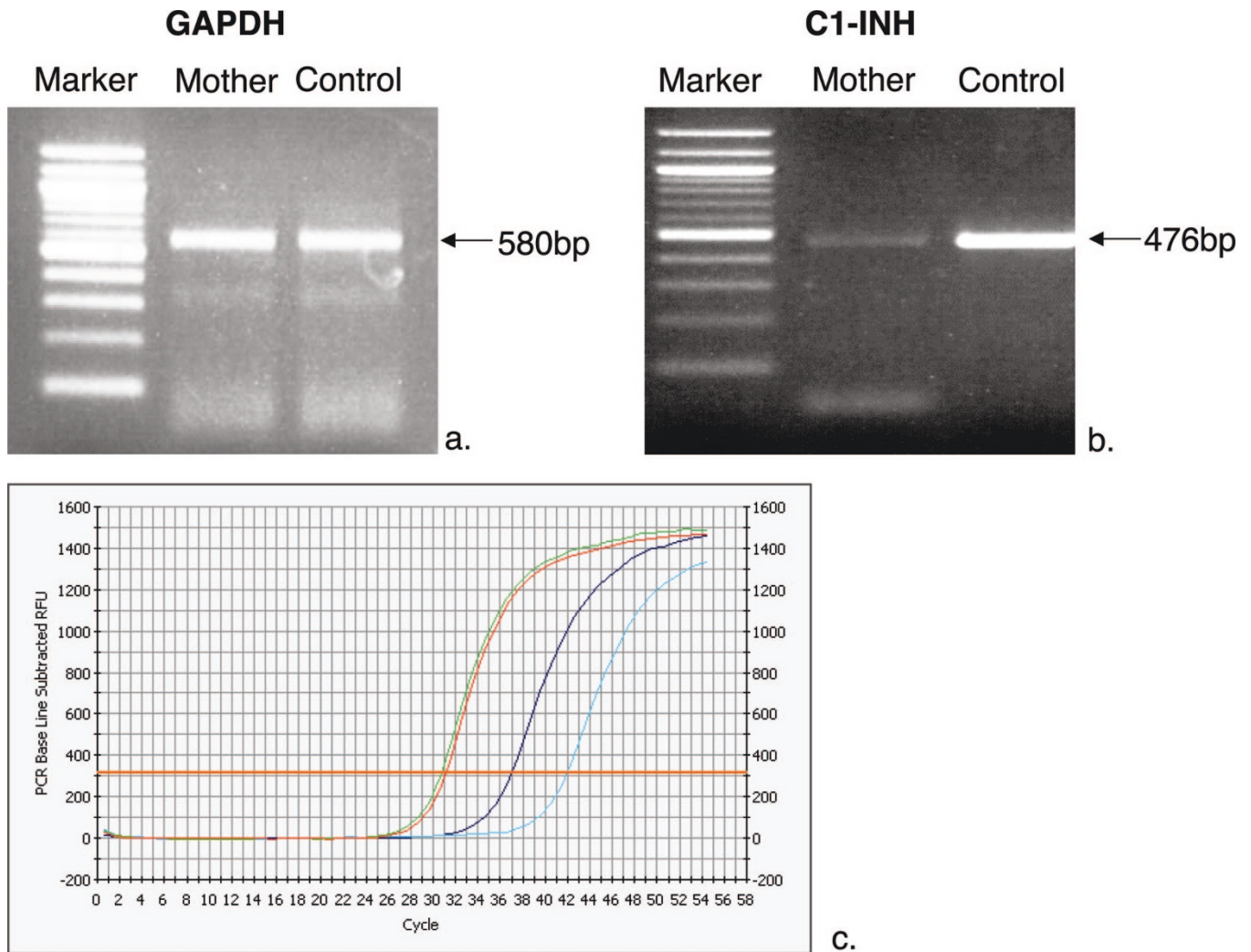


Fig. 3. After calibration with the 580bp PCR product of the housekeeping gene GAPDH as reference to assess mRNA expression level (A), cDNA-PCR amplification using C1-INH gene specific primers for exon 7 and 8 resulting in a 476bp PCR product, is markedly reduced in comparison to control sample (5'-gtg ggg cag ctg cag ctc t-3' [sense, nucleotide position 15213-15231]; 5'-tca ggc cct ggg gtc ata tac tc-3' [antisense, nucleotide position 18065-18077]). (100bp marker) (B). The reduction of C1-INH mRNA is confirmed and quantified by RT-PCR (C). Calibration with housekeeping gene GAPDH reveals threshold cycle (Ct) levels at 30,7 and 31,1 in control cDNA (green curve) and patient cDNA (mother, red curve), respectively. C1-INH Ct levels at 37 in control cDNA (black curve) and 42 in patient cDNA (blue curve) show an estimated difference of 4.6 cycles and a calculated 25-fold reduction of mRNA level in the patient.

Marker Control Mother

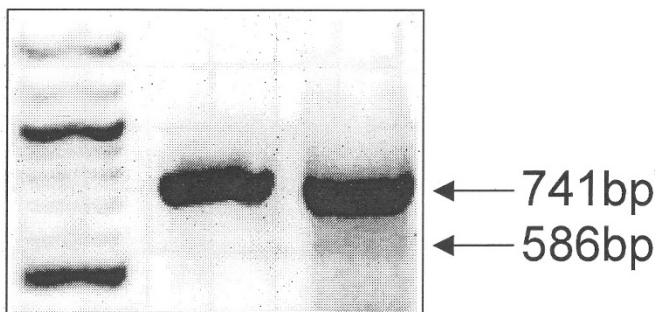


Fig. 4. Detection of the 155bp deletion at cDNA level using a reverse primer located 3' of the deletion (5'-ttt ttt ttt atg gtc tgt cag g-3'; antisense, nucleotide position 18329-18341). As a consequence of mRNA decay only a very faint 586bp PCR band is loomed in the patient's sample (mother) after concentration of 4 tubes of 30 μ l PCR with a Microcentrifugal Filter. The loading volume is approximately 20-fold concentrated to visualize the banding pattern in reverse illustration.

ready at the mRNA level (Fig. 3C). Apart from physiologically-biasing effects on level and steady state specific for each mRNA at the moment of measurement (like stability, transport, transactive factors, rate of degradation), it is also possible that abnormal mRNA may mediate/trigger a suppressive effect on either transcription, processing or translation of the second, normal allele (transinhibition), thus with molecular sequences that are significantly influenced by 3'UTR motifs and their aberrations.^{31,32}

Because inactivation of proteases by serpins like C1-INH depends upon a trapping mechanism finally resulting in cleavage and inactivation of the inhibitor,^{1,2} consumption of C1-INH upon activation of complement/contact/fibrinolytic system leads to its further reduction that probably accounts for the initiation of the attacks of angioedema. Obviously, the following further stimulated activation of complement and con-

tact system would then again result in enhanced consumption of C1-INH.

In summary, this report provides conclusive data on the pathogenicity of molecular alterations within the 3'UTR and correlates its importance for regular gene expression.

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