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Mutational spectrum of the *SERPING1* gene in Swiss patients with hereditary angioedema

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Summary

Hereditary angioedema with C1 inhibitor deficiency (C1-INH-HAE) is a rare autosomal dominant disease caused by mutations in the C1 inhibitor gene *SERPING1*. Phenotype and clinical features of the disease are extremely heterogeneous, varying even within the same family. Compared to HAE cohorts in other countries, the genetic background of the Swiss HAE patients has not yet been elucidated. In the present study we investigated the mutational spectrum of the *SERPING1* gene in 19 patients of nine unrelated Swiss families. The families comprise a total of 111 HAE-affected subjects which corresponds to approximately 70% of all HAE-affected patients living in Switzerland. Three of the identified mutations are newly described. Members of family A with a nucleotide duplication as genetic background seem to have a more intense disease manifestation with a higher attack frequency compared to the other families. Newly designed genetic screening tests allow a fast and cost-efficient testing for HAE in other family members.

Keywords: families, genetic analysis, hereditary angioedema (HAE), Swiss HAE cohort

Introduction

Hereditary angioedema with C1 inhibitor deficiency (C1-INH-HAE) is a rare autosomal dominant disease (OMIM 106100), characterized clinically by recurrent acute swelling episodes on the extremities, abdomen, face, trunk or airways, resulting from increased vascular permeability [1,2]. The disease is caused by mutations in the C1 inhibitor gene *SERPING1*, located on chromosome 11q12–q13.1. The gene involves eight exons and seven introns distributed over 17 kb, with introns containing repetitive *Alu* sequences [3]. Mutations in *SERPING1* result in reduced plasma levels (type I) or impaired function (type II) of the C1-INH protein. More than 300 different mutations in *SERPING1* have been described so far in the HAE database (http://hae.enzim.hu/) [4], ranging from small insertions, deletions and nucleotide substitutions to large deletions and duplications [5–8].

The genetic background of several HAE cohorts from different countries has already been documented [9–14]. A clinical description of the Swiss HAE cohort was published recently, describing 135 patients living in the German part of Switzerland [15]. However, the genetic background of Swiss HAE patients has not yet been elucidated.

In the present study we investigated the mutational spectrum of the *SERPING1* gene in 19 patients out of nine unrelated Swiss families, comprising a total of 111 HAE-affected subjects. According to the prevalence of 1 : 50 000 to 1 : 100 000 [16–18], this corresponds to approximately 70% of all HAE-affected patients living in Switzerland. Three of the identified mutations have not yet been described. Newly designed genetic screening tests allow a fast and cost-efficient method to identify selectively the presence of HAE in other family members.

Material and methods

The protocol of this study received ethics committee approval and was conducted according to the principles of good clinical practice and adhered strictly to the ethical standards outlined in the Declaration of Helsinki [19].

Patients and data collection

Nineteen patients receiving medical care at the Division of Haematology at the Cantonal Hospital Lucerne were recruited for genetic analysis. They belong to nine unrelated Swiss families comprising 556 members, of whom 111 are affected by HAE. Seventy of these patients were described clinically in the previous Swiss HAE cohort study [15]. All patients fulfilled the diagnostic criteria for HAE according the guidelines [2]. Data of patient's birthday, sex, date of first symptoms, frequency of angioedema and if they took prophylactic treatment were assessed from the questionnaire concerning clinical patient characteristics described previously [15]. Family affiliations have been determined with the collaboration of the Swiss HAE patient organization (www.hae-vereinigung.ch). All participants provided written informed consent.

Data handling

Clinical information was collected at the administration site of the Swiss HAE cohort (Haematology of the Cantonal Hospital Lucerne). Genetic analysis was performed at the Central Haematology Laboratory of the Cantonal Hospital Lucerne.

Numbering and nomenclature

The systematic numbering recommended by the Human Genome Variation Society (HGVS) (http://www.hgvs.org/mutnomen/), that starts with one at the first nucleotide of the coding sequence, was used to describe mutations at the DNA level (RefSeq_NG_009625.1). For amino acid numbering, the systematic numbering recommended by the HGVS was used. The translation initiator methionine was considered number one.

Mutations identified in this study that had already been published under a different nomenclature are named here according to the current standard. The original name is given in addition as an alternative.

DNA and RNA isolation

Genomic DNA was extracted from ethylenediamine tetraacetic acid (EDTA) anti-coagulated whole blood samples using the QIAamp DNA Mini Kit Qiagen (Hombrechtikon, Switzerland), according to the manufacturer's instructions. Total RNA was extracted with the MagNA Pure Compact RNA Isolation Kit (Roche Diagnostics, Rotkreuz, Switzerland). Reverse transcription was performed with the ipsogen RT Kit (Qiagen), according to the manufacturer's instructions.

Genotyping

Mutation analysis by next-generation sequencing (NGS). For the detection of *SERPING1* mutations in the non-coding exon 1 and in the seven coding exons 2–8 [including 150 base pairs (bp) flanking intronic sequence for each exon], NGS was performed using the Ion Torrent Personal Genome machine (PGM) (LifeTechnologies, Thermo Fischer, Switzerland). Ion AmpliSeq™ Designer version 2.0 (LifeTechnologies) web application was used to design custom primer pools. The final primer pool covered 94·98% (total bases: 3865; missed bases: 194) of the *SERPING1* input sequence. Sanger sequencing was performed to cover the missing bases. Library preparation was performed according to the user guide (publication part number MAN0006735, 10 September 2012) after genomic DNA had been quantified with the Qubit dsDNA HS Assay Kit (Life Technologies).

All thermal cycling reactions were carried out in a 0·2 ml non-polystyrene polymerase chain reaction (PCR) tube in a Techne TC-PLUS (Bibby Scientific, Stone, UK) thermal cycler. The Ion Xpress Barcode Kit 1- 16 (Life Technologies) was used to run more than one library multiplexed on a single chip.

The Ion PGM Template OT2 200 Kit (Life Technologies) was used to prepare template-positive ion sphere particles according to the user guide (MAN0007220 2.0).

Sequencing reactions were performed with an Ion 314™ Chip (version 2.0) according to protocol (MAN0007517 1.0).

Mutation analysis by Sanger sequencing. Sanger sequencing was used to cover parts of exons 3 and 8 that were not covered by NGS. Six primers (Supporting information, Table S1) were used for amplification and sequencing of these exons. Additionally, the detected variants in NGS were confirmed by Sanger sequencing. DNA amplification was carried out in a 10 µl reaction volume using HotStarTaq DNA polymerase (Qiagen) with 100 ng of genomic DNA and 0·4 µM of each primer. An initial denaturation (95°C, 15 min) was followed by 40 cycles (94°C, 30 s, 58°C, 30 s, 72°C, 60 s) and a final extension step (72°C, 5 min, 20°C, 5 s). One µl of ExoSAP-IT (Affimetrix) was mixed with 2·5 µl of PCR product in a 0·2 ml PCR reaction tube and placed in a thermal cycler (37°C, 15 min, 80°C, 15 min, 20°C, 5 s) for product clean-up. Cycle sequencing was carried out in a 10 µl reaction volume using the BigDye Terminator Chemie version 1.1 (Life Technologies) in a 0·2 ml PCR reaction tube. Products were then cleaned with the DyeEx 2.0 spin kit (Qiagen), and analysed on a Genetic Analyzer 3130 (Life Technologies).

Screening for large deletions and insertions. To spot large deletions and insertions that are not detected by the sequencing strategies used (NGS amplicon size limited to ∼200 bp), an *in-silico* method was established for relative exon

Table 1. Families with hereditary angioedema (HAE)

Family	HAE type	Family members	Affected people	Patients recorded in swiss HAE cohort [15]	Genetically analysed
A	Type I	435	74	40	4
B	Type I	15	7	7	2
C	Type I	18	8	8	3
D	Type I	47	14	9	5
E	Type I	9	3	1	1
F	Type I	7	1	1	1
G	Type I	6	1	1	1
H	Type I	5	2	2	1
I	Type II	14	1	1	1

quantification based on the raw data in the amplicon coverage analysis file. This *in-silico* quantification was performed after every run on the Ion Torrent PGM, regardless of a potential disease causing single nucleotide mutation called simultaneously by the Variant Caller. From the Ion Torrent PGM Torrent Suite (version 3.4.2), the amplicon coverage analysis file containing the total number of reads for each amplicon was downloaded and inserted into a prepared Microsoft Excel worksheet (Microsoft Office 2010; Microsoft, Redmond, WA, USA). The number of reads of the *SERPING1* amplicons associated with specific exons of the sample was then divided by the number of reads of a control sample (not affected by HAE). This value was then normalized by the mean value of the reads of two reference genes (ALB and F2; $n = 73$) that were analysed on the same chip. The mean values and standard deviations (s.d.) for the amplicons covering the corresponding exon (exons 1 and 5: $n = 3$, exons 2, 4, 6 and 7: $n = 2$, exon 3: $n = 5$, exon 8: $n = 4$) were calculated from the normalized values. A plot containing the mean value and s.d. was generated for data interpretation (Supporting information, Fig. S1). Samples where the *in-silico* quantification gave evidence for an entire exon deletion/duplication were analysed further for relative exon quantification by quantitative PCR (Supporting information, Fig. S2). Specific primer pairs for each exon were designed with the Primer-BLAST. As the deletion of exons 1 and 2 is highly unlikely, the Cp value of exon 1 was used as the reference exon and exon 2 quantification was not performed (Supporting information, Table S2). Additional intron quantification (primers shown in Supporting information, Table S3) was performed to narrow the expected break-point of the large deletion of exon 4 described in family C (Supporting information, Fig. S3). PCR analysis was performed with the LightCylcer 480. The data evaluation was carried out with Light Cycler® 480 software (release 1.5.0 SP4) (Roche).

Long-range PCR on suspicious samples was then performed and the actual break-point was determined by sequence analysis (Supporting information, Fig. S4).

Family-specific screening methods. For the mutations identified in families A, B and C genotyping methods were established for screening other family members and their offspring. Methods include DNA fragment analysis by capillary electrophoresis (Supporting information, Fig. S5), DNA fragment analysis by gel electrophoresis (Supporting information, Fig. S6) and Sanger sequencing assay (Supporting information, Fig. S7).

Results

Clinical patient characteristics

The 19 patients included into this study belong to nine unrelated Swiss families A–I (Table 1). Families A–H are affected by HAE type I, family I by HAE type II. These families comprise 556 members, 111 of whom are affected by HAE, representing approximately 70% of all patients with HAE living in Switzerland. Clinical data from 70 patients (38 women and 32 men) are known and have already been described [15]. Mean age was 45 years (s.d. = 19·3; range = 6–81). Symptom onset of the 64 symptomatic patients was at a mean age of 10 years (s.d. = 7·1). Family A comprised 40 patients (18 women and 22 men) with a mean age of 46 years (s.d. = 20; range = 6–81). Mean age at symptom onset of the 39 symptomatic patients was 8 years (s.d. = 5). Family B included seven patients (five women, two men) with a mean age of 35 years (s.d. = 14; range 14–57). Two patients were not symptomatic. Symptom onset was at a mean age of 11 years (s.d. = 7). In family C the mean age of the eight patients (four women, four men) was 37 years (s.d. = 19·8; range = 18–78), symptom onset of the seven symptomatic patients was at a mean age of 9 years (s.d. = 3·6) and in family D the six women and three men had a mean age of 55 years (s.d. = 16·5; range = 31–79). Symptom onset was at 14 years (s.d. = 8·2); two patients were not symptomatic. Prophylactic treatment was prescribed most often in family A. Attack frequency in patients without prophylaxis was higher in families with nucleotide duplications (A, E) and deletions (C, F) compared to families with nucleotide substitutions (B, G, H, I) (Table 2).

Genetic analysis

Clinically relevant mutations in the *SERPING1* gene were identified in eight families (Table 3). Mutations included

Table 2. Family-specific clinical characteristics

Family	Number F/M	Mean age (years)	Symptom onset	Prophylaxis yes/no	Number of attacks without prophylaxis
A	40	46	8	19/21	Frequent: 3
	18/22	s.d. 20	s.d. 5		Intermediate: 6
					Rare: 9
					Very rare: 3
B	7	35	11	1/6	Frequent: 0
	5/2	s.d. 14	s.d. 7		Intermediate: 1
					Rare: 3
					Very rare: 2
C	8	37	9	2/6	Frequent: 0
	4/4	s.d. 19.8	s.d. 3.6		Intermediate: 2
					Rare: 3
					Very rare: 1
D	9	55	14	2/7	Frequent: 0
	6/3	s.d. 16.5	s.d. 8.2		Intermediate 3
					Rare: 1
					Very rare: 3
E	1	25	2	1/0	–
	1/0				
F	1	47	9	0/1	Frequent:1
	1/0				
G	1	43	5	1/0	–
	1/0				
H	2	50	27	0/2	Frequent: 1
	1/1	s.d. 24	s.d. 8		Rare: 1
I	1	45	27	0/1	Intermediate:1
	1/0				

F = female; M = male; s.d. = standard deviation. Frequent number of attacks: $\geq$ 1/week. Intermediate number of attacks: $\leq$ 1/week; $\geq$ 1/month. Rare number of attacks: $\leq$ 1/month; $\geq$ 1/year. Very rare number of attacks: $<$ 1/year.

four nucleotide substitutions (families B, G, H and I), two nucleotide duplications (families A and E), one small deletion (family F) and one large deletion (family C). The mutations in families A, E, F, H and I have already been described [5,7,20,21]. The two nucleotide substitutions g.9518T > A (p.IIe196Asn) on exon 4 (family B) and g.13918C > G (p.Ser318Ter) on exon 6 (family G) and the large nucleotide deletion g.8569_9924del (p.Gly184_-Pro228del) (family C) have not been described previously. They have been deposited as GenBank Accession numbers SCV000297729 to SCV000297731. In family D the analysis showed no significant reduction or increase in the fold coverage in any of the eight exons. The molecular basis of clinical HAE is still unknown in family D.

The mutations in families A, B, E, F, H and I were identified by the implementation of the variant caller software after sequencing on the Ion Torrent PGM. Further analyses were necessary in three families (C, D and G), where the variant caller did not identify a mutation. Visualization of the aligned amplicons in the IGV browser showed a variant C > G at position g.13918 in exon 6 in family G. This nucleotide change is located in the second position of a TCA codon (encoding serine), and leads to a stop codon (TGA; p.Ser318Ter). All observed relevant variants were

Table 3. Identified mutations in the *SERPING1* gene

Family	Localization	Nucleotide change (DNA)	Predicted effect on transcription	Nucleotide change (RNA)	Ref
A	Exon 3	g.7807_7808dupTT	p.Thr179fs	n.d.	5
B	Exon 4	g.9518T>A^{N}	p.IIe196Asn	n.d.	This study
C	Introns 3–4	g.8569_9924del^{N}	p.Gly184_Pro228del	r.742_867del	This study
E	Exon 8	g.21875dupA	p.Glu451fs	n.d.	21
F	Exon 3	g.7625delC	p.Pro117fs	n.d.	20
G	Exon 6	g.13918>G^{N}	p.Ser318Ter	n.d.	This study
H	Exon 8	g.22026T>C	p.Ter501ArgexTer46	n.d.	21
I	Exon 8	g.21922G>A	p.Arg466His	n.d.	7

n.d. = not done; ^{N} = novel mutation.

confirmed by Sanger sequencing. *In-silico* relative exon quantification in family C revealed a significant reduction of exon 4 compared to the other exons (Supporting information, Figs S1–S4).

Based on negative family history, the mutations identified in the participants of families E, F, G, H and I were considered to be *de-novo* mutations.

Discussion

This is the first large genetic characterization of the Swiss HAE population. It represents a comprehensive picture of this rare disease at a national level. In eight of the nine families investigated with a diagnosis of HAE, mutations in the *SERPING1* gene were identified. The diversity of the identified mutations confirms the heterogeneity of mutations observed in other European countries [12,20,22,23]. Missense mutations and nucleotide duplications with consecutive frameshift constitute the largest groups of mutations, which is in line with studies of other populations [24]. Two of the reported missense mutations in family H (g.22026T > C) and I (g.21922G > A) are located in exon 8, which appears to be a hot-spot for mutations according to the literature, due to the two hinge regions surrounding the active site [21]. Mutations within the active centre loop site is a major cause of C1-INH-HAE type II [7]. The novel missense mutation g.9518 T > A (family B) with predicted amino acid exchange Ile196Asn replaces a hydrophobic and non-polar isoleucine with a hydrophilic and acidic aspartic acid. Ile196 is located in a region that does not have a direct role in the conformation transition of serpins and is described as being benign [25]. However, as in this family no other mutation in the *SERPING1* gene was found, we assume that it is the most plausible candidate for the described phenotype. The change Ter501Arg (family H) modifies the C-terminal portion of C1-INH, in that a thymine to cytosine transition in the termination codon allows abnormal elongation of C1-INH's C-terminal end. This mutation has already been described and it is hypothesized that the resulting mRNA transcript is not synthesized or that the mutant protein cannot be secreted normally or, alternatively, is degraded rapidly in plasma [21]. The large in-frame deletion g.8569_9924del (family C) excludes the entire exon 4, as such gross deletions result in unequal cross-overs between Alu repeats within this gene. Gross alterations of chromosomes or large chromosomal regions constitute an important cause of inherited disease and can take the form of deletion, duplication, insertions, inversions or translocations [26]. The duplication of two Ts in exon 4 between nucleotide 7807 and 7808 (g.7807_7808dupTT) in family A changes the sequence from ACC to TTAC. This would cause a frameshift, leading to a premature stop codon and therefore a premature termination of mRNA. The same occurs in family E (g.21875dupA), when the duplication of an A in exon 8 between nucleotide 21874 and 21875 changes the sequence from GAG to AGAG. Errors here may cause improper folding of the protein leading to a loss of function. A novel non-sense mutation (exon 6) was detected in family G (g.13918 > G).

As the phenotype of HAE is extremely heterogeneous, even within the same family, mutations in *SERPING1* are not the only responsible factor determining the clinical manifestations [24]. The number of family members in this study was too small to conclude strong associations from different genotypes to distinct phenotypes. However, it is conspicuous that patients with nucleotide duplications (A, E) or deletions (F, C) seem to have a more severe clinical course, needing more prophylactic treatment and suffering from more angioedema attacks, compared to the patients with missense mutations (B, G, H, I), as shown in Table 2. This confirms the observation from Speletas and colleagues [24]. Family D differs from the other families by a late symptom onset and the elevated median age. No molecular basis was found in the analysis of *SERPING1* in this family. This may be due to a number of reasons; for instance, that genomic defects are located in intronic or untranslated regions and possibly modifying *SERPING1* expression was not covered by the performed analyses. An effort needs to be made to elucidate the genetic background in this family with expanded analyses, such as investigation of the intronic and promotor regions.

The established genetic screening tests allow, in combination with the existing plasma determinations, a fast and cost-efficient method to identify selectively the presence of HAE in other family members.

A limitation of the study is that we have not yet verified that the mutations found in this study are not prevalent in healthy relatives and healthy people of the general population (true negative controls). However, we would like to emphasize that each family-specific variant was identified exclusively in this particular family only, and was not detected in samples of any other family in our study. This fact is already strong evidence that the novel-family specific variants are of high clinical relevance.

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Disclosure

The authors declare no competing financial or commercial interests that could be construed as a potential conflict of interest.

Author contributions

U. C. S. carried out concept and design of the study; accumulation of data; analysis and interpretation of the data. Performed writing and critical revision of the manuscript. M. K. designed genetic tests; performed genetic analysis; analysed and interpreted the data, writing and critical revision of the manuscript; P. S. designed genetic tests and *in-silico* relative quantification; performed genetic analysis; analysis and interpretation of the data and critical revision of the manuscript. S. C. carried out analysis and interpretation of the data; critical revision of the manuscript. W. A. W. carried out concept and design of the study; critical revision of the manuscript; all authors read and approved the final manuscript.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site

Fig. S1. *In-silico* relative exon quantification method to screen for large deletions and insertions based on the next-generation sequencing (NGS) raw data.
Fig. S2. Relative exon quantification [quantitative polymerase chain reaction (PCR)] to confirm *in-silico* quantification results.
Fig. S3. Relative intron quantification [quantitative polymerase chain reaction (PCR)] used to narrow the region to be sequenced for breakpoint elucidation.
Fig. S4. Long-range polymerase chain reaction (PCR).
Fig. S5. Screening in family A for mutation g.7807_7808dupTT.
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Table S1. Primers used in Sanger sequencing of exons 3 and 8.
Table S2. Primers used in relative exon quantification.
Table S3. Primers used in relative intron quantification.

