Aspects of hereditary angioedema genotyping in the era of NGS: The case of *F12* gene

Wybrane aspekty genotypowania wrodzonego obrzęku naczynioruchowego w erze NGS: gen F12

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Summary

Objective. To screen a cohort of patients diagnosed with non-FXII angioedema for carriage of variants of *F12* gene.

Material and methods. DNA samples from 191 patients suffering from primary angioedema with normal C1-INH, 54 samples from non--affected family members, and 161 samples from C1-INH-HAE (154 type I, 7 type II) patients were included in the study. The *F12* gene was genotyped by targeted NGS (100% coverage of translated regions). Sanger sequencing was performed for the verification of all identified variants and family segregation studies.

Results. The pathogenic *F12* variant c.983C>A was detected in three patients from two unrelated families initially diagnosed as U-HAE. Six additional mutations were identified, four of which were characterized as benign (c.41T>C, c.418C>G, c.1025C>T, c.530C>T) and two of uncertain significance (c.1530G>C, c.1768T>G). Two synonymous variants (c.756C>T and c.711C>T), the common polymorphism c.619G>C, and the functional polymorphism c.-4T>C were detected in allele frequencies similar to those presented in the ExAC database for the European population. One more not yet reported synonymous variant (c. 1599A>G) was also found.

Conclusion. Analyzing the entire translated region of *F12* gene is important in order to identify new variants that possibly affect HAE expressivity. Interestingly, genetic analysis of *F12* supports not only the diagnosis of FXII-HAE but also the correct exclusion diagnosis of U-HAE.

Keywords: *F12* gene, F12 mutations, hereditary angioedema, next-generation sequencing

Streszczenie

Cel. Przesiewowe badanie kohorty pacjentów z rozpoznanym obrzękiem naczynioruchowym innym niż zależny od FXII w kierunku nosicielstwa wariantów genu F12.

Materiał i metody. Do badania włączono próbki DNA 191 pacjentów cierpiących na pierwotny obrzęk naczynioruchowy z prawidłowym C1-INH, 54 zdrowych członków rodzin oraz 161 pacjentów z C1-INH-HAE (154 typ I, 7 typ II). Gen F12 był genotypowany metodą NGS (obejmującą cały rejon poddany translacji). Sekwencjonowanie metodą Sangera zostało wykonane celem weryfikacji wszystkich zidentyfikowanych wariantów i badań segregacyjnych rodzin.

Wyniki. U trzech pacjentów z dwóch niespokrewnionych rodzin pierwotnie zdiagnozowanych jako U-HAE wykryto patogenny wariant F12: c.983C>A. Zidentyfikowano sześć dodatkowych mutacji, z których cztery zostały określone jako łagodne (c.41T>C, c.418C>G, c.1025C>T, c.530C>T), a dwie jako mutacje o niepewnym znaczeniu (c.1530G>C, c.1768T>G). Stwierdzono dwa warianty synonimiczne (c.756C>T oraz c.711C>T), pospolity polimorfizm (c.619G>C) oraz czynnościowy polimorfizm c.-4T>C z częstością alleliczną podobną do podawanej w bazie ExAC dla populacji europejskiej. Wykryto również jeden nie raportowany dotychczas wariant synonimiczny (c. 1599A>G).

Wnioski. Analiza całego rejonu genu F12 poddawanego translacji jest ważna dla identyfikowania nowych wariantów, które mogą oddziaływać na ekspresję HAE. Ponadto, analiza genetyczna F12 pozwala nie tylko na potwierdzenie rozpoznania FXII-HAE, ale również prawidłowe wykluczenie rozpoznania U-HAE.

Słowa kluczowe: gen F12, mutacje F12, wrodzony obrzęk naczynioruchowy, sekwencjonowanie następnej generacji

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Abbreviations:

ACEi-AAE – acquired angioedema related to angiotensin-converting enzyme inhibitor

C1-INH – C1-inhibitor

C1-INH-HAE – hereditary angioedema due to C1-INH deficiency

FXII-HAE – hereditary angioedema with *F12* mutation

HAE – hereditary angioedema

INH-AAE – idiopathic non-histaminergic acquired angioedema

ISPs – enriched template-positive Ion Sphere Particles

n-C1-INH-HAE – hereditary angioedema with normal C1-esterase inhibitor

NGS – next generation sequencing

PGM – personal genome machine

U-HAE – unknown hereditary angioedema

VUS – variant of uncertain significance

INTRODUCTION

HAE is a rare and potentially life-threatening genetic disorder, manifested by recurrent attacks of subcutaneous and/or submucosal edemas which develop as a result of transient release of vasoactive peptides and increased permeability of blood vessels [1]. Its pathogenicity, until now, has been associated with mutations in four genes, *SERPING1* [1], *F12* [1], *PLG* [2, 3] and *ANGPT1* [4]. Mutations in *SERPING1* result in deficiencies of C1-INH causing C1-INH-HAE, whereas the other mutations do not affect concentration or function of C1-INH. These types of HAE are called nC1-INH-HAE, regardless if a mutation is known (FXII-HAE, PLG-HAE, ANGPT1-HAE) or not (U-HAE). The conventional analysis of these genes is cumbersome, time consuming, and unable to support parallel investigation of multiple targets. Recent progress in massive parallel sequencing, termed NGS, has increased the speed and efficiency of mutation testing. As NGS overcomes many of the obstacles that have been faced by traditional approaches, we proceeded to the development and validation of a custom NGS platform (NGS-HAE panel) that targets the entire 11q12-q13.1 locus, including the promoter, coding, intron-exon boundary, intronic regions of the *SERPING1* gene [5]. Aiming to examine the possible involvement of other genes in the manifestation of HAE, we expanded this panel by introducing a number of genes encoding for proteins involved in bradykinin metabolism and function, including *F12*.

As it is known, four *F12* pathogenic alterations have been identified, so far: two missense mutations (c.983C>A and c.983C>G) [6], a deletion of 72 bp (c.971_1018+ 24del72) [7] and a duplication of 18 bp (c.892_909dup) [8]. Furthermore, the functional promoter polymorphism *F12* c.-4T>C (rs1801020) acts as an independent modifier of C1-INH-HAE severity [9]. Finally, in a recent study a patient bearing two co-existing pathogenic mutations in *SERPING1* and *F12* gene has been described [10].

The aim of this article is to present the data obtained from genotyping a cohort of patients with different types of angioedema using the above mentioned NGS-HAE panel, as far as, the *F12* gene is regarded.

MATERIAL AND METHODS

Study subjects

A total of 191 DNA samples with normal C1-inhibitor (139 U-HAE, 14 INH-AAE, 3 ACEi-AAE, 8 CPN1 deficiency, 27 samples from patients with recurrent angioedema and normal C1-INH but with unknown family history and not fulfilling the criteria for INH-AAE) belonging to 160 families of European origin (52 Hungarian, 33 Greek, 3 German, 28 Spanish, 8 Polish, 31 Italian, 3 French, 2 Bulgarian) were genotyped along with 54 samples from non-affected family members. Additionally, 161 C1-INH-HAE (154 type I, 7 type II) samples bearing various *SERPING1* pathogenic mutations were screened for the presence of a mutation in *F12* gene. The local institutional review boards approved this study, and written informed consent was obtained from each individual or an accompanying relative.

Next-Generation Sequencing

A NGS custom panel was designed using the Ion AmpliSeq Thermo Fisher Scientific Designer, in order to analyze all the exonic, exon-intron junctions and untranslated (5' and 3'-UTR) regions of the *F12* gene. 16 amplicons divided in two primer pools provide 100% coverage of all translated regions of the gene. Additional amplicons (809 in total) for different genes (n=55) selected among those encoding for proteins involved in bradykinin metabolism, including *SERPING1* gene, were introduced to the panel. Amplicon libraries of the *F12* regions were prepared using an Ion AmpliSeq Library Kit 2.0 (Thermo Scientific) and Ion Xpress™ Barcode Adapter 1-96 Kit (Thermo Scientific). Pooled, barcoded libraries were clonally amplified using the Ion OneTouch™ system and Ion OT2 HI-Q Template kit (Thermo Scientific). ISPs were enriched with the Dynabeads® MyOne™ Streptavidin C1 Beads (Thermo Scientific) and washed with the Ion OneTouch Wash Solution included in the kit using the Ion One Touch ES system (Thermo Scientific). The subsequently enriched template-positive ISPs were loaded onto Ion 318 chips and sequenced on the PGM using the Ion PGM Hi-Q

sequencing kit (Thermo Scientific). All procedures were performed according to the manufacturer's instructions.

Sequencing raw data were analyzed for base calling, demultiplexing, alignment to hg19 reference genome (GRCh37), coverage analysis and variant calling on Torrent Suite 5.2 software (Ion Torrent) using default parameters. The plugins used for the analysis were CoverageAnalysis v.5.2.1.2 and VariantCaller v.5.2. The reference sequences of *F12* and *SERPING1* genes were NM_000505.3 and NM_000062.2, respectively. Annotation of variants was performed on Ion Reporter software v.5.2 (Thermo Scientific). PolyPhen2 (http://genetics.bwh.harvard.edu/pph2/) and SIFT (http://sift.jcvi.org/) bioinformatic tools were used to predict the effect of amino acid substitution. The integrative genomics viewer v2.2 (IGV, Broad Institute) was used for visualization.

Sanger sequencing was performed for the verification of all identified variants and family segregation studies, as previously described [11].

RESULTS

All variants detected in the *F12* gene are presented in Figure 1, while their allele frequencies are shown in Table I.

The missense pathogenic mutation c.983C>A (p.Thr-328Lys, rs118204456) of the *F12* gene was identified in three subjects, belonging to two unrelated Spanish families, initially diagnosed with U-HAE.

Beyond this, six *F12* missense mutations were found in our patients (Fig. 1). In two different patients with type I C1-INH-HAE the mutations c.41T>C (p.Leu14Ser, rs143809932) and c.1025C>T (p.Pro342Leu, rs2230939) were detected. The mutation c.418C>G (p.Leu140Val, rs35515200) was detected in one German patient with type I C1-INH-HAE and in two patients with U-HAE, one of Italian and the oth-

er of Polish origin. The mutation c.530C>T (p.Ala177Val, rs144821595) was detected in two U-HAE patients, while the c.1768T>G (p.Cys590Gly) and the c.1530G>C (p.Glu-510Asp) were detected in two different patients with normal C1-INH.

Carriers of combined *SERPING1* and *F12* pathogenic mutations were not detected.

Two common synonymous mutations were identified (c.756C>T, rs41309752 and c.711C>T, rs17876047) and their allele frequencies in individuals of European origin are 0.97% and 4.02% respectively (ExAC database). Additionally, one rare synonymous mutation (c.1599A>G), not published in NCBI and ExAC databases, was detected in a single U-HAE patient of Hungarian origin.

Finally, the common *F12* polymorphism rs17876030 (c.619G>C, p.Ala207Pro, exon 7) was detected in 11 samples (5 C1-INH-HAE, 6 nC1-INH-HAE) while the functional promoter polymorphism *F12* c.-4T>C (rs1801020) was detected in 73 samples (13 homozygous) with normal C1-INH belonging to 60 families and 52 samples with C1-INH-HAE (9 homozygous).

DISCUSSION

Approximately 25% of angioedema cases with normal C1-INH are associated with a mutation in the *F12* gene [12]. The mutations described until now as causative for FXII-HAE are located in exon 9 of the *F12* gene. The majority of patients bear the c.983C>A substitution which results in a threonine-to-lysine aminoacid change (p.Thr328Lys) in factor XII [12]. Another mutation predicting a threonine-to-arginine substitution (c.983C>G, p.Thr328Arg) in the same codon has been described in two German families [6]. There has also been a report of an 18-bp duplication (c.892_909dup) in a Hungarian family and a 72-bp deletion (c.971_1018 + 24del72) in a Turkish family [7, 8]. All these

Table I. The allele frequencies of all mutations

|	*F12* alteration			Allele frequencies	
Exon	Characterization of mutation	Nucleotide	ExAC (EU)	nC1-INH-HAE (n=160)	C1-INH-HAE (n=161)
utr_5	Functional Polymorphism	c.-4T>C	24.02%	60 (22.81%)	52 (18.94%)
Exon 1	Likely Benign	c.41T>C	0.01%	0 (<0.001%)	1 (0.31%)
Exon 6	Benign	c.418C>G	0.32%	2 (0.62%)	1 (0.31%)
Exon 7	Benign	c.530C>T	0.01%	2 (0.62%)	0 (<0.001%)
Exon 7	Polymorphism	c.619G>C	1.38%	6 (2.5%)	5 (1.55%)
Exon 8	Synonymous	c.711C>T	4.02%	8 (2.5%)	6 (1.86%)
Exon 8	Synonymous	c.756C>T	0.97%	4 (1.25%)	5 (1.55%)
Exon 9	Pathogenic	c.983C>A	<0.001%	2 (0.62%)	0 (<0.001%)
Exon 10	Benign	c.1025C>T	0.11%	0 (<0.001%)	1 (0.31%)
Exon 12	VUS	c.1530G>C	<0.001%	1 (0.31%)	0 (<0.001%)
Exon 13	Synonymous	c.1599A>G	<0.001%	1 (0.31%)	0 (<0.001%)
Exon 14	VUS	c.1768T>G	<0.001%	1 (0.31%)	0 (<0.001%)
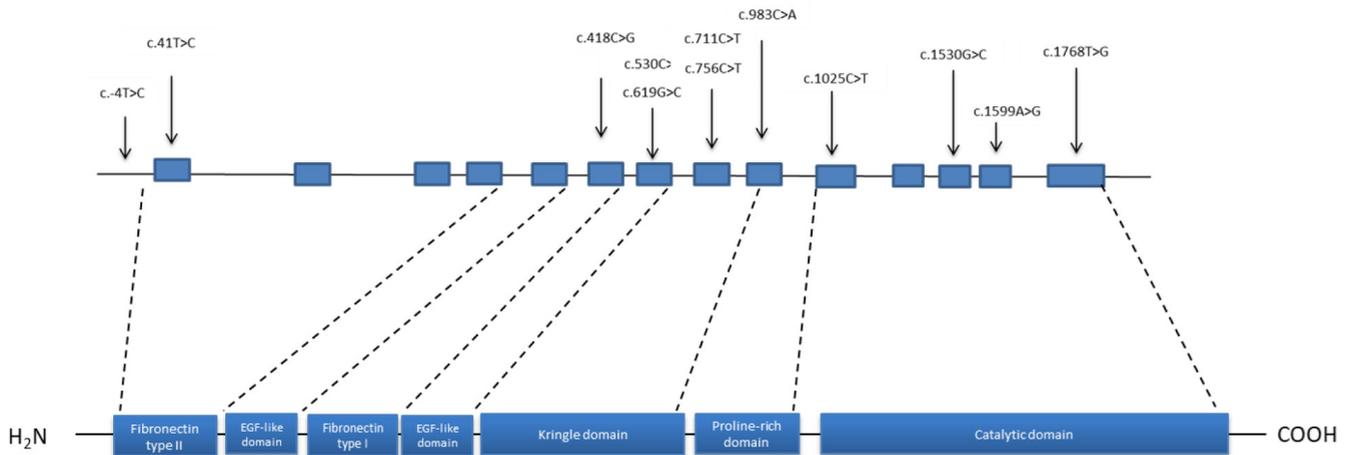
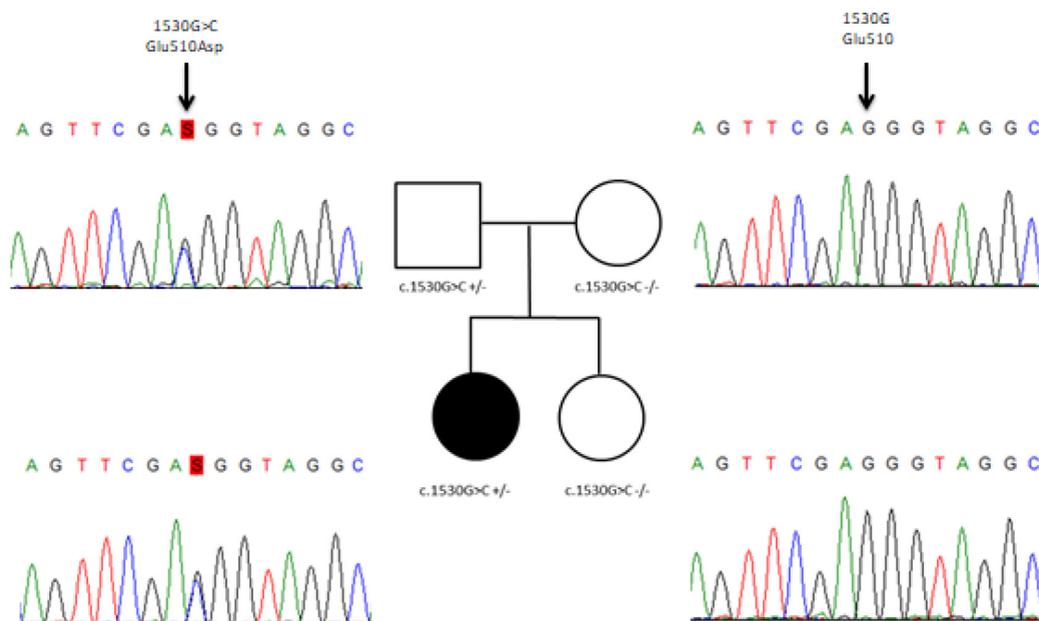Fig. 1. Detected mutations in the *F12* gene

Fig. 2. Family segregation study for the c.1530G>C (p.Glu510Asp) in the *F12* gene

mutations are located in the proline-rich linker peptide between the Kringle and trypsin-like serine protease (Tryp-SPc) domains of the FXII protein.

The mechanism of function of FXII-HAE is not fully understood. It has been proposed that the p.Thr328Lys increases enzymatic plasma activity in female mutation carriers that leads to enhanced kinin production resulting in angioedema [13]. Contrary to this study, Bork et al. have shown that there is no difference between FXII-HAE patients bearing the p.Thr328Lys mutation and their healthy probands in regard to FXII surface activation concluding that the mutation does not cause a 'gain-of-function' of FXIIa [14]. Subsequent *in vivo* and *in vitro* experiments in mice suggest that p.Thr328Lys and p.Thr328Arg result to the loss of O-linked glycosylation [15]. As a result, the proline-rich region is less negatively charged and the overall protein size of the mutant FXII molecule is reduced causing an increase in the susceptibility of FXII zymogen autoactivation that leads to excessive activation of bradykinin formation through the kallikrein-kinin pathway [16]. Furthermore, these mutations lead in accelerated activation of

the FXII by a plasmin, a natural activator of contact system [17]. However, further investigation is necessary to determine the pathway by which these alterations contribute to the formation of hereditary angioedema.

In this study, the mutation c.983C>A was identified in three women from two unrelated Spanish families initially misdiagnosed as U-HAE. These patients demonstrate features indicative of FXII-HAE in that attacks were appeared either during gestational periods or after the administration of estrogens.

Additionally, we identified six rare or not yet reported mutations in the *F12* gene (c.41T>C, c.418C>G, c.530C>T, c.1025C>T, c.1530G>C, c.1768T>G). The mutation c.41T>C (p.Leu14Ser), located in exon 1 of the *F12* gene, was identified in a single type I C1-INH-HAE Bulgarian patient co-carrying a pathogenic frameshift mutation in *SERPING1* gene. This variant is predicted to be deleterious by SIFT and probably damaging by PolyPhen2 analysis. Unfortunately, family segregation was impossible. Nevertheless, considering that the mutation has been detected in 0.01507% individuals of European origin (ExAC database)

and that its allele frequency is greater than that expected for FXII-HAE and *F12* deficiency, according to ACMG guidelines it is interpreted as likely benign [18].

The c.418C>G (p.Leu140Val) mutation is located in exon 6 of the *F12* gene and was detected in one German patient with type I C1-INH-HAE and two (an Italian and a Polish) U-HAE patients. Bioinformatic analysis by SIFT and PolyPhen2 algorithms predicted this mutation as tolerated and possibly damaging, respectively. The mutation is characterized as likely benign in ClinVar database in association to hereditary angioedema and factor XII deficiency. It has been detected in 0.3283% individuals of European origin and 0.2215% worldwide. Family segregation study performed in the two families of U-HAE revealed the mutation in three healthy family members, a fact supporting its previous characterization as benign.

The alteration c.530C>T (p.Ala177Val) is located in exon 7 of the *F12* gene and was detected in one Spanish and one Italian U-HAE patient. It presents with an allele frequency of 0.01413% and 0.00907% (ExAC database) in the European and worldwide population, respectively, and is predicted to be benign by both SIFT and PolyPhen2 bioinformatic tools. Family segregation in the Italian family supported that this alteration is benign for FXII-HAE, as three healthy members of the family were also carriers of the mutation.

Another alteration (c.1025C>T, p.Pro342Leu), was detected in exon 10 of the *F12* gene in one type I C1-INH-HAE Polish patient bearing a pathogenic *SERPING1* mutation. c.1025C>T is reported as likely benign in ClinVar database (rs2230939) in patients with HAE and FXII deficiency. This mutation is detected in 0.1804% individuals of European origin and was predicted to be benign by both SIFT and PolyPhen2 bioinformatic tools. Our data support the previous characterization of the mutation as benign.

The alteration c.1530G>C (p.Glu510Asp) was identified in a 12-year old girl with hereditary angioedema presenting with five to six angioedema attacks per month located in the face. This mutation was predicted as probably damaging by both the bioinformatic tools used in this study (SIFT and PolyPhen2). There is no report of this mutation in ClinVar database and it is not detected amongst 115000 exomes of the ExAC database. Family segregation study identified the mutation in the father of the patient, who had no angioedema attacks during his life (Figure 2). However, FXII-HAE has a very low penetrance, particularly in males (over 90% of male carriers are asymptomatic compared with 40% of females) [19]. This substitution occurs at the catalytic domain of the FXII protein. As the available evidence is currently insufficient to determine the role of this variant in FXII-HAE, we classify this mutation as a variant of uncertain significance (VUS). Nevertheless, it would be very interesting to test female probands of the patient's father, if any, in order to clarify its pathogenicity.

In one German patient the mutation c.1768T>G (p.Cys-590Gly) in exon 14 of the *F12* gene was detected. The patient is presenting with recurrent angioedema of unclear origin. c.1768T>G is predicted deleterious by SIFT and possibly damaging by PolyPhen2 analysis. There is no report of this mutation in ClinVar database and is not detected amongst 115000 exomes of the ExAC database. The substitution occurs at the catalytic domain of the FXII protein and in the same nucleotide position a substitution has been described (c.1768T>A, p.Cys590Ser, rs1157280571) leading to loss of enzyme activity [20]. However, it is currently unclear if c.1768T>G is a pathogenic variant and thus it is characterized as a VUS for FXII-HAE.

The allele frequencies of all common polymorphisms (rs17876030, rs41309752 and rs17876047 and rs1801020) are similar with the corresponding allele frequencies presented in the ExAC database for the European population (Table I). The rare (c.1599A>G) synonymous variant, according to Human Splicing Finder 3 bionformatic tool, is not predicted to have an effect in the splicing of the transcript.

It is concluded that high-throughput genomic technologies facilitate an extended analysis of genes involved in the pathogenesis of angioedema. However, the interpretation of their results represents a rather challenging attempt. In many genes, including *SERPING1*, a loss of function is a known disease mechanism and clearly deleterious variants (nonsense, frameshift, indels, splice defects) can be considered pathogenic. In *F12* gene, however, only specific variants lead to FXII-HAE, as causative variants must induce a gain of function in the encoded protein. This applies in missense or synonymous *F12* variants posing great challenges to interpretation. Loss of function variants in the gene are associated with Factor XII deficiency (OMIM: 234000), and thus are not expected to be identified in FXII-HAE patients. Furthermore, *F12* variants have been shown to have low penetrance, particularly in males, adding another level of difficulty. In the absence of functional and conclusive family segregation studies all missense variants, predicted to modify the structure of the protein and located outside exon 9 are characterized as VUS.

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