ORIGINAL ARTICLE

Study of angiopoietin and plasminogen genes in hereditary angioedema

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SUMMARY

OBJECTIVE: *To investigate the presence of the Angiopoietin 1 (ANGPT1) and Plasminogen (PLG) mutations in patients with Hereditary Angioedema (HAE) and normal C1 esterase inhibitor (C1-INH) levels, who do not harbor the F12 gene mutation.*

METHODS: *Patients clinically diagnosed with HAE but without C1-INH deficiency or dysfunction and F12 gene mutation were evaluated. DNA extraction, quantification, and dilution were performed at a concentration of 100 ng/µL, followed by a DNA amplification (PCR) for molecular evaluation of exon 2 of the ANGPT1 gene and exon 9 of the PLG gene for identification of mutations c.807G>T / p.A119S and c.988A>G / p.K330E, respectively. The PCR product was evaluated in 1% agarose gel electrophoresis. Sequencing was performed using the Sanger method. The electropherograms were analyzed using the FASTA® program.*

RESULTS: *DNA samples from 15 women were sequenced. Their ages ranged from 10 to 60 years and the normal C1 esterase and C4 inhibitor serum levels ranged from 22 to 39 mg/dL and from 10 to 40 mg/dL, respectively. No mutations were detected in the analyzed exons of ANGPT1 and PLG. However, a single-nucleotide polymorphism (SNP) was detected in two homozygotic and five heterozygotic patients.*

CONCLUSION: *Further studies are needed to evaluate these SNPs and scrutinize their potential for use as molecular markers of HAE and as novel therapeutic targets.*

KEYWORDS: *Bradykinin, Mutation, Angiopoietin-1, Plasminogen.*

INTRODUCTION

Hereditary Angioedema (HAE) is a rare and severe genetic disease of the kallikrein-kinin system caused by a deficiency of the C1-esterase inhibitor (C1-INH), which is inherited in an autosomal dominant manner. It affects approximately 1 in 60,000 inhabitants (ranging from 1:10,000 to 1:160,000) belonging to different

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ethnic groups. Although it is an autosomal dominant disease, it is detected more frequently and severely in women.[1-5]

Family history with similar clinical manifestations reinforces the HAE diagnosis. About 75-80% of HAE cases occur in the same family while 20-25% of cases are due to novel spontaneous mutations.[1-3]

HAE can be classified into three phenotypes: (1) quantitative C1-INH deficiency due to mutations, located throughout the *SERPING1* gene, that affect the structure of the protein and impair its secretion; (2) C1-INH dysfunction due to a mutation in exon 8 of the *SERPING1 gene* that results in the secretion of a non-functional protein; and (3) normal C1-INH that may be associated with mutations in the *F12*, *ANGPT1*, *PLG,* and Kininogen 1 (*KNG1*) genes, mainly found in female patients with normal C1-INH protein levels and activity.[1-14]

A variant of the *ANGPT1* gene was reported to interfere with the interaction between the angiopoietin protein and its natural receptor, endothelial tunica of the kinase cell– (TIE2), in endothelial cells leading to increased vascular permeability and edema. [12,15,16]

A mutation in the *PLG* gene has also been reported to increase fibrinolysis, which in turn causes plasmin formation and increased levels of bradykinin, leading to edema (*PLG*). [11,13,14]

The presence of genetic alterations in different genes emphasizes the importance of molecular genetic analysis in Brazilian patients to expand our knowledge on HAE and identify new strategies for the treatment of this disease.[11,12]

The objective of this study was to investigate the presence of mutations in the *ANGPT1* and *PLG genes* of HAE patients without C1-INH deficiency or dysfunction and *F12* gene mutations.

METHODS

A 5 mL blood sample was collected from each patient via venous puncture, followed by genomic DNA extraction using the Wizard Genomic DNA Purification® kit (Promega). DNA was quantified in a spectrophotometer and, when necessary, diluted to a concentration of 100 ng/µL for sequencing. After extraction, the DNA was used to amplify exon 2 of the *ANGPT1* gene and exon 9 of the *PLG* gene by polymerase chain reaction (PCR). The PCR products were resolved by 1% agarose gel electrophoresis and subjected to Sanger sequencing for detecting the mutations c.807 G>T / p.A119S and c.988 A>G / p.K330E in exon 2 of the *ANGPT1* gene and exon 9 of the *PLG* gene, respectively.

The forward primer of the *ANGPT1* gene was 5'GTTGACAACTGGATTCCTGTG3', and that of the *PLG* was 5'CTTAGTTTAGTACTGGAACGCAGG3', and reverse primer of the *ANGPT1* gene was 5'CGCATAGCATGTCAGGCAGTC3', and that of the *PLG* was 5'CAGGCTTTCTGACCACAATAGC3'. The sequencing reaction was performed using the Big Dye Terminator® v.3.1 kit (Thermo Fischer Scientific, Waltham, MA, USA).

Samples of purified sequencing reaction products were heated at 80°C for 15 minutes to inactivate the ExoSAP enzyme and subsequently, the products were precipitated.

After the precipitation of the DNA in the plates, it was resuspended in a specific buffer and DNA sequencing was performed by the Sanger method.

The electropherograms were analyzed by FASTA®, a software package for the alignment of DNA and protein sequences.[17]

STATISTICS

The data were inserted in an Excel® spreadsheet. The allele frequencies were combined on alleles I and II. Then the frequencies of the mutations in both alleles were added and compared with the relative frequencies reported in the specific literature using the Chi-square test with a result of $p < 0.05$ was not considered as significant.

RESULTS

A total of 69 patients with HAE, including both genders, were treated at the Allergy and Immunology Clinic of the Hospital de Clínicas Complex, Federal University of Paraná - Curitiba (CHC-UFPR). Of these, 27 patients diagnosed with normal C1-INH HAE were evaluated; as all of them were women, this study focused on female patients.

Patients with C1-INH deficiency or dysfunction, patients with mutations involving the *SERPIN*G1 gene and other forms of non-hereditary angioedema were also excluded.

The clinical features were recorded using a questionnaire form. The 27 patients were evaluated for *F12 gene* mutations and 12 of them were excluded

TABLE 1. CHARACTERISTICS OF PATIENTS STUDIED

FAMILY	PATIENT	RELATIONSHIP	GENDER	BIRTHDATE	AGE SYMPTONS STARTED	FAMILY HISTORY	TREATMENT
1	HAE001 HAE003	SISTER (HAE003) SISTER (HAE001)	F F	06/19/1979 12/27/1961	36 YEARS OLD 52 YEARS OLD	YES YES	NO NO
2	HAE005	XXXXX	F	11/16/1981	18 YEARS OLD	YES	NO
3	HAE006	XXXXX	F	07/13/2009	6 YEARS OLD	YES	NO
4	HAE007	XXXXX	F	02/12/1979	15 YEARS OLD	YES	NO
5	HAE008	XXXXX	F	02/02/1987	17 YEARS OLD	YES	NO
6	HAE009	XXXXX	F	06/22/1986	25 YEARS OLD	YES	NO
7	HAE011	XXXXX	F	11/12/1982	20 YEARS OLD	YES	NO
8	HAE012	XXXXX	F	03/24/1980	16 YEARS OLD	YES	NO
9	HAE013	XXXXX	F	01/10/1981	04 YEARS OLD	YES	NO
10	HAE014	XXXXX	F	06/22/1969	30 YEARS OLD	YES	NO
11	HAE015 HAE016	SISTER (HAE016) SISTER (HAE015)	F F	08/12/1983 05/03/1988	15 YEARS OLD 05 YEARS OLD	YES YES	NO NO
12	HAE019	XXXXX	F	11/13/1975	39 YEARS OLD	YES	NO
13	HAE021	XXXXX	F	10/08/1985	22 YEARS OLD	YES	NO

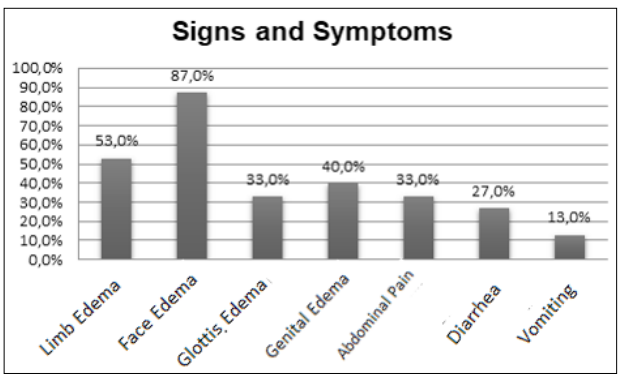
from the study because they presented positive results. Therefore, 15 patients without *F12* gene mutations were examined for the presence of mutations in exon 9 of the *ANGPT1 gene* and exon 2 of the *PLG gene*.

Biochemical tests revealed normal C1-INH and C4 serum levels ranging from 22 to 39 mg/dL and 10 to 40 mg/dL, respectively in the patients with HAE, who were aged between 10 and 60 years, and had 1 to 4 flares lasting for 3 days on an average per month. All patients reported a family history of angioedema (TABLE 1).

The patients reported flare symptoms in during emotional stress and menstrual cycle, leading to emotional problems and depression in some cases as well as financial burden. Chart 1 shows the most common signs and symptoms of HAE.

DNA sequence analysis did not detect the mutation c.807G>T / p.Ala119Ser in the *ANGPT1* gene and the mutation c.988 A>G / p. lys330Glu in the *PLG* gene.

GRAPH 1. MOST FREQUENT SIGNS AND SYMPTOMS



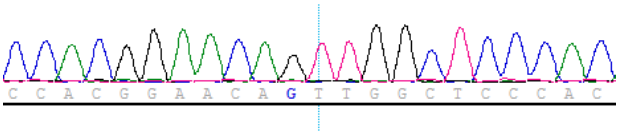
However, a single-nucleotide polymorphism (SNP) in the *PLG* gene was identified.

The electropherograms were evaluated by FASTA[17] and are shown below:

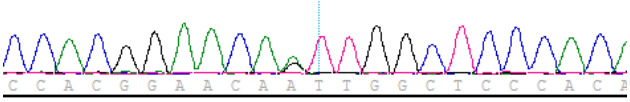
NORMAL SEQUENCE:
CAATCCTGACGGAAAAGGGCCATGGTG

MUTATED SEQUENCE:
GCAATCCTGACGGARAAAGCCATGGTG

HOMOZYGOUS SEQUENCE:
CCACGGAACAGTTGGCTCCCAC



HETEROZYGOUS SEQUENCE
CCACGGAACAA/GTTGGCTCCCAC



SNP silent c.1083A>G p.Gln 361= (CAA CAG→)

DISCUSSION

Bork and Binkley have described a type of HAE with normal C1 inhibitor, in which the functional activity of C1-INH and C4 levels remained normal, suggesting that HAE diagnosis would be more accurate through genetic analysis.[18,19]

HAE with normal C1-INH levels is the rarest among

the HAE phenotypes described in literature, occurring in about 30% of patients.[11] *F12* gene mutation was present in 44% of the 27 patients included in this study, although this prevalence is higher than that cited in other studies and may be related to ethnic differences among the populations studied.

Initially, the symptoms of HAEs with normal C1-INH were shown to affect predominantly women and to be associated with estrogen usage; however, because it is an autosomal dominant disease, men are also affected but are often asymptomatic.[9] The 15 patients who participated in this study reported clinical features that corroborate the literature such as limb, face and genital edema, abdominal pain, diarrhea, and vomiting. Of these, 87% reported face edema as the most common symptom of HAE with normal C1-INH. [1,3,9,11]

Of the two mutations that were evaluated in the 15 patients, the c.807G>T/ p.Ala119Ser in exon 2 of the *ANGPT1* gene has been described in an Italian family;[12] however, in this study, this mutation has been detected in a Brazilian family of non-Italian origin.[16] The c.988 A>G / p. lys330Glu mutation in exon 9 of the *PLG* gene has been described in 13 families in Europe,[11] including 3 in France and 2 families in Japan. [14,15] Considering the colonization and the population brazilian composition of 47.5% of European descendants according to IBGE[23], this research in Brazilian patients is justified.

The mutations described were not identified in the patients. However, a silent homozygous SNP c.1083A>G / p. Gln 361= (CAA CAG→) in the *PLG* gene was detected in two unrelated patients, where the presence of base G occurred on both alleles. Furthermore, five patients were heterozygous for this SNP . This SNP is identified as silent since it does not alter the protein sequence and has no clear effect on the gene function or the phenotype of the individual carrying the mutation. [20-22]

According to the literature,[24] the incidence of the SNP rs13231 flanking the *PLG* gene mutation c.1083A>G p.Gln 361= CAA CAG→ is 0.78% and 0.21 (or 21%) for alleles A and G, respectively. According to the ABRAOM[25] database, the incidence of this SNP in the Brazilian population was reported for the A allele 0.74% and for the G allele 0.26%.. In the sample studied, the frequency of the SNP was 30% (9/30), higher than that described for Brazilian and European populations; however, this is not a statistically significant difference (p>0.05).

All patients who participated in the study were clinically evaluated and diagnosed with Hereditary Angioedema of unknown cause (HAE-U), according to the criteria established by the Brazilian Guidelines for the diagnosis and treatment of hereditary angioedema.[1]

These Brazilian Guidelines were published in 2017 and therefore, cite only the *F12* gene mutations. After that, three more mutations have been identified as related to HAE with normal C1-inhibitor, including the two mutations analyzed in this study.[9,10]

This study has some limitations, particularly the small sample size. Nevertheless, the incidence of HAE in the Brazilian population is 1/160,000 and of these, only 30% have HAE with normal C1-INH.[1-3]

CONCLUSION

No mutations were detected in the evaluated exons in the 15 patients studied; however, 7 patients had a silent SNP, c.1083A>G / p.Gln 361= (CAA CAG→).

Further studies on SNPs are needed to clarify whether they can be used as molecular markers of HAE and as therapeutic targets for new treatments.

RESUMO

OBJETIVO: *Investigar a presença das mutações no gene Angiopoietina (ANGPT1) e gene Plasminogênio (PLG) em pacientes com Angioedema Hereditário (AEH) com inibidor C1 esterase (C1-INH) normal e negativos para mutação do gene F12.*

MÉTODOS: *Foram avaliados pacientes com diagnóstico clínico de AEH sem deficiência ou disfunção de C1-INH e negativos para mutação do gene F12. Realizou-se extração, quantificação e diluição do DNA a uma concentração de 100 ng/uL, em seguida amplificação do DNA (PCR) para avaliação molecular do exon 2 do gene ANGPT1 e do exon 9 do gene PLG para identificação das mutações c.807G>T.p.A119S e c.988A>G p.K330E, respectivamente. O produto da PCR foi avaliado em eletroforese em gel de agarose 1%. Foi realizado o sequenciamento pelo método de Sanger. As análises dos eletroferogramas foram realizadas pelo programa FASTA®.*

RESULTADOS: *Foram sequenciadas amostras de 15 mulheres, idade entre 10 e 60 anos, com níveis séricos de inibidor de C1 esterase e C4 normais variando de 22 a 39mg/dL e 10 a 40mg/dL, respectivamente. Não foram identificadas mutações nos éxons analisados dos genes ANGPT1 e PLG. Entretanto no gene PLG foram encontrados polimorfismo de nucleotídeo único (SNP), em duas pacientes homozigotas e cinco heterozigotas.*

CONCLUSÃO: *Mais estudos sobre SNP são necessários para esclarecer estes achados pois eles podem ser utilizados como marcadores moleculares do AEH e alvo para novos tratamentos.*

PALAVRA CHAVE: *Bradicinina, Mutação, Angiopoietina-1, Plasminogênio.*

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