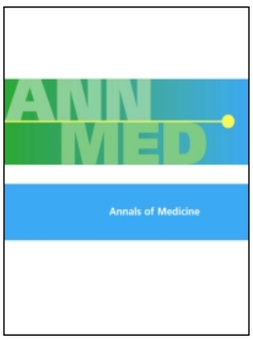

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Hereditary angioedema due to C1-inhibitor deficiency in Macedonia: clinical characteristics, novel *SERPING1* mutations, and genetic factors modifying the clinical phenotype

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Running title: **C1-INH-HAE in Macedonia**

ABSTRACT

Objective: Hereditary angioedema due to C1 inhibitor deficiency (C1-INH-HAE) is a rare disease, characterized by swellings. We aimed to characterize on a clinical and molecular basis C1-INH-HAE patients in the Republic of Macedonia.

Results: All 15 patients from six unrelated families were diagnosed with C1-INH-HAE type I, with a mean age of symptom onset of 11 years and an average delay of diagnosis of 7 years. Patients reported on average 31 angioedema attacks/year, with a median clinical severity score (CSS) of 7. We identified three known mutations, and two mutations (c.813_818delCAACAA and c.1488T>G) were reported for the first time. To address the genotype-phenotype association, a pooled analysis including 78 C1-INH-HAE south-eastern European patients was performed, with additional analysis of F12-46C/T and KLKB1-428G/A polymorphisms. We demonstrated that patients with nonsense and frameshift mutations, large deletions/insertions, splicing defects, and mutations at Arg444 exhibited an increased CSS compared with missense mutations, excluding mutations at Arg444. In addition, the CC F12-46C/T polymorphism was suggestive of earlier disease onset.

Discussion: Genetic analysis helped identify the molecular basis of C1-INH-HAE given that causative mutations in *SERPING1* were detected in all patients, including an infant before the appearance of clinical symptoms. We identified two novel mutations and further corroborated the genotype-phenotype relationship, wherein mutations with a clear effect on C1-INH function predispose patients to a more severe disease phenotype and CC F12-46C/T predisposes patients to earlier disease onset.

KEYWORDS: hereditary angioedema, C1 inhibitor, *SERPING1* gene, Macedonia.

KEY MESSAGES:

- In the present nationwide study, we aimed to characterize on a clinical and molecular basis patients with hereditary angioedema due to C1 inhibitor deficiency (C1-INH-HAE) in the Republic of Macedonia.
- Causative mutations in *SERPING1* were detected in all 15 C1-INH-HAE patients from six Macedonian families, including an infant, before the appearance of clinical symptoms.
- We identified three known mutations and two novel mutations (c.813_818delCAACAA and c.1488T>G). These findings further corroborated the genotype-phenotype relationship, wherein mutations with a clear effect on C1-INH function predispose patients to a more severe disease phenotype and the CC F12-46C/T polymorphism predisposes patients to earlier disease onset.

Introduction

Hereditary angioedema due to C1 inhibitor deficiency (C1-INH-HAE) is characterized by swelling of the face, lips, tongue, larynx, abdomen, genitalia or extremities(1–4). C1-INH-HAE (OMIM 106100) is a rare autosomal dominant disease with few cases of homozygous mutations reported in consanguineous patients. The condition is caused by mutations in the *SERPING1* gene, which codes for C1-INH(1–4). *SERPING1* is located on chromosome 11q12-q13.1 and involves eight exons and seven introns distributed over 17 kb, with introns containing 17 repetitive *Alu* sequences(1,5,6). More than 450 different mutations in *SERPING1* have been described to date, ranging from nucleotide substitutions and small insertions and deletions to large deletions and duplications (HAEdb, http://www.hae.enzim.hu)(7), resulting in low levels of C1-INH (C1-INH-HAE type I, 85% of cases) or normal levels of non-functional C1-INH (C1-INH-HAE type II, 15% of cases)(1,2,4,6). C1-INH is a serine protease inhibitor and represents a key regulator of several immune and inflammatory pathways, given that it inhibits classical and lectin complement pathways, the mannose-binding protein-associated serine protease system, kallikrein, coagulation factors XIa and XIIa, plasmin, and tissue plasminogen activator. Therefore, deficient C1-INH causes overactivation of cascades, generating several vasoactive substances. Bradykinin represents the most important vasoactive substance generated, resulting in increased vascular permeability and leading to angioedema attacks(1,2). In addition to C1-INH-HAE, other types of HAE include F12-HAE caused by specific mutations in the factor XII (*F12*) gene(8) and recently identified ANGPT1-HAE and PLG-HAE, which are caused by mutations in angiopoietin-1 (*ANGPT1*) gene(9) and plasminogen (*PLG*)(10), respectively. In several families with HAE, the genetic background and pathophysiology of disease remain unknown (U-HAE)(4,8–11).

The aim of the present study was to characterize the spectrum of mutations of the *SERPING1* gene in 15 patients from six unrelated Macedonian families with C1-INH-HAE. This is the first genetic study on C1-INH-HAE in the Republic of Macedonia. The awareness and recognition of this potentially life-threatening disease still needs to be improved in Macedonia as well as in other countries. In addition, we wanted to further investigate the possible relationship between the type of mutation in the *SERPING1* gene and the clinical presentation of C1-INH-HAE. To evaluate the association between genotype and phenotype in C1-INH-HAE, patients from Macedonia were pooled with 65 C1-INH-HAE patients from 35 unrelated south-eastern European (SEE) families, specifically from Slovenia(12), Serbia(13) and the Šibenik-Knin region of Croatia(14), who were previously genotyped for the *SERPING1* gene mutations. In addition to *SERPING1* gene mutations, recently published research indicates that functional alterations in genes encoding for proteins involved in bradykinin metabolism and function could affect the clinical phenotype of C1-INH-HAE(6,15–17). Specifically, the functional promoter polymorphism F12-46C/T that results in reduced translation efficiency and low plasma levels of FXII activity(15) and the G allele of the KLKB1-428G/A variant that is associated with reduced plasma kallikrein activity(16) might affect the disease phenotype of C1-INH-HAE patients. Therefore, we also examined the association of F12-46C/T (rs1801020) and KLKB1-428G/A (rs3733402) polymorphisms with clinical presentation of the disease. These polymorphisms were previously shown to be associated with age at disease onset(15–17).

Materials and methods

Patients

The patients were recruited at the Dermatology Clinic, School of Medicine, Ss. Cyril and Methodius University, Skopje, Republic of Macedonia, which is a tertiary medical centre responsible for the diagnosis and treatment of hereditary angioedema in the Republic of Macedonia. For the genetic analysis, which was performed at the University Clinic of Respiratory and Allergic Diseases Golnik, Slovenia, 14 patients, including one asymptomatic adult, from 6 unrelated families were recruited. Furthermore, two babies younger than 1 year with a family history of C1-INH-HAE but without any symptoms were also recruited for complement and genetic testing. The diagnosis of HAE was established in the presence of at least one major clinical criterion (subcutaneous angioedema, abdominal pain, or laryngeal oedema) and one laboratory criterion (C1 inhibitor antigenic levels, C1 inhibitor function) followed up with positive family history, as proposed in the guidelines for the diagnosis of HAE(1,2). C1-INH-HAE severity was calculated according to a scoring system proposed by Bygum et al.(18) This clinical severity score is based on age of disease onset, organs affected, and the need for long-term prophylaxis. The maximum score is 10. The study was conducted in accordance with the amended Declaration of Helsinki, and all participants or the relatives of minor participants provided written informed consent.

Complement testing

Serum protein concentrations of C1 inhibitor (normal range: 0.20–0.35 g/l) and C4 (normal range: 0.16–0.31 g/l) (Siemens, Marburg, Germany) were quantified by means of radial immunodiffusion and C1 inhibitor function (C1 inhibitor functional levels ≤ 40% of

normal are considered decreased) were measured using an enzyme immunoassay (Quidel Corporation, California, USA) in accordance with the manufacturer's instructions.

Genotyping

Genomic DNA was extracted from EDTA-containing whole blood samples using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

The detection of *SERPING1* mutations in the promoter, noncoding exon 1, 7 coding exons and exon-intron boundaries were performed as described previously (12). To identify mutations, all sequences were compared with the *SERPING1* reference sequence in GenBank (GenBank accession number X54486.1). *SERPING1* variations were numbered in two ways. Traditional genomic numbering denotes the first nucleotide of exon 1 as number one [5], whereas the systematic cDNA numbering recommended by the Human Genome Variation Society (HGVS) considers the first nucleotide (A) of the initiation methionine (ATG) of the cDNA sequence (GenBank accession number NM_000062.2) as nucleotide number one. Regarding protein amino acid positions, the study used traditional numbering based on the mature protein of 478 amino acids. The first 22 amino acids of the N-terminal residue of the signal peptide were denoted using negative numbers and systematic amino acid numbering, where the translation initiator methionine was considered number one. To identify large deletions/duplications, multiplex ligation-dependent probe amplification (MLPA) was performed using the SALSA MLPA P243-A2 SERPING1 kit (MRC-Holland, The Netherlands), and data were analysed using Coffalyser MLPA data analysis software (MCR-Holland).

The detection of F12-46C/T (rs1801020) and KLKB1-428G/A (rs3733402) polymorphisms was performed by Sanger sequencing, using primers F12-46C/T-F 5'-

GCTTTCCACAAACAGCCTGT-3', F12-46C/T-R 5'-CCACAGCACTCACCGAAAG-3', KLKB1-528G/A-F 5'-AACCCAAATGGTAGTGGGTA-3', and KLKB1-528G/A-R 5'-AGCGCGACTCCATCTCAATA-3' as described previously(12,16,17). F12-46C/T and KLKB1-428G/A polymorphisms were determined in this patient group and in previously published patients from south-eastern Europe, specifically from Slovenia(12), Serbia(13) and the Šibenik-Knin region of Croatia(14).

Statistical Analysis

Data distribution was evaluated using the D'Agostino–Pearson test. Parametric statistics (unpaired t-tests) were used on normally distributed data, whereas non-parametric statistics (the Mann–Whitney) were used if the distribution deviated from normal. To increase the power of the statistical genotype-phenotype association analysis, patients from Macedonia were pooled with 65 C1-INH-HAE patients from south-eastern Europe, specifically from Slovenia(12), Serbia(13) and the Šibenik-Knin region of Croatia(14). Two-sided Fisher's exact test was used to calculate the significance of the differences in patient's clinical characteristics between the two mutation groups in *SERPING1* as well as F12-46C/T and KLKB1-428G/A polymorphisms. Spearman's rank correlation test was used to analyse the correlation between the two mutation groups as well as F12-46C/T and KLKB1-428G/A polymorphisms and the patient's clinical characteristics. A generalized estimating equation (GEE) with a robust covariate matrix and exchangeable correlation structure was implemented to model the association of response variables (clinical severity score, age at onset of symptoms, oedema on different body parts and need for long-term prophylaxis) with explanatory variables (mutation group, polymorphisms, age, and gender) because our patient population consisted of related subjects. Clinical severity score and age at disease onset were modelled as continuous variables in linear GEE models, whereas oedema on different body

parts and the need for long-term prophylaxis were entered as binary variables in the logistic GEE model. GraphPad Prism 5.0 software (San Diego, CA, USA) and SPSS 21.0 software (Chicago, IL, USA) were used for statistical analysis. A P-value of less than 0.05 was considered statistically significant.

Results

Clinical details

Clinical and laboratory data are presented in Table 1. All recruited patients were diagnosed with C1-INH-HAE type I with reduced C1-INH serum concentrations and function.

The mean age at onset of clinical symptoms was 11 years (range 1–20 years), and the average delay from the first symptoms until diagnosis was 7 years (range 1–33 years). The delay until diagnosis was especially long in three patients, at 20, 26 and 33 years. Patients reported on average 31 angioedema attacks annually, and the number of attacks ranged from 4 to 66 episodes per year. One adult patient was asymptomatic (Table 1). During the previous two years, angioedema attacks most often occurred on the abdomen (39% attacks), followed by skin (36%) and facial oedema (21%). In contrast, laryngeal oedema events were rather rare (4% of attacks). All but one of the symptomatic patients experienced more than one angioedema attack per month, and three patients experienced at least one attack per week (Table 2). The median clinical severity was 7.0 (out of 10.0; range 7–9). We also tested two babies younger than 1 year both belonging to family 1 without any symptoms. One infant exhibited reduced C1 inhibitor serum concentration and function and harboured the family mutation in *SERPING1*, whereas normal complement and genetic testing results were noted in the other infant.

Genetic analysis

In all patients with C1-INH-HAE from six families, a mutation in *SERPING1* responsible for the disease was identified, whereas no mutations were present in healthy relatives and controls. Two families carried known mutations (c.481A>T(19) and c.550G>A(20)), and two sporadic cases harboured the same know mutation (deletion of exon 4(21)). Two mutations were reported for the first time (Table 3). The novel mutations included a deletion of six nucleotides (c.813_818delCAACAA), located in exon 5, in which two asparagine residues were removed (Asn271_Asn272del) and a nonsense substitution (c.1488T>G), located in exon 8, causing a premature termination of the protein at amino acid 496 (Tyr496Stop). In unaffected relatives or controls, these mutations were absent, confirming that the identified mutations are indeed responsible for the disease.

Genotype-phenotype association

To address the question of a possible association between the type of mutation in the *SERPING1* gene and clinical presentation of the disease, patients were divided into two groups based on mutation type, as described previously(13). However, our C1-INH-HAE cohort was too small to permit any relevant genotype-phenotype association analysis. Therefore, to increase the power of the statistical analysis, patients from Macedonia were pooled with 65 C1-INH-HAE patients from Slovenia(12), Serbia(13) and the Šibenik-Knin region of Croatia(14). Using a generalized estimating equation as described previously(13) and including 78 C1-INH-HAE patients, we found that patients with nonsense and frameshift mutations, large deletions/insertions, splicing defects, and mutations at Arg444 (group 1) exhibited an increased clinical severity score compared with those with missense mutations, excluding mutations at Arg444 (group 2; mean: 6.9 vs. 5.7; Mann-Whitney: P = 0.009;

Spearman: P = 0.008, $r_s = -0.299$; GEE: P = 0.03, β coefficient = 1.32, unadjusted; P = 0.02, β coefficient = 1.22, adjusted for gender and age).

Next, we addressed the possible association of F12-46C/T (rs1801020) and KLKB1-428G/A (rs3733402) polymorphisms and clinical presentation of the disease in 78 C1-INH-HAE patients. The F12-46C/T (rs1801020) polymorphism was suggestively associated with the age of disease onset but not with the clinical severity score. In patients with the CC F12-46C/T genotype, symptoms appeared 5 years earlier than in patients with CT and TT genotypes (median: 10 vs. 15 years; Mann-Whitney: P = 0.035; Spearman: P = 0.033, rs = 0.241; GEE: P = 0.05, β coefficient = -3.91, unadjusted; P = 0.14, β coefficient = -3.26, adjusted for gender and age). Although significance was lost after correction for gender and age, an underlying association between the F12-46C/T genotype and disease onset was highly suggestive. On the other hand, no association was noted between KLKB1-428G/A (rs3733402) polymorphisms and age of disease onset, clinical severity score or any other clinical presentation.

Discussion

In the first genetic nationwide characterization of the Macedonian C1-INH-HAE population, we identified mutations in the *SERPING1* gene responsible for the disease in all 15 patients from six unrelated families. Interestingly, all patients have C1-INH-HAE type I. The minimum C1-INH-HAE prevalence in the Republic of Macedonia is 1:140,000 inhabitants. This rate is lower than that reported in Denmark(18,22), Italy(23), Hungary(24), Norway(25), Sweden(26), and Switzerland(27), where the calculated prevalence is 1:65,000 to 1:70,000 inhabitants. The rate is also slightly lower than reported in Greece(24), Serbia(13), Slovenia(12), and Spain(21,28), where the prevalence is reported to be approximately 1:90,000 to 1:100,000 inhabitants. These data suggest that this rare disease still remains underdiagnosed, given that six to up to twenty-seven additional C1-INH-HAE patients living in Republic of Macedonia expected to be diagnosed with HAE based on the prevalence rate of 1:50,000 to 1:100,000(12,13,18,21–28). Another argument that further supports the indication that HAE is underdiagnosed in the Republic of Macedonia is that we did not identify any patient with C1-INH-HAE type II, where according to previous reports, C1-INH-HAE type II accounts for 15% of C1-INH-HAE cases(1,2,29).

In our cohort of Macedonian patients, the mean delay from symptom onset to establishment of correct diagnosis of 7 years is comparable to that reported in other European countries; however, substantial differences are noted in the observed delay among different countries. Speletas et al.(24) studied 265 patients from Greece, Romania, Germany and Hungary and found that the mean delay was 12 years. Among these countries, the shortest mean delay was noted in Germany (8.7 years), and the longest was in Greece (17 years). Similarly, Zanichelli et al.(30) found a median delay of 9 years in 150 patients from six countries, with the shortest again being in Germany (2 years) and the longest in Italy (15 years). The mean delay in diagnosis was 12 years in Sweden(26), 16 years in Denmark(18)

and 11 years in Serbia(13). Although the mean delay in the Republic of Macedonia is among the shortest in Europe, it is still inadequate given that three patients required more than 20 years to obtain a correct diagnosis of C1-INH-HAE. In addition, based on the low calculated prevalence, several patients are still awaiting the correct diagnosis. Therefore, the awareness and recognition of this rare disease in the Republic of Macedonia still needs to be increased, including continuous education of primary care physicians and raising awareness of HAE among both medical professionals and the general population.

In C1-INH-HAE patients, we have identified five different mutations, namely, 1 missense mutation (c.550G>A), 2 nonsense mutations (c.481A>T and c.1488T>G), 1 small deletion (c.813_818delCAACAA) and one large deletion (deletion of exon 4). The mutations were distributed across several exons, specifically exons 3, 4, 5 and 8 (Table 3). Two mutations (c.813_818delCAACAA and c.1488T>G) were reported for the first time. The in-frame deletion c.813_818delCAACAA is located in exon 5 and is probably caused by the presence of three direct repeats of the triplet CAA, making this region susceptible to mutation by a slipped mispairing mechanism(31). The deletion of the two triplets was described here for the first time; however, the deletion of a single triplet CAA in this region was previously described as disease causative(21,31). The c.813_818delCAACAA mutation was identified in two patients from the same family and was absent in a healthy relative and controls, which further confirms that the identified mutation is indeed responsible for C1-INH-HAE. The newly identified nonsense mutation (c.1488T>G) located in exon 8 causes a premature termination of the protein at the 496th amino acid, resulting in the synthesis of a protein that is five amino acids shorter than the normal protein. As a result, this truncated protein is probably rapidly intracellularly degraded in the endoplasmic reticulum as previously described for the mutation in the C-terminal domain of *SERPING1* (i.e., Arg494Stop)(32).

This mutation was present in two patients from the same family and absent from controls, which further supports the causative nature of this mutation.

High variability in the clinical expression of C1-INH-HAE remains an unsolved issue despite great efforts to unveil the genotype-phenotype association. Our results indicate that different types of mutations might be responsible for the high variability of C1-INH-HAE clinical expression, where mutations with a clear effect on C1-INH function predispose individuals to a more severe disease phenotype given that patients with nonsense and frameshift mutations, large deletions/insertions, splicing defects, and mutations at Arg444 (group 1) exhibited an increased clinical severity score compared with patients with missense mutations, excluding mutations at Arg444 (group 2). Additionally, the rationale of our modified categorization of mutations into two groups(13) was further confirmed in additional populations and with a larger sample size. In this analysis, 78 C1-INH-HAE patients were pooled from south-eastern Europe, specifically Macedonia, Slovenia(12), Serbia(13) and the Šibenik-Knin region of Croatia(14). Our results are consistent with some reports that have suggested an association of disease severity and disease onset with mutation type(13,17,24), whereas several other studies have failed to demonstrate the genotype-phenotype association in C1-INH-HAE(21,22,33–35). One reason for this discrepancy is that different reports used different categorizations for mutation grouping; therefore, we propose that our modified categorization into two groups, as discussed in greater detail elsewhere(13), should be more frequently adopted in genotype-phenotype association analyses. We also provide additional confirmation of previous findings suggesting that patients with the CC F12-46C/T (rs1801020) genotype have earlier disease onset than other C1-INH-HAE patients(15–17). Although, we did not identify any association between the KLKB1-428G/A (rs3733402) polymorphism and disease onset or any other clinical presentation of C1-INH-HAE, this polymorphism needs to be further investigated to confirm its role in C1-INH-HAE. Reporting

of *SERPING1* mutation categorization and the F12-46C/T polymorphism upon detection could contribute to improvements in the early management of this potentially life-threatening oedema as some precautionary measures could be taken prior to the appearance of clinical symptoms. However, we must be aware that other disease-modifying factors may play important roles in determining the clinical variability of C1-INH-HAE(3,6).

In conclusion, established genetic analysis helped identify the molecular basis of C1-INH-HAE given that causative mutations in *SERPING1* were detected in all patients from six Macedonian families. Molecular analysis allowed the detection of a causative mutation in an infant before the appearance of clinical symptoms, which is essential in the prevention and adequate treatment of life-threatening oedema. Although more than 450 different mutations in *SERPING1* have been described to date(7), our study identified two novel mutations, highlighting the heterogeneity of mutations causing C1-INH-HAE. Furthermore, our results corroborated the genotype-phenotype relationship, where mutations with a clear effect on C1-INH function predispose individuals to a more severe disease phenotype and the CC F12-46C/T polymorphism predisposes individuals to earlier disease onset.

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Disclosure statement

The authors report no conflicts of interest.

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Table 1. Clinical features of Macedonian patients with C1-INH-HAE.

Age at onset of symptoms (years)	Age at diagnosis (years)	C1-INH-HAE type	Clinical severity score	Skin oedema	Facial oedema	Abdominal oedema	Laryngeal oedema	Number of attacks		Family history	C1-INH, g/l	C1-INH function, %	C4, g/l
								in 2015	in 2016				
18	51	Type I	7	+	+	+	+	46	39	+	<0.05	25	<0.05
12	13	Type I	7	+	+	+	+	20	21	+	<0.05	<24	<0.05
ND	ND	Asympt	0	ND	ND	ND	ND	ND	ND	+	0.12	61	0.06
4	4	Type I	9	+	+	+	+	31	28	+	0.05	12	0.05
4	4	Type I	9	+	+	+	+	42	60	+	<0.05	14	<0.05
1	1	Type I	9	+	+	+	+	56	59	+	0.11	60	<0.05
ND	ND	Asympt	0	ND	ND	ND	ND	ND	ND	+	0.12	16	0.08
3	3	Type I	9	+	+	+	+	18	24	NA	<0.05	24	<0.05
20	20	Type I	7	+	+	+	+	34	34	-	0.10	17	0.07
20	40	Type I	7	+	+	+	+	57	66	-	0.10	45	0.09
14	17	Type I	7	+	+	+	+	16	23	+	0.12	48	0.11
19	19	Type I	7	+	+	+	+	27	19	+	<0.05	27	<0.05
6	6	Type I	8	+	+	+	+	14	21	+	<0.05	<24	<0.05
12	21	Type I	7	+	+	+	+	19	14	+	<0.05	24	<0.05
15	41	Type I	7	+	+	+	+	4	11	-	0.06	9	<0.05

Asympt: Asymptomatic; ND: Not determined; NA: Not Available

Table 2. Frequency of angioedema attacks in 2015 and 2016.

	2015		2016	
	No. of attacks	No. of patients	No. of attacks	No. of patients
Patients				
Skin oedema	134	13	155	13
Facial oedema	86	13	86	13
Abdominal oedema	148	13	164	13
Laryngeal oedema	16	8	14	8
≥1/week		2		3
≤1/week to ≥1/month		10		9
≤1/month to ≥1/year		1		1

Table 3. Mutations identified in Macedonian patients with C1-INH-HAE.

Family	No. of patients	Traditional genomic numbering	Systematic (HGVS) genomic numbering	cDNA numbering	Exon	Predicted effect on protein (traditional numbering)	Predicted effect on protein (systematic numbering)	Reference
1	7	2625A>T	g.3807A>T	c.481A>T	3	Lys139Stop	Lys161Ter	(19)
2	1			Deletion of exon 4				(21)
3	1			Deletion of exon 4				(21)
4	**2**	**8452_8457del CAACAA**	**g.9634_9639del CAACAA**	**c.813_818del CAACAA**	**5**	**Asn249_Asn250del**	**Asn271_Asn272del**	**this study**
5	**2**	**16880T>C**	**g.18062T>G**	**c.1488T>G**	**8**	**Tyr474Stop**	**Tyr496Ter**	**this study**
6	2	2694G>A	g.3876G>A	c.550G>A	3	Gly164Arg	Gly184Arg	(20)

New mutations are indicated in boldface type.