

Mutational spectrum and geno-phenotype correlation in Chinese families with Hereditary Angioedema

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Abstract

Background: Hereditary angioedema is a rare autosomal dominant disease, and its correlation between genotype and phenotype seems not to exist. So far, there are very few studies on Chinese population. We aimed to establish a Chinese genetic database of hereditary angioedema and investigated the potential correlation between genotype and phenotype.

Method: All the eight exons and intron–exon boundaries of C1 inhibitor gene were detected in 48 unrelated families with HAE. The correlations between genotype and clinical parameters were evaluated by R statistical software.

Results: Thirty-five different mutations (25 of them were novel) and 7 SNPs (3 of them were novel) were identified. Significant difference was found in the level of C1 inhibitor antigen ($P = 0.01793$) between different groups of mutational types. The correlation between different groups of mutational types and the level of C1 inhibitor antigen (0.5047, $P = 0.00027$) was significant. The different groups of mutational types showed neither difference nor correlations of clinical parameters (severity score and the level of C1 inhibitor function).

Conclusion: It appears that nonsense, frameshift, and mutations on Arg466 can cause lower level of C1 inhibitor antigen than missense and in-frame mutations; however, it does not affect severity of symptoms.

Hereditary angioedema (HAE), a relatively rare autosomal dominant disease with a prevalence of 1 : 10000–50000(1,2), is characterized by recurrent attacks of swelling involving subcutaneous or submucous tissue of the face, extremities, genitalia, the upper airway, and gastrointestinal tract(3). It is caused by a deficiency of C1 inhibitor (C1-INH). C1-INH is the only known inhibitor for activated protease C1r and C1s and through this inhibition prevents the activation of the classical complement pathway (4). It also plays a key role in the inhibition of the contact system. C1-INH can inhibit all the abilities of kallikrein and FXII_a and prevent the activation of prekallikrein. All these three, prekallikrein, kallikrein,

and FXII_a, are the main protein in the bradykinin-forming cascade. Thus, in the deficiency of C1-INH, the bradykinin-forming cascade simplifies inappropriately and generates over the level of bradykinin that can lead to the angioedema by increasing the vascular permeability (5–8). Besides these, C1-INH is also the main inhibitor for the lectin pathway of the complement system and fibrinolytic system (4–8). C1-INH consists of nine α -helixs, three β -sheets, and one reactive central loop (RCL) (9). RCL is the crucial structure for C1-INH function to interact with the target protease (4). The deficiency of C1-INH leads to an increase in the activity of target serpin proteases, which presents as a decrease in C4 concentration, increasing activation of kallikrein and production of bradykinin. The over production of bradykinin results in the angioedema typical of HAE.

To date, more than 238 different mutations have been reported by C1 inhibitor gene mutation database (<http://hae.enzim.hu/>); however, only a few of them are from a Chinese

Abbreviation

C1-INH, C1 inhibitor; sC1-INH, serum C1 inhibitor; HAE, Hereditary angioedema; RCL, reactive central loop; SNP, single nucleotide polymorphism.

population (10). For this reason, we established a Chinese genetic database and investigated the potential correlation between different groups of mutational types and clinical presentation of HAE. Our hypothesis is that compared with missense and in-frame mutations, nonsense, frameshift mutations and missense mutations on Arg466 might result in lower levels of sC1-INH antigen, and this, in turn, might affect the clinical severity of HAE.

Method

Subject

Forty-eight patients from 48 unrelated Chinese families with HAE were recruited from the Department of Allergy, Peking Union Medical College Hospital. The diagnosis of HAE was based on the expected medical history and laboratory tests consistent with HAE. Patients required low levels of C4, and either low levels of C1-INH antigen and function (type I) or normal or high levels of C1-INH antigen with low function (type II). Fifty-three unrelated normal subjects were utilized as controls. The study was approved by the ethics committee of Peking Union Medical College Hospital, and all participants gave written informed consent. Two milliliter EDTA anticoagulated peripheral blood was collected for genetic analysis.

Measurement of C4 and C1-INH antigen and function

Single immunodiffusion assay was used to test the levels of C4 and sC1-INH antigen (Shensuo, China) before 2001. Since 2001, immunonephelometry was used to qualify C4 concentration by C4 (Complement 4) Reagent, 2×100 Test Cartridge (Beckman Coulter, CA, USA). The level of sC1-INH antigen was measured by BNTM II System (Dade Behring Marburg GmbH, Marburg, Germany). The consistency of these two methods was evaluated when single immunodiffusion was replaced by immunonephelometry (11). Good correlations in terms of sC1-INH antigen and C4 were found (11). Given this, we used a percentage of normal mean to express the levels of sC1-INH antigen and C4 to perform the statistical analysis. The level of C1-INH function was tested by Chromogenic Kit (Immuno Chrom, Vienna, Austria).

Genetic analysis

The extraction of DNA from peripheral leukocytes was performed by the salting-out method. The concentration was measured by spectrophotometer at 260 nm and 280 nm. All the eight exons and intron-exon boundaries were amplified by PCR on a T-professional Standar 96 thermocycler (Biometra GmbH, Göttingen, Germany). Oligonucleotide primers were designed using Primer 5.0. The primers' sequences are provided in supplement (Table S1). The amplicons were purified from 1.5% agarose gel by QIAquick Gel Extraction Kit (Qiagen, CA, USA) and sequenced by BigDye Terminator DNA Sequencing Kit (Applied Biosystems, CA, USA) on an ABI-PRISM 377-96 sequencer (Applied Biosystems).

Nucleotide sequence X54486 from GenBank (BA123456) and amino acid sequence P05155 from UniProt (Q9H0H5)

were used to perform the sequence alignment. Compared with the reference sequence, mutations were identified by NCBI BLAST. Nucleotides were numbered in two types: (i) the traditional system using the first nucleotide of exon 1 as position 1 and (ii) the recommended CDS numbering system by the Human Genome Variation Society (HGVS), which considers the A of the ATG translation initiation codon as CDS + 1(X54486). On the basis of the recommendation by HGVS, position 1 of the amino acid sequence was the first-translated residue (Met) of the premature protein including the signal peptide sequence.

Missense mutation prediction

The amino acid sequences of C1-INH in other species were aligned by ClustalW software (<http://www.ebi.ac.uk/Tool/clustalw2/index.html>). The effects of missense mutations with single amino acid change on protein were predicted by three protein prediction web servers: PolyPhen-2 (<http://genetics.bwh.harvard.edu/pph2/>), SIFT (<http://sift.bii.a-star.edu.sg/>), and PSIPRED (<http://bioinf.cs.ucl.ac.uk/psipred/>).

Clinical severity evaluation of HAE

For the reason that a validated severity scoring system was not available at the time of this study, we established a severity scoring system to evaluate the severity of HAE (shown in Table 1). All clinical data were obtained from medical records and patients' questionnaire.

Patients grouping

Patients were divided into two groups: Group A included patients with disease causative mutations (nonsense, frameshift, and mutations on Arg466) and group B included those with potential deleterious mutations (in-frame and missense not including those on Arg466). Patients with splicing defects were not enrolled for it was not sure whether these defects would impact the amino acid sequence of C1-INH.

Statistical analysis

Two-sample *t*-test was used to evaluate the differences for the levels of C1-INH antigen and function, and clinical severity score between groups A and B. Pearson's correlation test was used to analyze the potential correlation between different groups of mutational types and clinical parameters. All the values were expressed as means with a 95% confidence interval. Statistical analysis was performed by the statistical software package R, version 2.14.0 (<http://cran.r-project.org/>).

Results

Nonsense and frameshift mutations

Six nonsense and 10 novel frameshift mutations were identified (see Table 2). Two of the nonsense mutations (c.322C>T, c.1042C>T) were previously reported (12). c.1111delG was

Table 1 Patients' data

	No. of family	C1-INHf* (U/ml)	sC1-INHa* (%)	C4 (%)	Severity score
Group A	2		0	0.0954	3
	3	0.3	0.33	0.4042	4
	5		0.3	0.4583	4
	7		0.1	0.1715	5
	9		0.1667	0.125	3
	14	0.16	0.1533	0	2
	17	0.18	0.1667	0.24	4
	22		0.1667	0.125	4
	23		0.0312	0.0618	2
	25	0.04	0.16	0	2
	27		0.1545	0.1731	5
	32	0.37	0	0.4575	4
	34		0.1333	0.1667	5
	35	0.07	0	0	3
	37		0.1667	0.2083	2
	39	0.11	0.1633	0.225	5
	42	0	0.1974	0	1
	43	0.32	0.2187	0.0409	4
	44	0	0.2197	0.2396	2
	47	0.16	0.1545	0	2
Group B	48	0	0.2034	0.1236	4
	4		0.3667	0.5417	3
	6		0.2333	0.25	2
	8		0.1333	0.375	2
	16	0.14	0.2533	0.4167	3
	18	0.1	0.2333	0.1177	3
	19	0.2	0.2667	0.56	6
	21	0.14	0.2	0.2916	4
	30	0.46	0.3	0.3479	3
	31		0.7725	0.7792	5
	33		0.2667	0.4583	5
	38		0.1	0.125	3
	41	0.2	0.6266	0.5564	3

*sC1-INHa – serum level of sC1-INH antigen; C1-INHf – the level of C1-INH function. Clinical severity score (0–7 points) system was performed as follow: frequency of attacks > 1/month, 1': skin edema of any area ever, 1': single submucous site involvement, 1': multiple submucous sites involvement, 2'; emergency visit ever, 1'; improper abdominal operation ever, 1'; tracheotomy, 1'.

present in four unrelated families. All other mutations were distinct for 15 families.

Splicing defect mutation

Two novel splicing defect mutations were found separately in two families (see Table 2). The mutation of g.4485 + 1G>A affected the position + 1 of the consensus donor site GT of intron 4, and g.14031-1delG involved the position -1 of the consensus accepted site AG of intron 6.

Missense mutation

Fifteen missense mutations were identified in 19 unrelated families, 9 of which were novel (see Table 2). Mutations c.550G>A, c.1396C>A, c.1396C>T, c.1397G>T, and c.1418T>G have been reported previously (13–17). c.352A>G occurred in two families, c.1340T>C in three families, and c.1396C>A in two families. The remaining 12 missense

mutations were distinct for 12 families. Except for mutations involving the reactive bond (c.1396C>A, c.1396C>T, and c.1397G>T), the other twelve variants were evaluated for evolutionary conservation by ClustalW and predicted potential impact on protein structure and function using the PolyPhen-2, SIFT, and PSIPRED. It showed that all of them occurred in well-conserved amino acid residue. With the exception of p.T118A, all the other concerned variants were predicted as deleterious mutations. The conservation alignment and prediction results were summarized in supplements (Table S2). All these variants were absent in controls. Based on these, we classified them as missense mutations.

In-frame mutation

In two patients, we detected c.806_808insCAC (p.Thr271ins) and 813_815delCAA (p.Asn272del) respectively (see Table 2). The latter one has been previously reported (13).

Table 2 Mutations in C1-INH in HAE patients

Nucleotide change		Molecular consequence	Position	No. of family	Reference
Traditional	CDS				
g.2287_2288 delCA	c.143_144delCA	p.T48fs55×	exon 3	23	
g.2350 delA	c.206delA	p.N69fs77×	exon 3	2	
g.2433 C>T	c.289C>T	p.Q97×	exon 3	34	
g.2440_2447 delCCATCCAA	c.296_303del CCATCCAA	p.T99fs129×	exon 3	35	
g.2466 C>T	c.322C>T	p.Q108×	exon 3	12	(11)
g.2496 A>G	c.352A>G	p.T118A	exon 3	36, 37	
g.2605 A>G	c.461A>G	p.Y154C	exon 3	20	
g.2653 C>T	c.509C>T	p.S170F	exon 3	30	
g.2694 G>A	c.550G>A	p.G184R	exon 3	19	(13)
g.4429_4445delTGAAGGGCTTCACGACC	c.629_645 delTGAAGGGCTTCACGACC	p.T210fs249×	exon 4	3	
g.4485 + 1 G>A		Splicing defect	intron 4	29	
g.8328 T>C	c.689T>C	p.L230P	exon 5	16	
g.8334 T>A	c.695T>A	p.I232K	exon 5	4	
g.8366_8367 delCT	c.727_728delCT	p.T243fs254×	exon 5	44	
g.8445_8447 insCAC	c.806_808insCAC	p.insT271	exon 5	45	
g.8453_8455 delCAA	c.813_815delCAA	p.delN272	exon 5	41	(13)
g.8521 C>A	c.882C>A	p.Y294×	exon 5	15	
g.8728 T>C	c.895T>C	p.W299R	exon 6	33	
g.8729 G>A	c.896G>A	p.W299×	exon 6	7	
g.14031-1 delG		Splicing defect	intron 6	46	
g.14033 C>T	c.1042C>T	p.Q348×	exon 7	9	(11)
g.14112delG	c.1111delG	p.E371fs397×	exon 7	14, 17, 27, 39	
g.14200_14201 insCC	c.1199_1200insCC	p.T404fs431×	exon 7	37	
g.16671delC	c.1279delC	p.I426fs428×	exon 8	47	
g.16681T>A	c.1289T>A	p.L430Q	exon 8	6	
g.16714T>C	c.1326T>C	p.M441T	exon 8	18	
g.16732 T>C	c.1340T>C	p.L447P	exon 8	8, 18, 21	
g.16734G>T	c.1342G>T	p.E448×	exon 8	25	
g.16757delC	c.1365delC	p.S422fs433×		43	
g.16788 C>A	c.1396C>A	p.R466S	exon 8	42, 48	(14)
g.16788 C>T	c.1396C>T	p.R466C	exon 8	22	(12)
g.16789G>T	c.1397G>T	p.R466L	exon 8	5	(15)
g.16810T>G	c.1418T>G	p.V473G	exon 8	31	(16)
g.16871delG	c.1479delG	p.G493fs500×	exon 8	32	
g.16882A>G	c.1490A>G	p.D497G	exon 8	38	

Polymorphism

Seven SNPs were tested in this study (shown in Table 3). Four of them (3248T>C, 3493T>C, 15193A>G, 18012G>A) have been indexed in SNP database – HapMap (<http://hap-map.ncbi.nlm.nih.gov/>) – which are related to Han Chinese, while the other three (g.738 G>A, g.4573 G>A, and g.8316 T>C) were submitted by us and have been incorporated in dbSNP recently.

Correlations between genotype and clinical data

As noted in the method, to quantify clinical manifestation severity, we established a severity scoring system. Clinical severity score was performed on patients enrolled in groups A and B. The clinical data before treatment were from

medical records, some of which were many years old. For this reason, complete data sets were not possible, and 5 of 38 medical records utilized in groups A and B had missed clinical information. In addition, only 18 records had the laboratory report of C1-INH function available. The original laboratory results were shown in Table 1.

Differences of severity score and the levels of C1-INH antigen and function before treatment were evaluated by statistical analysis (two-sample *t*-test) (results in Table 4).

The level of sC1-INH antigen ($P = 0.01793$, <0.05) was significantly different between groups A and B. The mean of level of sC1-INH antigen (15.17%) in group A was much lower than in group B (31.27%). There was a significant positive correlation between the level of sC1-INH antigen and genotype (0.5047, $P = 0.00027$). Despite these differences, other clinical parameters were not different between

Table 3 SNP and polymorphism in patients and control subjects

Nucleotide change					Allele frequency		
Traditional	CDS	Mol cons	Position	Number of family	Major allele	Minor allele	SNP
g.738 G>A			intron 2	5 [*] (1P [†] ,4C [‡])	G(197 [§])97.52%	A(5 [*]) 2.48%	rs199473715 [¶]
g.2066T>C			intron 2	26(15P,11C)	T(173)85.64%	C(29) 14.36%	rs1005510
g.2311 T>C	c.167T>C	p.V56A	exon 3	12(4P,8C)	T(190)94.06%	C(12) 5.94%	rs11546660
g.4573 G>A			intron 4	16(6P,10C)	G(183)90.59%	A(19) 9.41%	rs11229063 [¶]
g.8316 T>C			intron 4	7(1P,6C)	T(195)96.53%	C(7) 3.47%	rs141593943 [¶]
g.14011 A>G			intron 6	41(18P,23C)	A(153)75.74%	G(49)24.26%	rs2511988
g.16830 G>A	c.1438GA	p.V480M	exon 8	23(6P,17C)	G(175)86.63%	A(27) 13.37%	rs4926

P, patient; C, control subject.

*Total number of the subjects with minor allele.

†Number of patients with minor allele.

‡Control subjects' number.

§Total number for major allele.

¶These three were submitted by us.

Table 4 Results of statistical analysis

	Mean of Group A	Mean of Group B	P-value [*]	P-value ^{**}	Cor
C1-INHf	0.14%	0.21%	0.3474	0.3399	0.2387
C1-INHa	0.15%	0.31%	0.0179	0.0027	0.5047
Severity score	3.3333	3.5	0.714	0.7192	0.0665

*P-value, preformed by *t*-test to evaluate the difference of each parameter between groups A and B.

**P-value, preformed by Pearson's correlation test. cor, the correlation coefficient.

the groups nor were there correlations found in severity score or the level of C1-INH function.

Discussion

Among the cohort of 48 unrelated Chinese families, we identified 35 different mutations comprising 6 nonsense, 10 frameshift, 2 splicing defect, 15 missense, and 2 in-frame mutations. Twenty-five of these mutations were novel. Additionally, 7 SNPs were described, 3 of which were first described by us and have been incorporated into dbSNP (shown in Table 3). The frequencies of each type mutation were listed in order: missense and in-frame (51.21%), frameshift (31.7%), nonsense (14.63%), and splicing defect (4.87%). Exon 8 (36.58%) was the most frequent site of mutations, followed by exon 3 (24.39%), exon 5 and 7 (14.63%, 14.63%), exon 6 (4.87%), exon 4 (2.43%), and intron 4 and 6 (2.43%, 2.43%). We failed to find causative mutations in the sequencing region of C1-INH in five families.

Nonsense mutation leading to the termination of translated mRNAs with premature stop codons is usually eliminated by nonsense-mediated mRNA decay (18). Even if proteins are translated, they will be truncated. In this study, all of premature stop codons were located in the upstream of RCL coding sequence, of which protein products would not cover RCL. Except for c.1479delG, other frameshift mutations shared the same mechanism as nonsense by creating

premature stop codons in the upstream of RCL. Mutation c.1479delG led to an alteration of amino acid sequence in the C-terminal region, but the total number of amino acids was not changed. Being responsible for anchoring the RCL (4), C-terminal region is so conserved that alterations in certain residues may impair folding, transporting of the protein, and proper exposure of RCL (19). Mutations on Arg466, the reactive bond, have been demonstrated to be causative (13–17). These three types of mutations were generally considered as disease-causing mutations. All the missense mutations occurred at conserved sites and with the exception of p.T118A, all the others were predicted as deleterious mutations. However, it is hard to determine the impact of single amino acid substitution on the function of C1-INH. For this reason, we preferred to regard missense mutations as potential disease-causing mutation. Given this, we divided patients into two groups according to the mutation they carried. Group A included nonsense, frameshift, and mutations on Arg466, while group B included missense and in-frame mutations.

The statistical analysis revealed our patients in group A had lower level of sC1-INH antigen than those in group B. This is reported for the first time. While the differences of severity score, and the level of C1-INH function between these two groups were not found to be significant, this lack of correlation concurred with the majority of previous reports (20–23). However, there are several problems limiting us to draw a conclusion that truncated mutations and those

affecting Arg466 are leading to sC1-INH antigen levels lower than missense and in-frame mutations, for example, our sample size was small – only 38 patients could be analyzed.

C4, as a surrogate parameter, was not analyzed for the reason that the method was not able to distinguish between native C4 and its degradation products.

In summary, this is the first study on a large number of C1-INH gene mutations in Chinese population. Here, we identified 25 novel mutations and uniquely described a difference of the level of sC1-INH antigen between different groups of mutational types.

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Authors contributions

Ying-Yang Xu, MS, performed the experiment, analyzed and interpreted the data, and drafted the article. Yu-Xiang Zhi, MD, made substantial contributions to conception and

design, made major contributions to the acquisition of the cases and gave final approval of the version to be published. Jia Yin, MS, Liang-Lu Wang, MD, Li-Ping Wen, MD, Jian-Qing Gu, MD, and Kai Guan, MD made substantial contributions to the acquisition of the cases. Timothy Craig, DO, helped in writing manuscript and reviewed presentation of data. Hong-Yu Zhang, MD, made substantial contributions to acquisition of the cases.

Conflicts of interest

All the authors except Timothy Craig have no conflicts of interest or financial disclosure. Timothy Craig has carried out research for CSL Behring, Dyax, Pharming, Shire, and Viropharma. He speaks for CSL Behring, Viropharma, Shire, and Dyax. He consults for CSL Behring and Dyax.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Primers sequence.

Table S2. Prediction results.

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Table S2. Prediction results

Mut	Conservation	PolyPhen			SIFT		PSIPRED
		Result1	spec(%)	sens(%)	Result2	probability	
p.T118A	R	benign	0.49	0.96	tolerated	0.39	changed
p.Y154C	F	pro dam	1.00	0.00	not	0.00	changed
p.S170F	F	pro dam	1.00	0.08	not	0.00	changed
p.G184R	F	pro dam	1.00	0.00	not	0.00	changed
p.L230P	R	pro dam	1.00	0.08	not	0.00	changed
p.I232K	R	pro dam	0.91	0.65	not	0.00	changed
p.W299R	F	pro dam	1.00	0.00	not	0.00	changed
p.L430Q	R	pro dam	0.96	0.48	not	0.00	changed
p.M441T	R	pos dam	0.90	0.70	not	0.01	changed
p.L447P	F	pro dam	1.00	0.00	not	0.00	changed

p.V473G	R	pro dam	0.95	0.52	not	0.01	changed
p.D497G	R	pro dam	0.88	0.71	not	0.03	changed

Abbreviations: Mut–mutation, R–relatively conserved, F–fully conserved, pro dam–probably damaging, pos dam–possible damaging, spec–specificity, sens–sensitivity. This table showed the results of three prediction programs and the conservation of each amino acid concerned. Resule1 in PolyPhen is classified as benign, possibly damaging, and probably damaging. Result2 in SIFT consists of tolerated substitution and not tolerated substitution. Probability refers to the fraction of an amino acid occurring at a particular position. Probability <0.5 is considered as “not tolerated substitution”. The smaller the probability is, the more a substitution is likely a deleterious mutation. With the exception of p.T118A, all the missense mutations were consistently predicted as deleterious mutations by PolyPhen-2, SIFT and PSIPRED.

Table S1. Primer sequence

	CCCACCTACCAGGGGATTT
Exon 1	GGGGTAGGAGGAGGCAGAA
	TTGGGGAAAACAAAACAGAGG
Exon 2	GGAGGAGTAGGCTGAGAAAAGTG
	CCACCCTCACCTGTATTGC
Exon 3	GCAATCGTGCCTATTACATCACT
	CTGGTCCCCAACCCTCAT
Exon 4	GCCTGGCTTTCTGCTTCTT
	TCTGGGTTTACCTTCTTTGGG
Exon 5	GAAGCCAGGAGGGGAAGAA
	GTCCTTCTTCCCCTCCTGG
Exon 6	AGGAGAAAAGATAGGGTGGAAAT
	CTTAGGTCTGACTGATGCTTGTTG
Exon 7	TGGGTCGGTGTTCTGGTTT
	TCAGGACAAAGGTCTCCATCA
Exon 8	TGAGATGGGAGGATTGTTTGA
