ORIGINAL ARTICLE

Genetic analysis of a novel missense mutation (Gly542Ser) with factor XII deficiency in a Chinese patient of consanguineous marriage

Anqing Zou[1] · Mingshan Wang[1] · Yanhui Jin[1] · Xiaoli Cheng[1] · Kankan Su[1] · Lihong Yang[1]

Abstract
Coagulation factor XII deficiency is a rare autosomal recessive disorder, which could be found in a consanguineous family. We studied a Chinese family in which the activated partial thromboplastin time (APTT) of the proband had clearly prolonged up to 101.7 s, associated with low FXII activity of 3% and FXII antigen < 1%. To analyze the gene mutation in this FXII-deficient patient, we performed FXII mutation screening, and analyzed the DNA sequence of the F12 gene. A ClustalX-2.1-win and four online bioinformatics software services were used to study the conservatism and effects of the mutation. A transient in vitro expression study was performed to elucidate the possible pathological mechanism. Sequence analysis revealed a homozygous c.1681 G > A point mutation in exon 14, causing a novel Gly542Ser mutation in the catalytic domain. The results of the conservatism and bioinformatics analyses both indicated that the mutation likely affects the function of the protein. Additional expression studies in COS-7 cells showed that the antigen level of mutant FXII (FXII-Gly542Ser) was lower than wild type in culture medium, whereas the corresponding level of FXII antigen in cell lysates was equivalent. These results suggest that the Gly542Ser mutation causes FXII deficiency through intracellular degradation.

Keywords Gene mutation · Factor XII deficiency · Genetic analysis · CRM · Expression study

Introduction

Coagulation factor XII (FXII), a single-chain glyco-protein, produced by hepatocytes and then secreted into plasma as an inactive serine protease precursor [1]. The molecular weight is 80 kDa, composed of 596 amino acid residues [2]. FXII is the initiator of intrinsic coagulation system. It is converted to activated FXII (FXIIa) by proteolytic cleavage at the Arg353-Val354 polypeptide bond, Then, FXIIa converted factor XI to activated factor XI (FXIa), initiating the rapid intrinsic coagulation pathway. In addition, it also plays a vital role in fibrinolysis regulation, complement activation, bradykinin production and inflammation [3–5].

Congenital FXII deficiency is an autosomal recessive disease due to mutations in the FXII gene (*F12*). It can be classified as three types: a cross-reacting material (CRM)-negative group (FXII:Ag cannot be detected), a CRM-positive group (FXII:Ag is normal), and a CRM-reduced group (FXII:Ag reduced). Most patients with FXII deficiency demonstrate no significant clinical symptoms, while several cases present with bleeding, thrombosis or recurrent abortions [6, 7]. And now several clinical investigations manifested which was a risk factor for thrombosis [8]. They are usually detected by chance for a prolonged activated partial thromboplastin time (APTT) on health checkup or preoperative screening, which always presents with a decreased FXII activity (FXII:C) [9]. The molecular mechanism for FXII deficiency has been described in only a few cases so far [10, 11].

In this paper, we detected a patient with FXII deficiency from a consanguineous marriage family, then studied the phenotype and genotype of the proband and his families. We also analyzed the conservative and harm of the mutation by bioinformatics software and performed a transient in vitro expression study to explore possible mechanisms responsible for this deficient phenotype.

✉ Lihong Yang
YLH91@163.com

1   Department of Clinical Laboratory, The First Affiliated Hospital of Wenzhou Medical University, Shangcai Village, Ouhai District, Wenzhou 325000, Zhejiang, China

Materials and methods

Patients

The proband was a 38-year-old Chinese man, who went to the First Affiliated Hospital of Wenzhou Medical University (China) for a bronchoscope examination. He had never experienced bleeding or thrombosis in the past. Pre-operative coagulation screening revealed activated partial thromboplastin time (APTT) was extremely prolonged at 101.7 s. Further assessment showed FXII:C decreased at 3%(normal: 72–113%) and FXII:Ag level was less than 1% (normal: 72–113%). Except the fibrinogen (FIB) and D-Dimer (D-D) were slightly high, other indexes, such as prothrombin time (PT), thrombin time (TT), lupus anticoagulant (LAC), factor VIII activity (FVIII:C), factor IX activity (FIX:C), and factor XI activity (FXI:C), were all within normal ranges. No evidence showed abnormalities in liver and kidney functions.

Family study showed that there was a consanguineous relationship between the proband's parents, as his paternal grandmother and maternal grandmother were sister-German (Fig. 1). A total of another six family members from three generations of his family participated in our research.

Our study was approved by the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University (China). Peripheral blood samples from the probands and their family members were collected after obtaining written informed consent.

Coagulations tests

Peripheral blood samples from the probands and their family members were collected into standard 0.129 mmol/l trisodium citrate tubes. Routine coagulation tests, including APTT, PT, TT and FIB, were tested by STAR analyzer

(Stago, France). D-Dimer was detected by immune turbidimetry method. Lupus anticoagulant was detected by clotting method. Coagulation activities of FXII, FVIII, FIX, and FXI were measured with a one-step clotting method. And the FXII:Ag was measured using enzyme-linked immunosorbent assay (ELISA). The reference ranges of these indexes in our laboratory were established with 100 healthy subjects.

Genetic analysis

The genomic DNA of all the participants was isolated from peripheral blood using the QIAamp DNA Blood Mini kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's protocol. All the 14 exons along with their intron–exon boundaries and 5′,3′-untranslated regions (reference sequence, GenBank: AF538691.1) were amplified by polymerase chain reaction (PCR) in a 25 µl reaction volume on a thermal cycler (Eppendorf, Hamburg, Germany). PCR products were then subjected to direct sequencing on an ABI PRISM 3700 (Applied Biosystems, Foster City, USA). The primers are as shown in Table 1.

When mutational sites were detected, they were identified by reverse sequencing. The corresponding mutational exons of the family members were amplified and sequenced to confirm whether they carry the same mutation.

Conservation analysis

The multiple sequence alignment software ClustalX-2.1-win (Science Foundation Ireland, Dublin, Ireland) was used to analyze the conservative property of Gly542 in *homo sapiens* and its seven homologous species (*Mus musculus*, *Rattus norvegicus*, *Pan troglodytes*, *Macaca mulatta*, *Canis lupus familiaris*, *Bos Taurus*, and *Xenopus tropicalis*) (HomoloGene, http://www.ncbi.nlm.nih.gov/homologene).

Bioinformatics prediction

We adopted four online bioinformatics software to predict whether the amino acid substitution could affect protein function. They were (1) Polymorphism Phenotyping v2 (PolyPhen-2, http://genetics.bwh.harvard.edu/pph2) (J. Craig Venter Institute, Tauranga, New Zealand), (2) Protein Variation Effect Analyzer (PROVEAN, http://provean.jcvi.org/seq_submit.php) (J. Craig Venter Institute, Rockville, USA), (3) Sorting Intolerant From Tolerant (SIFT, http://sift.jcvi.org) (the Genome Institute of Singapore, Singapore, Singapore), (4) Mutation Taster (http://http://www.mutationtaster.org) (Charité-Universitätsmedizin Berlin, Berlin, Germany). The reference protein ID of FXII was 'P00748' and the Ensembl transcript ID was 'ENST00000253496'.
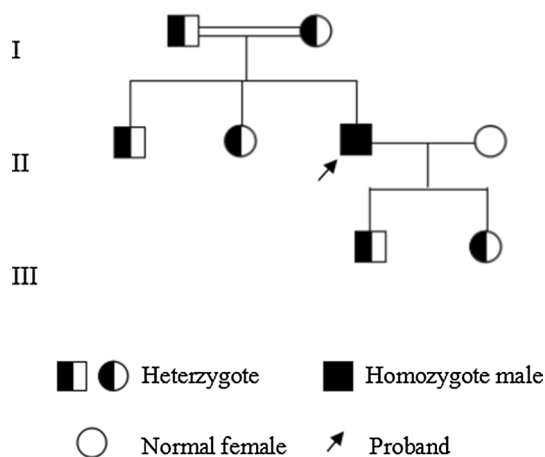

Fig. 1 The family tree of the inherited coagulation FXII deficiency

Table 1 Polymerase chain reaction (PCR) primers for amplification of exons, exon–intron boundaries and 5′,3′-untranslated regions of FXII gene

Exons	Primers	Product length (bp)	Annealing temperature (°C)
E1 + 2	F: 5′-AGGGCAGCTTGACCAATC-3′	594	60
	R: 5′-GAGGAGCCAGGCCACTTA-3′		
E3 + 4	F: 5′-GTGCCTGAGCAGTTGAGT-3′	437	60
	R: 5′-AGTAATGAGGCGGGAGGA-3′		
E5 + 6	F: 5′-CTGCTCTTCCTTCCACCAT-3′	434	60
	R: 5′-CCGTTCCCAACCATCTGC-3′		
E7 + 8	F: 5′-CACTGCGTTCCCTCCCCAAG-3′	600	58
	R: 5′-CGTTAGAGCGCCGGGGAG-3′		
E9 + 10	F: 5′-ATCCGCCCGTGGTGCTT-3′	703	58
	R: 5′-CCCAATCCCGTGTTCCAG-3′		
E11 + 12	F: 5′-CTGGAACACGGGATTGG-3′	530	56
	R: 5′-CGGGCTTCTTCCGCCTAAC-3′		
E13 + 14	F: 5′-CGGCAAGCGTTGTCAGA-3′	638	54
	R: 5′-GCGGGACCCATGAAGAAA-3′		

Transient in vitro expression study

The *c.1681G > A* nucleotide substitution was introduced into the pIRES2-EGFP expression vector (the wild type plasmid, containing the full-length FXII cDNA, provided by Ruijin Hospital, Shanghai Jiaotong University School of Medicine) using a QuickChange Site-Directed Mutagenesis Kit (Stratagene, USA). The resulting mutant plasmid was checked by sequencing subsequently.

The wild type and mutant plasmids were transfected into African green monkey kidney cells (COS-7) using the Lipofectamine 2000 reagent (Invitrogen, USA). Both culture medium and cells were collected following 48 h culture of the transfected cells in serum-free DMEM.

Culture supernatants were concentrated approximately 10 times using Centricon Plus-20 centrifugal filters (Millipore, USA). After centrifugated at 12,000 rpm for 10 min, the supernatants were subjected to measure the activities of FXII with a one-step clotting assay and the antigens of FXII by ELISA. The cells were washed twice with phosphate-buffered saline (PBS) and then lysed in 120 μl M-ER mammalian protein extraction reagent (Pierce Biotechnology Inc., USA) with 20 μl protease inhibitor (Roche Diagnostics GmbH, Germany), ELISA was used to evaluate FXII antigens of cell lysates.

Results

Routine coagulation indices analysis

The phenotypic detection of family members showed that his elder sister had decreased FXII:C of 52% and FXII:Ag of 50.6%, and his father, mother, elder brother, son and daughter all had decreased FXII:C and FXII:Ag to approximately 30%. We also tested the APTT, PT, TT, FIB, D-D and LAC of the six family members and found that APTT of his son was slightly high, FIB of his father and mother were slightly high, D-D of his elder brother and sister were slightly high, other indices were no abnormal results (Table 2).

Table 2 Results of coagulation tests in the family

	PT (s)	APTT (s)	TT (s)	FIB (g/l)	D-D (mg/l)	LAC	FXII:C (%)	FXII:Ag (%)
Proband	13.0	101.7	15.3	4.07	0.66	0.87	3	<1.0
Father	13.3	43.0	15.8	4.76	0.54	0.98	29	28.0
Mother	13.7	39.6	16.2	4.81	0.32	0.97	35	32.4
Brother	12.5	44.7	15.0	3.88	0.70	1.06	33	30.7
Sister	13.2	40.4	14.8	3.33	0.75	1.00	52	50.6
Son	15.0	52.6	14.8	2.99	0.26	0.98	25	25.2
Daughter	12.6	46.4	14.8	3.41	0.26	0.99	31	30.3
Normal range	11.7–14.8	29.0–43.0	14.0–20.0	2.0–4.0	0–0.5	0.75–1.25	72–113	72–113

DNA sequencing analysis

DNA sequencing analysis revealed that the proband carried a homozygous *c.1681G > A* point mutation in exon 14 of FXII resulting in a substitution of glycine 542 by serine (Gly341Ser) (Fig. 2①, ②). His father, mother, elder brother, elder sister, son and daughter all took a heterozygous Gly542Ser (Fig. 2③). Genotype analysis of 100 healthy subjects excluded the possibility of Gly542Ser as a polymorphism.

Gly542 conservation analysis

Homologous sequence alignment results showed that the Gly542 was highly conserved among the homologous species: *Mus musculus*, *Rattus norvegicus*, *Pan troglodytes*, *Macaca mulatta*, *Canis lupus familiaris*, *Bos taurus*, and *Xenopus tropicalis* (Fig. 3).
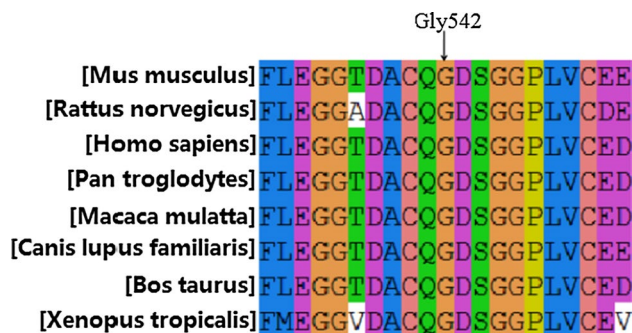

Fig. 3 The conservative analysis diagrams of Gly542Ser. The targeted amino acid was indicated with an arrow, this line are all G (Gly) which indicated that the G (Gly) are conserved in the homologous species

Bioinformatics prediction of Gly542Ser

The forecasting results of the four bioinformatics softwares were 'Probably damaging', 'Deleterious', 'Affect protein

Fig. 2 The sequence diagram of Gly542Ser. ① is the forward sequencing of homozygous *c.1681G > A*; ② is the backward sequencing of homozygous *c.1681C > T*; ③ is the forward sequencing of heterozygous *c.1681G* and ④ is the forward sequencing of wild type. The position of mutational base is indicated with an arrow
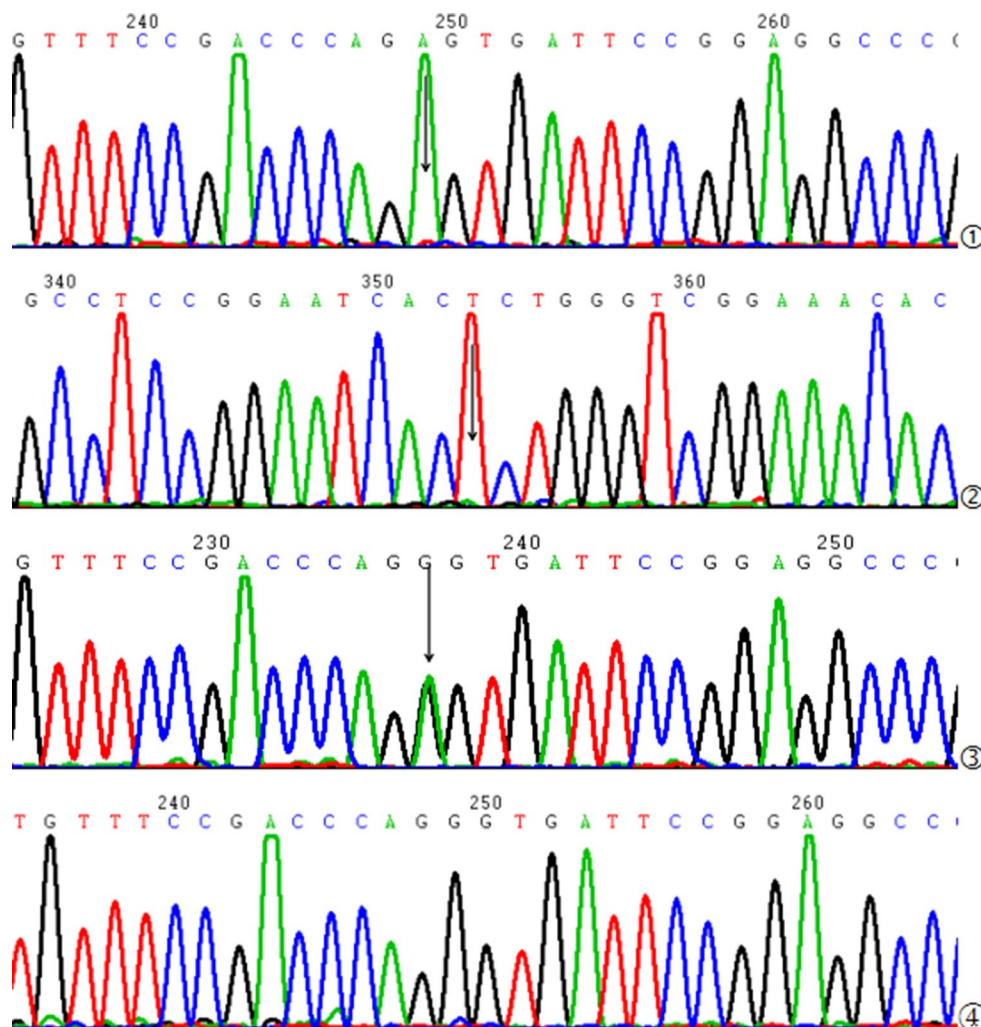
function', and 'Disease causing' corresponding to 'Poly-Phen-2', 'PROVEAN', 'SIFT', and 'Mutation Taster' with the scores '1.000', '− 4.975', '0.00', and '1.000', respectively (Table 3).

Transient in vitro expression results

As shown in Table 4 and Fig. 4, the FXII:Ag level in culture medium independently expressing the pIRES2-EGFP/FXII-Gly542Ser was reduced to 26.5% of the wild type recombinant protein (set as 100%), whereas the corresponding level of FXII:Ag in cell lysates was 86.8% of the wild type FXII protein. The mean values of wild-type FXII were set as 100%.

Discussion

In this study, DNA sequencing analysis and mutational screening revealed one genetic variation in the FXII gene of the proband, who took the homozygous Gly542Ser mutation. This homozygous mutation was found in another male patient in this area in the previous study by our research group [12]. Family study of the proband indicated that the proband had FXII:C and FXII:Ag level approximately reduced to 3% and less than 1%,and took the homozygous Gly542Ser mutation. And the FXII:C and FXII:Ag of his father, mother, elder brother, son and daughter were all decreased to around 30%, who carried the heterozygous Gly542Ser mutation. Because the proband's parents were consanguineous marriage, we could speculate that the homozygous Gly542Ser alleles of the proband were derived from his father and mother, and the mode of its inheritance is in accordance with that of autosomal recessive trait. And we

Table 3 The bioinformatics software's prediction results of Gly542Ser

Software name	Prediction results	Score
PolyPhen-2	Probably damaging	1.000
PROVEAN	Deleterious	− 4.975
SIFT	Affect protein function	0.00
MutationTaster	Disease causing	1.000

The results of prediction are as in the table, and the meaning of scores is as follows:

1. PolyPhen-2: Scores are evaluated as 0.000 (most probably benign) to 1.000 (most probably damaging)

2. PROVEAN: The predefined threshold of score is − 2.5, the score ≤ − 2.5 (deleterious), > − 2.5 (neutral)

3. SIFT: Scores range from 0 to 1, the score ≤ 0.05 (damaging), > 0.05 (tolerated)

4. MutationTaster: A probability close to one indicates a high security of prediction

Table 4 Transient expression results of wild-type and mutant FXII (pIRES2-EGFP/FXII-Gly542Ser)

	FXII:Ag (%)		FXII:C (%)
	Culture medium	Cell lysates	Culture medium
Wild type	92.0	103.2	96
Gly542Ser	24.4	89.6	30
Gly542Ser/wild type	26.5	86.8	31.3

could see this mutation belongs to the CRM⁻ group because the FXII:C and FXII:Ag level was reduced near to zero.

To further research the biological characteristics of Gly542Ser mutation, we analyzed its conservatism and possible effects on the protein. We found that Gly542 was a highly conservative site in the eight homologous species, which indicated that it was an important functional site of FXII, and played a key role in the normal function of the protein. The four bioinformatics prediction softwares all manifested the baneful effect of Gly542Ser, which was a deleterious mutation and could affect the FXII protein function and cause disease.

Gly542 lies in the catalytic domain of FXII. When the homozygous *c.1681G > A* mutation of *F12* occurred, which led to a novel Gly542Ser mutation in FXII. It would confer a change in the catalytic domain and alter its local interactions. There were some other mutations which had been reported near or in this region: Leu519Arg [13], Asp538Asn [14] and Cys571Ser [15]. Miyata T et al. once demonstrated that the change of Cys571Ser can break the disulfide bond of Cys540–Cys571, which results in the change of serine active site or enzyme action substrate-binding site, and lose the
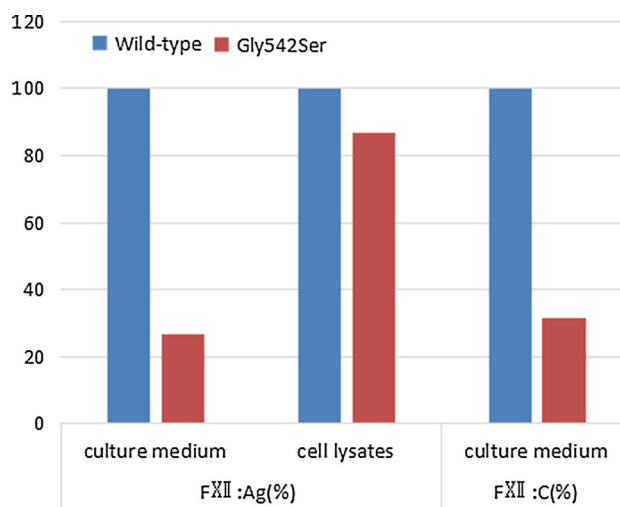

Fig. 4 Transient expression of wild-type and mutant FXII (pIRES2-EGFP/FXII-Gly542Ser) in COS-7 cells

enzyme activity [15]. Because there was no X-ray 3D structure file of the catalytic domain of FXII, we were not able to do the spatial structure analysis of the mutant protein. But, we knew that Gly542 is a nonpolar hydrophobic amino acid, at the same time, it has acidic and basic functional groups, while Ser is a polar non-charged hydrophilic amino acids. Hence, when the Gly542 mutant into Ser542, located between Cys540 and Cys571, it probably induces partial structural changes. As a consequence, the variant might be unstable and prone to intracellular proteolysis. To investigate the pathogenicity of this Gly542Ser missense mutation, we conducted the transient in vitro expression in African green monkey kidney (COS-7) cells. The results demonstrated accumulation of mutant FXII-Gly542Ser in the cell similar with wild type, while the mutant FXII-Gly542Ser showed lower levels than wild type in culture medium. From these results, we conclude that Gly542Ser substitution is susceptible to intracellular degradation,which was similar to Asp538Asn and many other reported FXII mutations [2, 14, 16, 17].

Moreover, the catalytic region was the key position of FXII for enzymatic function, which contains the activation domain and serine protease domain, and the activity center of serine protease consisted of His393, Asp442 and Ser544 residues [18]. There were only 2 amino acids range from Gly542 to Ser544, the Gly542Ser mutation may directly affect the activation of the Ser544 site. It may affect the FXII normal biological function because the catalytic domain of FXII protein could not be fully activated. Wang XF et al. had reported the Gly542Ser heterozygous mutation [19].

In summary, we reported a Gly542Ser mutation causing CRM-negative FXII deficiency in a Chinese patient family, which was a deleterious mutation analyzed through bioinformatics prediction softwares. And we also discussed its possible pathogenic mechanism that the mutation was the important reason for the decrease of FXII:C by intracellular degradation.

Compliance with ethical standards

Conflict of interests No potential conflict of interest was reported by the authors.

References

1. Citarella F, Ravon DM, Pascucci B, Felici A, Fantoni A, Hack CE. Structure/function analysis of human factor XII using recombinant deletion mutants. Eur J Biochem. 1996;238:240–9.

2. Ishii K, Oguchi S, Moriki T, Yatabe Y, Takeshita E, Murata M, et al. Genetic analyses and expression studies identified a novel mutation (W486C) as a molecular basis of congenital coagulation factor XII deficiency. Blood Coagul Fibrin. 2004;15:367–73.

3. Kaplan AP, Silverberg M. The coagulation-kinin pathway of human plasma. Blood. 1987;70:1–15.

4. McMullen BA, Fujikawa K. Amino acid sequence of the heavy chain of human a-factor XIIa (activated hageman factor). J Biol Chem. 1985;260:5328–41.

5. Iijima K, Arakawa Y, Sugahara Y, Matsushita M, Moriguchi Y, Shimohiro H, et al. Factor XII Osaka: abnormal factor XII with partially defective prekallikrein cleavage activity. Thromb Haemost. 2011;105:473–8.

6. Pauer HU, Burfeind P, Köstering H, Emons G, Hinney B. Factor XII deficiency is strongly associated with primary recurrent abortions. Fertil Steril. 2003;80:590–4.

7. Mordillo C, Martinez-Marchαn E, Fontcuberta J, Soria JM. Molecular analysis of multiple genetic variants in Spanish FXII-deficient families. Haematologica. 2007;92:1569–72.

8. Renné T, Schmaier AH, Nickel KF, Blombäck M, Maas C. In vivo roles of factor XII. Blood. 2012;120(22):4296–303.

9. Singhamatr P, Kanjanapongkul S, Rojnuckarin P. Molecular analysis of factor XII gene in Thai patients with factor XII deficiency. Blood Coagul Fibrinolysis. 2013;24:599–604.

10. Ishii K, Oguchi S, Moriki T, Yatabe Y, Takeshita E, Murata M, et al. Genetic analyses and expression studies identified a novel mutation (W486C) as a molecular basis of congenital coagulation factor XII deficiency. Blood Coagul Fibrinolysis. 2004;15:367–73.

11. Suzuki K, Murai K, Suwabe A, Ishida Y. Factor XII Ofunato: Lys346Asn mutation associated with blood coagulation factor XII deficiency causes impaired secretion through a proteasome-mediated degradation. Thromb Res. 2010;125:438–43.

12. Yang LH, Wang YY, Zhou JP, Cheng XL, Hao XP, Xie HX, et al. Identification of genetic defects underlying FXII deficiency in four unrelated Chinese patients. Acta Haematol. 2016;135:238–40.

13. Dai LY, Zhang DT, Lin J, Wang YY, Tong Y, Li J, et al. A pedigree analysis of Hereditary coagulation factor XII deficiency caused by a novel homozygous mutation: Leu519Arg. Chin J Lab Med. 2015;38:466–9 **(China)**.

14. Li M, Xie HX, Wang MS, Ding HX. Molecular characterization of a novel missense mutation (Asp538Asn) in a Chinese patient with factor XII deficiency. Clin Lab. 2015;61:1967–71.

15. Miyata T, Kawabata S, Iwanaga S, Takahashi I, Alving B, Saito H. Coagulation factor XII (Hageman factor) Washington DC: inactive factor XIIa results from Cys-571-Ser substitution. Proc Natl Acad Sci USA. 1989;86:8319–22.

16. Kondo S, Tokunaga F, Kawano S, Oono Y, Kumagai S, Koide T. Factor XII Tenri, a novel cross-reacting material negative factor XII deficiency, occurs through a proteasome-mediated degradation. Blood. 1999;93:4300–8.

17. Matsuki E, Miyakawa Y, Okamoto S. A novel factor XII mutation, FXII R84P, causing factor XII deficiency in a patient with hereditary spastic paraplegia. Blood Coagul Fibrinolysis. 2011;22:227–30.

18. Yarovaya GA, Blokhina TB, Neshkova EA. Contact system. New concepts on activation mechanisms and bioregulatory functions. Biochemistry. 2002;67:13–24.

19. Wang XF, Dai J, Wang MS, Ding QL, Wang HL. Analysis of the factor XII gene mutations in two Chinese pedigrees with congenital clotting factor XII deficiency. J Diagn Concepts Pract. 2005;4:447–50 **(Shanghai)**.