Factor IX alteration p.Arg338Gln (FIX Shanghai) potentiates FIX clotting activity and causes thrombosis

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Mutation analysis

The coding sequence and flanking regions of F9, SERPINC1, PROC and PROS1 genes were amplified by polymerase chain reaction (PCR). Primers used for PCR were listed in Supplement Table 1. The reaction mixture for amplification of F9, SERPINC1, PROC (E6, 8, 9), PROS1 (E2-3, 5-6, 7-8, 9, 10, 11-12, 13, 14) contained 1×PCR buffer, 50 ng genomic DNA, 0.2μM of each primer, 0.2 mM dNTP, 5 mM MgCl2 and 0.3 U HotStar Taq DNA polymerase (Qiagen, Germany) in a final volume of 10 μL. As to the rest exons of PROC and PROS1 genes, a 20μl mixture was prepared for each reaction and included 1x GC buffer I (TAKARA, Japan), 0.2 mM dNTP, 0.2μM of each primer, 1 U HotStarTaq polymerase (Qiagen, Germany) and 50 ng template DNA. Amplification conditions for Exon 5 of PROC was 95℃ for 15 min, 38 cycles of 96℃ for 10 s, 68℃ for 1min. The cycling program for Exon5-6, Exon7-8 and Exon12-13 of PORS1 was 95℃ for 15 min, 35 cycles of 96℃ for 10 s, 68℃ for 6 min, 72℃ for 2 min. Rest reactions were amplified using the following conditions: initial denaturation at 95℃ for 15 min, 11 cycles of 94℃ for 20 s, 62℃ (decreasing by 1℃ per cycle) 40 s, 72℃ 2 min, then 24 cycles of 94℃ 20 s, 56℃ 30 s, 72℃ 2 min, and a final elongation step at 72℃ for 2 min. PCR products were analysed by direct DNA sequencing.

Supplement Table 1

Primers used for amplification of F9, SERPINC1, PROC and PROS1 genes

Gene	Primer	Forward (5'to 3')	Reverse(5'to 3')
F9	E1	CAACCTTA AGAATCTGACAGTAAAAA	TGCTGTCAAATCATGTAATCAAAA
F9	E2-3	GATTTTGGCTCCATGCCCTA	GTTCCCACACTGGCATAACC
F9	E4	CAGCTGGCTTCCAGGTCAGT	GCTTCTTGAACTCATATCCTGAAA
F9	E5	AACATGAATGCCCCCAATGT	TGATTTCAAAAGGAAGCAGATTCA
F9	E6	AGGATGGGCCTCAATCTCAA	GGAGGCCTTCTCACATTGGT
F9	E7	CATTCCATTTCTGCCAGCAC	TGACCCTTCTGCCTTTAGCC
F9	E8	GGTCAGTGGTCCCAAGTAGTCA	GGCTGGGCCCTTAGAAATG
SERPINC1	E1	CAGGTGTAACATTGTGTTTTCC	CCTTTGGAGGTCACAAAACC
SERPINC1	E2	GGGCTGGAATCCTCTGCTTTA	CTAGTGGCCTGCAGTGTTGGT

SERPINC1	E3	CCCAGGTACTGTGCTTGAAGG	TTGGATGCTGTTTCTCCACCT
SERPINC1	E4	TATGTGAGGCTTCCCCAATTT	TGTAGCTTTCGGCAGTCCATT
SERPINC1	E5	GTGCATATCCCGCCAGTCTT	CCAACTCTTCCACTTTTGGTCA
SERPINC1	E6	TTTCAAAAAGCCCCAAAGGAT	AAGAGGTAGTGGGAGGGAAGG
SERPINC1	E7	AGGATTCTGGAGGGAATCCAA	TTTACCATGTGCCCCAATAGC
PROC	E1	GTCCACAAAAGGGGCCAAAT	TGACAGCCTGGAGTTCGAGTT
PROC	E2	CAAGGGTTTTGCCCTCACCTC	GCTGGAGGATTCAGCCCTCATC
PROC	E3	GCTTTCTAGGCAGGCAGTGT	AGGAGGGAGCTTTAGGAGGTC
PROC	E4	CATCTCAGAGCAAGGCTTCG	ACTCCTAAGAGGGCCTCAGC
PROC	E5	TCGGGCGTCGATCCCTGTTTG	CCGCTGCCCCAAGGCTCAACT
PROC	E6	GACTGACTGGAGGGGGTTTGT	GGTTTCTGCACCCTGAGCATA
PROC	E7	AGGAAGACACCCTGGGACAG	GGTCTGTGTAGCCCCTCACAA
PROC	E8	TCACCGTTGATAGGGTTCCAC	ATGAAGTTGAGGACGAAGGTGC
PROC	E9	GCAGACCATAGTGCCCATCTG	GGGATGGAAGGACAGAACAGC
PROS1	E1-1	ATTCCCTTCTTTTCTTCATGTTCT	CCACCCAGGACCCTCATTT
PROS1	E1-2	TCCGAAAAGCTTCCTGGAAATG	CGCTGGGTGTCTGTCGGTACT
PROS1	E2-3	CACCAAACCCGCTCTCTGAATTGTT	AACAGTGGAATTCACCACACATGCCTA
PROS1	E4	ATTTGAGGGCAACATCTTGTTTCTG	TTGGGAATAAAAATTCTGAAGCTGATA
PROS1	E5-6	ATTTAAAGAAAGGAGTTGTGTGTTTTT	AGATGAAGGGCTGATGTTGGA
PROS1	E7-8	TGGTCCAAAGGCCAATCTGTT	CAGAACGTCTGTATTTTCCTGACTTAGC
PROS1	E9	ACGTAATTCTCCTCAAAATATCCTCA	CTTCATTTCTGAACTCAATCTAAGTACG
PROS1	E10	CCTGGGGAAAAGATTCCAAGA	TTGGCAATTGAGGAGTTTTCC
PROS1	E11	GGCGGGTGGATCATGAGG	GGCACACAGTAGATACTCAATAATGTTTCACA
PROS1	E12-13	GTGAATGTGCAAGGGAGAAGG	GTACTCGGATGGCTCCAAGC
PROS1	E14	TCCGGTTGTTTTACGGAATTT	TCGCAAGGTCAGGAGATCAAG
PROS1	E15-1	TCGGCGTCACTTAACATGTATTT	GCAATCTTACCTCCTTACTTCTTTG
PROS1	E15-2	CTGCAGTCTGTCAGGATGAGAT	AATGGCCCTGTTATTTGGAGT

E, exon

> The coding DNA sequence of codon-optimized human FIX (hFIXco)

atgcagagggtgaacatgatcatggctgagagccctggcctgatcaccatctgcctgctgggctacctgctgtctgctgagtgc
actgtgttcctggaccatgagaatgccaacaagatcctgaacaggcccaagagatacaactctggcaagctggaggagtttgt
gcagggcaacctggagagggagtgcatggaggagaagtgcagctttgaggaggccagggaggtgtttgagaacactgag
aggaccactgagttctggaagcagtatgtggatggggaccagtgtgagagcaacccctgcctgaatgggggcagctgcaag
gatgacatcaacagctatgagtgctggtgcccctttggctttgagggcaagaactgtgagctggatgtgacctgcaacatcaag
aatggcagatgtgagcagttctgcaagaactctgctgacaacaaggtggtgtgcagctgcactgagggctacaggctggctg
agaaccagaagagctgtgagcctgctgtgccattcccatgtggcagagtgtctgtgagccagaccagcaagctgaccagggc
tgaggctgtgttccctgatgtggactatgtgaacagcactgaggctgaaaccatcctggacaacatcacccagagcacccaga
gcttcaatgacttcaccagggtggtgggggggggaggatgccaagcctggccagttcccctggcaagtggtgctgaatggcaa
ggtggatgccttctgtgggggcagcattgtgaatgagaagtggattgtgactgctgcccactgtgtggagactggggtgaag
atcactgtggtggctggggagcacaacattgaggagactgagcacactgagcagaagaggaatgtgatcaggatcatccccc
accacaactacaatgctgccatcaacaagtacaaccatgacattgccctgctggagctggatgagcccctggtgctgaacagct
atgtgacccccatctgcattgctgacaaggagtacaccaacatcttcctgaagtttggctctggctatgtgtctggctggggcag
ggtgttccacaagggcaggtctgccctggtgctgcagtacctgagggtgcccctggtggacagggccacctgcctgaggagc
accaagttcaccatctacaacaacatgttctgtgctggcttccatgaggggggcagggacagctgccaggggactctgggg
gcccccatgtgactgaggtggagggcaccagcttcctgactggcatcatcagctgggggggaggagtgtgccatgaagggca
agtatggcatctacaccaaagtctccagatatgtgaactggatcaaggagaagaccaagctgacctga

>The sequence of human promoter transthyretin (TTR)

gtgtctgtctgcacatttcgtagagcgagtgttccgatactctaatctccctaggcaaggttcatatttgtgtaggttacttattctcct
tttgttgactaagtcaataatcagaatcagcaggtttggagtcagcttggcagggatcagcagcctgggttggaaggaggggg
tataaaagccccttcaccaggagaagccgtcacacagatccacaagctcctg

Coagulation function assay

The plasma was collected in sodium citrate (final concentration 0.38%) and one stage activated partial thromboplastin time (APTT) and prothrombin time (PT) were carried out on a Sysmex CA7000 analyzer (Sysmex Corporation, Tokyo, Japan) using APTT reagent constituted with ellagic acid (Dade Actin FSL) and PT reagent (Thromborel S) (Siemens Healthineers, Erlangen, Germany). The coagulation factor activity was determined using the commercially available factor deficient substrate plasma (Instrumentation Laboratory, Bedford, MA) based on either the partial prothrombin time (PTT) or the APTT assay. The FIX antigen in plasma was measured by ELISA with a goat anti-human FIX polyclonal antibody as the primary antibody and a peroxidase-conjugated antibody for detection (GAFIX-APHRP, Affinity Biological Inc., Ancaster, Canada). A standard curve was generated with serial dilutions of pooled normal human plasma.

Genetic analysis

Whole genome DNA was extracted from isolated peripheral leukocyte using the QIAamp DNA mini kit (QIAGEN, Hilden, Germany). The coding sequence and flanking regions of the *F9* gene were amplified by PCR and directly sequenced (the detailed conditions and primers for PCR are showed above). The common genetic thrombotic risk factors, including the factor V (FV) Leiden mutation and prothrombin G20210G/A, were screened. Due to the concern of warfarin interference on the accuracy of measurements of protein C (PC), protein S (PS) and antithrombin III (AT III), the coding genes of these established thrombotic risk factors were also amplified and sequenced (methods and results provided above).

In vitro expression of FIX Shanghai

The *F9* cDNA containing p.R338Q mutation were inserted in the mammalian expression vector pCDNA3.1 and transfected into HEK293T cell using the lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). The cell clone stably expressing FIX was selected by gentamicin (G418) (Invitrogen, Carlsbad, CA). The cells were maintained in a cell free media supplemented with ITS (Invitrogen, Carlsbad, CA), containing insulin, transferrin and selenium, and soybean trypsin inhibitor (Sigma, Missouri, USA). Vitamin K1 (1mg/L) was added to the media to enable the efficient carboxylation of FIX Gla domain. The conditioned media was harvested after 48 hours and subjected to semi-affinity purification. Briefly, the filtered media was allowed to flow through a sepherose Q column and the bound carboxylated FIX were eluted by calcium containing HEPES buffer. The FIX quantity and coagulation activity were determined by the ELISA assay and APTT-based clotting assay. The recombinant FIX Shanghai was subjected to SDS-PAGE analysis and detected by western blot using the FIX specific antibody (PAHFIX-S, Haematologic technologies, Essex Junction, VT).

Generation of recombinant adeno-associated virus vectors

The plasmids AAV-hFIXco, AAV-hFIXco-R338Q or AAV-hFIXco-R338L, AAV8 helper plasmid, and the mini adenovirus function helper plasmid pFΔ6, were co-transfected into HEK293 cells cultured in roller bottles at a ratio of 1:2:1. The transfected cells were harvested 3 days later. rAAVs were purified by two rounds of cesium chloride gradient ultracentrifugation and draw by a 28G needle. After buffer exchange against phosphate-buffered saline with 5% D-sorbitol for three times,

the viral vectors were stored at −80 °C before administration. The titer of purified vectors was determined by qPCR, and the viral preparations were stored at -80°C.

Animal procedures

The FIX-deficient mice (HB mice) were obtained from the Jackson Laboratory (Farmington, CT) and housed in a specific pathogen-free environment on normal diet. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Sichuan University. Approximately, 5×10^{10} or 4×10^{11} rAAV8 viral genomes (vg), which were equivalent to 2×10^{12} or 1.6×10^{13} vg/kg in a clinical setting, were diluted into 200μl of PBS and injected into 4-8 week-old HB mice via the tail vein (n=5 mice per group). Blood samples were collected from the retro-orbital plexus in capillary tubes without heparin at the designated time points. Sodium citrate was used as an anticoagulant at a final concentration of 0.38% (wt/vol). The blood samples were then centrifuged at 4 °C for 10 minutes at 2,000g in a microcentrifuge. The plasma samples were collected and stored at −80 °C prior to further analysis. The protein quantity of human FIX and FIX clotting activity (FIX:C) in mouse were determined by ELISA and the APTT-based assay, using the pooled normal human plasma as the standard.