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Key words: factor VIII, hemophilia A, exon 13, duplication, skipping.

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Disorders of Hemostasis

Factor XI deficiency with a novel homozygous mutation Trp599Arg near the C-terminal region

We identified a novel mutation in an asymptomatic 72-year old Japanese woman with severe factor XI (FXI) deficiency. Sequence analysis showed a homozygous missense mutation Trp599Arg (g.234T→C according to Genbank accession number M20218). This residue belongs to a region conserved in human FXI and the FXI of several animals. Molecular modeling showed that the Trp599 residue is positioned in an alpha helix in the C-terminal region of the FXI molecule.

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The factor XI gene (*F11*) (23 Kb in size) is located on chromosome 4q35, and contains 15 exons plus 14 introns.[1] To date, more than 40 different FXI gene lesions have been documented in the factor XI mutation database (*http://uwcmml1s.uwcm.ac.uk/uwcm/mg/search/119891.htm*). We identified a novel causative homozygotic mutation in a Japanese woman with severe factor XI (FXI) deficiency, which resulted in a Trp599 to Arg substitution near the carboxyl-terminal region of FXI. The proband was a 72-year old Japanese woman who consulted Iki public hospital after she had been bitten by a mamushi pit viper. She was immediately treated by serotherapy and coagulation tests were performed. Her platelet count was decreased for a time, but was within the normal range after a few hours. She had no bleeding tendency in that time. While results showed a remarkably prolonged activated partial thromboplastin time (89 s, reference range; 28-32s), her bleeding time, prothrombin time and fibrinogen levels were within normal ranges. No inhibitor of the intrinsic coagulation pathway was found in her plasma. Her parents were first cousins. After obtaining signed documents of informed consent, blood specimens were drawn from the proband and her son. FXI coagulant activity (FXI:C) was assayed by a one-stage method based on the activated partial thromboplastin time using FXI-deficient plasma. FXI antigen (FXI:Ag) concentrations were determined using goat anti-FXI-IgG (Cedarlane, Ontario, Canada), biotin labeled anti-FXI-IgG and peroxidase-conjugated streptavidin (DAKO A/S, Glostrup, Denmark) by an in-house enzyme-linked immunosorbent assay (ELISA). Both FXI:C and FXI:Ag levels of the proband were below 1% of normal control values. Coagulation factors, apart from FXI, were within normal ranges. FXI:C and FXI:Ag for her son were 56.6% and 62.0%, respectively. Polymerase chain reaction (PCR) amplification of exons 1 through 15 of the *F11* gene was performed with the use of genomic DNA as a template, with primer and conditions as described previously.[2] Purified PCR products were directly sequenced using a *Taq* Dye Deoxy Terminator Sequencing Kit (Applied Biosystems, Foster City, USA). Sequence analysis showed a T→C mutation at codon 599 in exon 15 (g.234T→C according to Genbank accession number M20218), resulting in a substitution of Trp599 (TGG) by Arg (CGG) in the catalytic domain. This point mutation created an *Msp* 1 restriction endonuclease recognition site in the exon 15 nucleotide sequences. Results of an *Msp* 1 digestion indicated that the proband was homozygous for the mutation, while her son was heterozygous for the mutation (Figure 1). No *Msp* 1 restriction endonuclease sites were detected in any of exon 15 sequences from 50 normal Japanese individuals.

Trp599 belongs to a structurally conserved region present in human FXI (Genbank accession number NM-000128), rabbit FXI (AF395821), bovine FXI (NM028066) and mouse FXI sequences (BC019485) (Figure 2A). It is

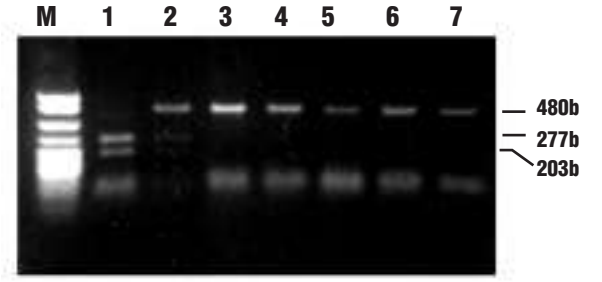

Figure 1. ***Msp 1*** **restriction analysis of the patient, her son and normal individuals. The normal individuals exhibit an undigested band at 480bp, while the variant gives digested bands at 203 and 277bp. This confirms the creation of an** ***Msp 1*** **cleavage site due to the altered sequence from CTGG to CCGG, and also demonstrates that the proband is homozygous for the observed mutation. Her son shows 480, 277 and 203bp bands, indicating that he is heterozygous for this mutation. PCR fragments spanning exon 15 were digested and analyzed on a 1.8% agarose gel. Lane 1, proband; lane 2, son; lane 3-7, normal individuals. The left lane contains DNA molecular weight markers.**

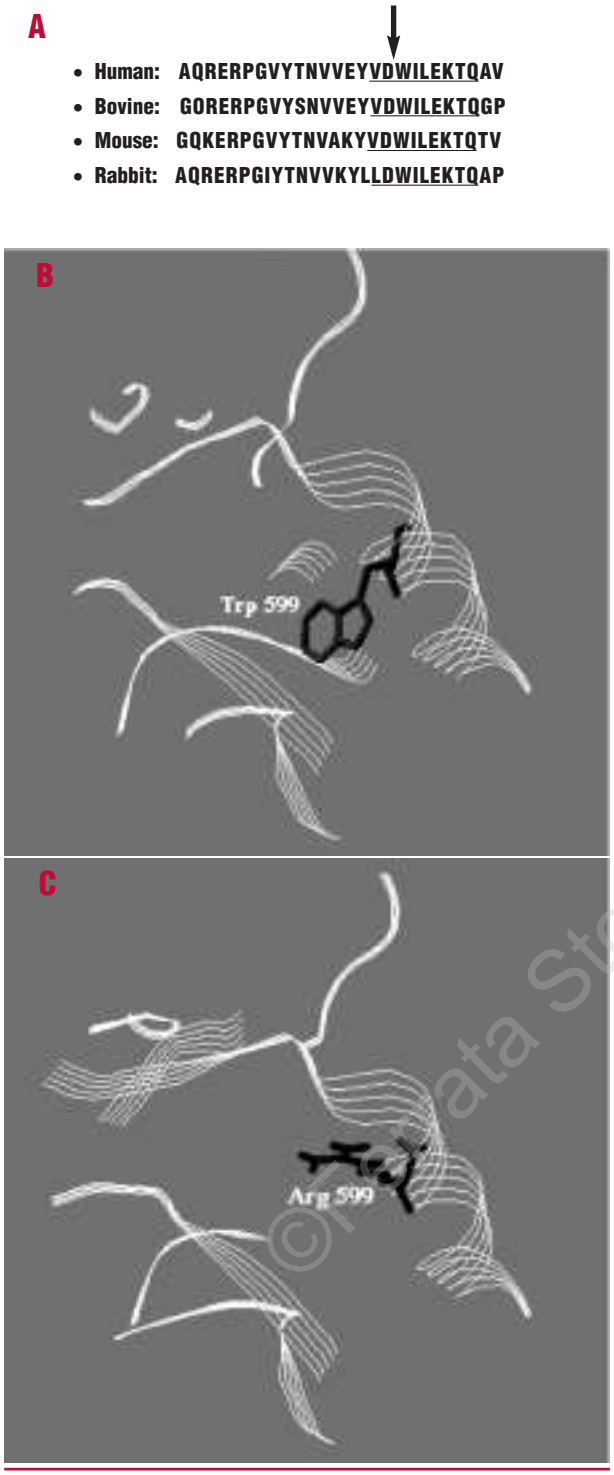

Figure 2. A. Alignment of sequences of the factor XI C-terminal region. Partial amino acid sequences from human (Genbank accession number NM_028066), mouse (AAH19485), rabbit (AAK82432) and bovine (NM_001008665) factor XI were aligned using the Clustal W program (*http://www.ddbj.nig.ac.jp/search/clustalw-j.html*). The Trp599 residue, which is conserved in all four species, is indicated by an arrow. **B.** Localization of site 599 in the 3D-structure of the α helix at the C-terminal region of factor XI. The main peptide chain is indicated by ribbons. The position of Trp599 is shown by a stick and ball. **C.** The mutation was built into the model by replacement, followed by energy minimization. The position of Arg599 is shown by a stick and ball.

also conserved in prekallikrein, which is thought to derive from a common molecular ancestor.[3] To examine the structural environment of the Trp599 residue in the FXI molecule, the co-ordinates of FXIa monomer were extracted from the rhFXI370-607-ecotin complex (1XXd.PBD), and were visualized using SwissPdbViewer version 3.5. The Trp599 residue was positioned in an α helix in the C-terminal region of the FXI molecule (Figure 2B). The α helix is positioned on the surface of the FXI molecule, although we note that the structural model utilized the co-ordinates of the FXIa monomer from the rhFXI370-607-ecotin complex, which did not contain the apple domains.

The substitution of Trp599 by Arg results in a change in charge and the absence of a bulky aromatic side chain on the surface of the region (Figure 2C). It has been reported that the carboxyl-terminal amino acids play important roles in the secretion of many proteins.[4-6] In coagulation factors, the carboxyl-terminal amino acid of both factor IX (FIX) and protein C were studied for their roles in protein secretion.[7-8] An exactly analogous mutation (FIXTrp407Arg) in the carboxyl-terminal region of FIX was expressed in Hep G2 cells, resulting in moderate reductions in intracellular FIX levels, but severely reduced levels of secreted FIX.[7] We suggest that Trp599 in the α helix at the C-terminal region FXI may be structurally important for secretion from cells. *In vitro* expression and characterization of the mutant protein is in progress in our laboratory in order to further define the functional effect of the mutation.

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Stem Cell Transplantation

Feasibility of early tapering of cyclosporine following reduced-intensity stem cell transplantation for advanced hematologic or solid malignancies

Although some researchers have reported that early tapering of cyclosporine is feasible and beneficial to augment graft-versus-leukemia effects after conventional stem-cell transplantation, there is little information on the feasibility of this strategy following reduced-intensity stem cell transplantation (RIST). We summarized outcomes of 17 patients who underwent early tapering of cyclosporine following RIST from HLA-identical siblings.

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There are some reports that early tapering of cyclosporine is feasible and beneficial to augment a graft-versus-leukemia (GVL) effect after conventional allogeneic stem cell transplantation (allo-SCT),[1,2] while there is little information on the feasibility of this procedure following reduced-intensity stem cell transplantation (RIST). We summarized the outcomes of 17 patients with a median age of 53 years (range, 25-61) who underwent early tapering of cyclosporine following RIST from HLA-identical siblings from 1999 to 2003 in our hospital. Nine had chemorefractory hematologic malignancies and eight had advanced solid tumors. Preparative regimens comprised fludarabine/busulfan (n=16),[3] and cladribine/busulfan (n=1).[4] Eight patients with solid tumors received rabbit antithymocyte globulin (ATG) 2.5 mg/kg for 4 or 2 days.[4] Graft-versus-host disease (GVHD) prophylaxis was continuous infusion of cyclosporine 3 mg/kg/day. Early tapering of cyclosporine was defined as completion of tapering by day 60. We intended to continue tapering even if the patients developed GVHD during this dose reduction. If GVHD was tolerable the patients received

Table 1. Clinical features of acute and chronic GVHD.

Case	Tapering period (days)	Acute GVHD (maximum stages and grade)	Onset of acute GVHD (Day after transplant)	Treatment of acute GVHD	Interval between the onset of GVHD and initiation of its treatment (days)	Duration of corticosteroid use*1(days)	Outcome of acute GVHD	Chronic GVHD	Type of chronic GVHD	Onset of chronic GVHD (day after transplant)	Treatment of chronic GVHD	Outcome of chronic GVHD
Preparative regimens without ATG												
1	25-35*2	skin 3 liver 0 gut 0 (II)	35	mPSL 1 mg/kg	2	9	CR					
		skin 2 liver 0 gut 1 (II)	54	mPSL 1 mg/kg	1	9	CR	NE				
2	29-55*2	skin 3 liver 0 gut 0 (II)	47	cyclosporine			CR	yes	de novo extensive	113	mPSL 1 mg/kg	NC
3	22-36	skin 1 liver 2 gut 1 (II)	57	mPSL 2 mg/kg, ATG, MMF	0	51	MR	no				
4	22-25	no GVHD						NE				
10	30-41	skin 3 liver 0 gut 2 (III)	41	mPSL 0.5 mg/kg	0	7	CR	yes	quiescent extensive	121	none	PD
12	19-25*2	skin 0 liver 0 gut 3 (III)	23	mPSL 2 mg/kg	3	39	PR	yes	quiescent extensive	327	mPSL 1 mg/kg	PR
		skin 0 liver 0 gut 3 (III)	82	mPSL 2 mg/kg	0	42	CR					
13	30-45	skin 0 liver 3 gut 0 (III)	71	PSL 1 mg/kg	15	14	CR	yes	quiescent extensive	148	PSL 1 mg/kg	PR
15	30-45	skin 1 liver 0 gut 2 (III)	63	PSL 1 mg/kg	9	15	PR	yes	progressive extensive	NE	PSL 1 mg/kg	PR
17	30-45	skin 2 liver 2 gut 3 (III)	58	PSL 1 mg/kg	15	8	PR	no				
Preparative regimens with ATG												
5	20-44	skin 2 liver 0 gut 3 (III)	21	none			CR	yes	progressive limited	NE	none	CR
6	43-45	skin 3 liver 0 gut 3 (III)	54	cyclosporine			CR	yes	quiescent extensive*3	150	none	CR
7	30-45	no GVHD						yes	*de novo* extensive*4	129	none	CR
8	32-46	no GVHD						no				
9	27-57	no GVHD						no				
11	31-44	no GVHD						no				
14	30-45	no GVHD						no				
16	30-45	no GVHD						yes	*de novo* extensive	117	none	NC

*mPSL: methylprednisolone; PSL: prednisolone; MMF: mycophenolate mofetil, CR, complete response; PR: partial response; NC: no change; MR: mixed response; NE: not evaluable. *1Use of more than 0.5 mg/kg methylprednisolone. (Doses of prednisolone were converted into those of methylprednisolone). *2Tapering of cyclosporine was discontinued because of the development of acute GVHD (Cases 1, 2, and 12). *3Case 6 developed GVHD after administration of interferon-α. *4Case 7 developed liver GVHD and autoimmune hepatitis after administration of interferon-α.*