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Letter to the Editors-in-Chief

Mutation of Factor IX Cys178 is intolerant and may cause severe hemophilia B

Hemophilia B (HB, OMIM: 300746) is a rare X-linked recessive hemorrhagic disorder caused by the deficiency of coagulation Factor IX (FIX), with an estimated prevalence of 5.7 cases per 100,000 births [1]. The *F9* gene, located on Xq27.1, comprises 8 exons and encodes the vitamin K-dependent FIX, a 461 amino acid precursor which is further cleaved and secreted into plasma as a mature, 415-residue activated protein. Upon activation, coagulation factor VIII (FVIII) and FIX form a tenase complex to activate coagulation factor X (FX) and the following coagulation pathway. HB could be classified into three subgroups based on the FIX activity (FIX:C): severe form (FIX:C < 1%), moderate form (FIX:C 1%–5%), and mild form (FIX:C 5%–30%). Patients with HB may experience spontaneous, trauma-induced, or deep-muscle bleeding episodes. Molecular genetic testing promotes the diagnosis of HB and genetic counseling [2]. The treatment of HB is mainly aimed to prevent joint bleeding and damage.

Up to date, 1094 and 1218 unique mutations of the *F9* gene have been reported in EAHAD *F9* variant database (http://www.factorix.org/) and HGMD (http://www.hgmd.cf.ac.uk/ac/index.php) databases respectively. The most common variants were missense variants (74%), followed by nonsense variants, small deletions, splice site, and gross deletions (http://www.hgmd.cf.ac.uk/ac/gene.php?gene=F9).

A Chinese family with a 11-year old boy suffered HB with excessive bleeding was admitted for HB genetic testing and genetic counseling for planning their second child. This boy was clinically diagnosed as severe HB with FIX:C < 1%. Sanger sequencing was performed for the boy with primers covering 8 exons, the promoter region, and adjacent splice site sequences of the *F9* gene (Table 1). Novel hemizygous *F9* c.534T > A (NM_000133.3 as reference transcript) nonsense mutation was identified in this boy, which was inherited from his mother who harbored heterozygous *F9* c.534T > A mutation (Fig. 1A). This novel *F9* c.534T > A nonsense mutation was predicted to generate a premature stop codon (p.Cys178Ter), and was absent from numerous databases including gnomAD (http://gnomad.broadinstitute.org/), ExAC (http://exac.broadinstitute.org/), 1000G (http://browser.1000genomes.org/), dbSNP (https://www.ncbi.nlm.nih.gov/snp/), Chinese Millionome Database (https://db.cngb.org/cmdb/), ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), EAHAD *F9* variant database, LOVD (https://databases.lovd.nl/shared/genes/F9), and HGMD database. Several algorithms (http://varcards.biols.ac.cn/) predicted deleterious effect of this mutation, including LRT, MutationTaster, CADD, DANN, FATHMM_MKL, GERP++, and phastCons. *F9* c.534T > A (p.Cys178Ter) nonsense mutation as determined as 'Likely Pathogenic' according to the ACMG guidelines on InterVar (http://wintervar.wglab.org/) using the following criteria: PVS1 (null variant), and PM2 (absent from control group in several databases) [3]. Therefore, *F9* c.534T > A (p.Cys178Ter) nonsense mutation may be the causal mutation for this Chinese family.

Although *F9* c.534T > A (p.Cys178Ter) nonsense mutation was novel, substitution of Cys178 has been widely documented in both EAHAD *F9* variant database and HGMD database, including p.Cys178Arg, p.Cys178Tyr, p.Cys178Ser, p.Cys178Ser, p.Cys178Phe, and p.Cys178Trp [4–7]. Among these missense mutations, all patients showed severe phenotype except for one moderate patient carrying p.Cys178Arg mutation from Spain with FIX:C of 1.5% [8]. These results indicate essential role of Cys178 in maintaining biological function of FIX. The conservation analysis of FIX Cys178 among different species including Human, Chimpanzee, Orangutan, Gorilla, Gibbon, Macaque, and Marmoset (Fig. 1B), showed that the Cys178 residue was highly conserved among these species, which further support the critical role of the Cys178 residue in the activity of FIX.

The wild-type FIX Cys178 residue is involved in a disulphide bridge, essential for stability of FIX. Only cysteine was able to make these types of bonds, substitutions of this residue may break the 3D-structure of FIX, leading to the loss of this interaction and destabilization of the structure [9]. These mutations may affect the characteristics of this residue, including amino acid type, cyclic, size, hydrophobicity, and charge. The POSITIVE Arg was bigger and less hydrophobic than NEUTRAL Cys. Tyr was bigger and less hydrophobic than Cys. Ser was less hydrophobic than Cys. Phe and Trp were both bigger than Cys. The structure difference was shown in Fig. 1C.

We further analyzed the mutation tolerance at FIX Cys178 using MetaDome (https://stuart.radboudumc.nl/metadome/). MetaDome integrated data from Pfam (https://pfam.xfam.org/), GENCODE (https://www.gencodegenes.org/), gnomAD and ClinVar, and analyzed all

Table 1
Primers used in this study.

Primer name	Sequence
F9 exon 1 forward (427 bp)	CAG AAG CCC ACG AAA TCA GAG G
F9 exon 1 reverse (427 bp)	TAC CAA CCT GCG TGC TGG CT
F9 exon 2 forward (550 bp)	ACA TCA CAG ATT TTG GCT CC
F9 exon 2 R (550 bp)	AGC TAG AGG ATA AAA TGA ATT GC
F9 exon 3 forward (481 bp)	GCC AAA GAG GTA TAA TTC AGG
F9 exon 3 reverse (481 bp)	CAT TCA ATA TGG GTT AGA GGG
F9 exon 4 forward (543 bp)	TTC CAG GTC AGT AGT TTT GCT CT
F9 exon 4 reverse (543 bp)	GTT GCG GTT TTC TCA TCT GC
F9 exon 5 forward (491 bp)	GCT CCA AAA TTT CTC TCC CC
F9 exon 5 reverse (491 bp)	GGA CAC AGA AAG AAT TCA GGT TG
F9 exon 6 forward (507 bp)	GGC CTG CTT CTC AGA AGT GA
F9 exon 6 reverse (507 bp)	GGT AGC TGG TTT TGA GCC TCT
F9 exon 7 forward (412 bp)	CTA ATT CAT CTG CAA AGC TCA C
F9 exon 7 reverse (412 bp)	CGT GGG TTC TGA AAT TAT GAC
F9 exon 8 1 forward (533 bp)	AAT ATG CAT TGG CTC TCA TTA C
F9 exon 8 1 reverse (533 bp)	TCT ACC TCC TTC ATG GAA GC
F9 exon 8 2 forward (552 bp)	TTG CTG ACA AGG AAT ACA CG
F9 exon 8 2 reverse (552 bp)	TTT CTA ATC AAT TTG CTC AGG T

The PCR reaction was performed using 2× Ex Mix (Cat No.: RR902A) from TAKARA (Tokyo, Japan).

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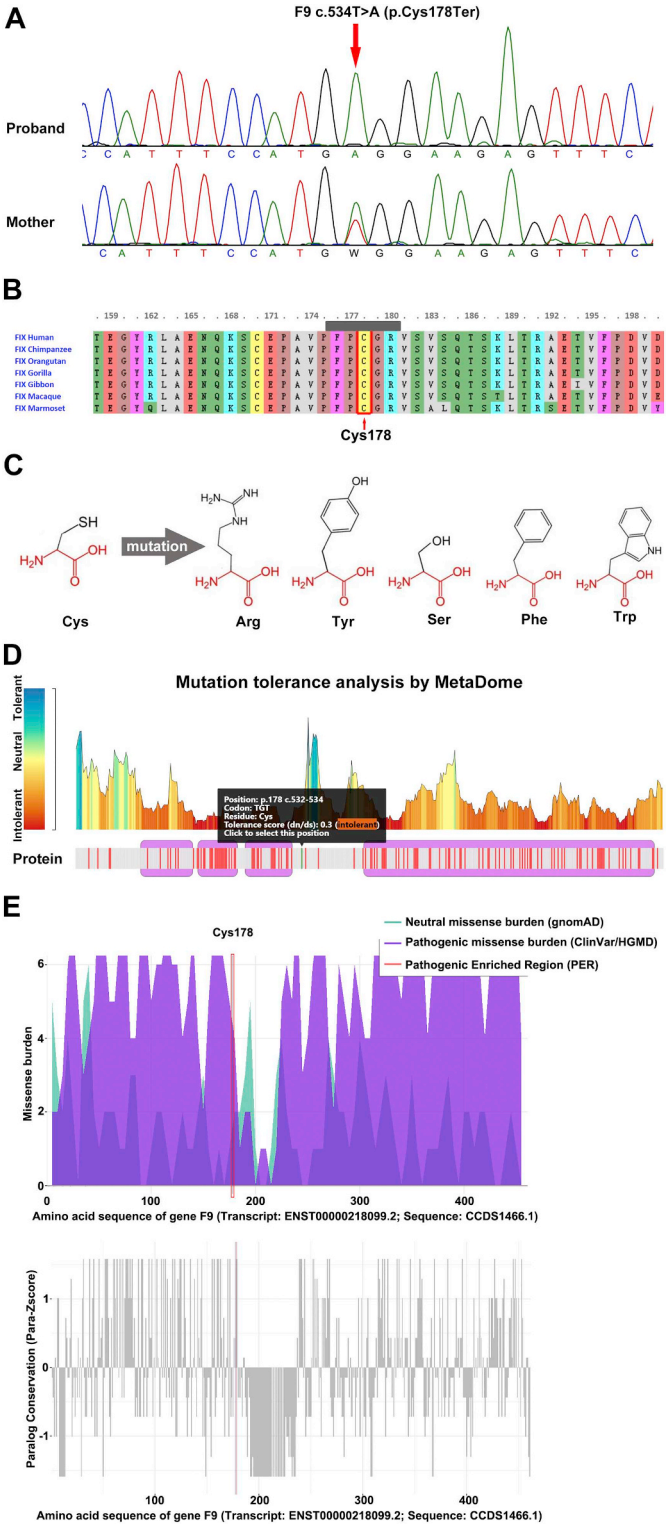

Fig. 1. Mutation of Factor IX Cys178 is intolerant and causes severe hemophilia B. A, *F9* c.534T > A nonsense mutation was identified in the boy and his mother. B, Conservation analysis of FIX Cys178 residue among different species including Human, Chimpanzee, Orangutan, Gorilla, Gibbon, Macaque, and Marmoset. C, The schematic structures of the original Cys residue (left) and the mutant (right) residues. The backbone represents the same for each residue and labeled in colored red. The side chain, which is unique for each amino acid, is labeled in colored black. D, Mutation tolerance analysis of FIX Cys178 with MetaDome. The color is based on missense over synonymous ratio over the whole protein for all proteins. The red (intolerant), yellow (neutral), and blue (tolerant) are used. E, missense burden and paralog conservation analysis of FIX Cys178 with PER viewer (http://per.broadinstitute.org/). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

homologous domains across the whole human genome simultaneously [10]. The definition of a tolerant or intolerant mutation is based on the Residual Variation Intolerance Score (RVIS). An RVIS < 0 means that a gene has fewer common functional mutations that expected; an RVIS > 0 indicates that a given gene has a comparatively high frequency of mutations that affect its function. A tolerance score of 0.3

was achieved for FIX Cys178, suggesting mutation intolerance for this residue (Fig. 1D). Moreover, we explored pathogenic variant enriched regions (PERs) across the *F9* gene and gene families with PER viewer (http://per.broadinstitute.org/). Conserved residues among gene-family members will be enriched and constrained from variants within the general population. The PER viewer showed similar results as MetaDome. The missense burden showed that FIX Cys178 was among the PERs, and was also highly conserved (Fig. 1E).

In conclusion, we have identified a novel *F9* c.534T > A (p.Cys178Ter) nonsense mutation in a Chinese family. Moreover, based on our study, literature review, and bioinformatics analysis, we may conclude that mutation of Factor IX Cys178 is intolerant and may cause severe hemophilia B.

Addendum

Xiong Wang and Linna Gao performed genetic tests. Na Shen performed laboratory test. Yanjun Lu performed genetic counseling. Xiong Wang wrote the manuscript. Aiguo Liu and Qun Hu cared the patient. Yanjun Lu and Qun Hu reviewed the manuscript.

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Compliance with ethical standards

The work was performed with an approval from the Ethics Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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