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A Novel Missense Mutation of *F9* Gene in Hemophilia B Patients

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Abstract

Background: Hemophilia B is an X-linked recessive disorder caused by mutations in the coding sequence of *F9* gene, leading to dysfunctional Factor IX (FIX) protein.

Objectives: This study is to identify novel and recurrent mutations in hemophilia B patients.

Method and Materials: In this study, 9 hemophilia B patients were screened on 8 exons using polymerase chain reaction (PCR) and direct sequencing.

Results: We identified 6 point mutations, including 4 missense mutations and 2 nonsense mutations. One of the six point mutations is a novel mutation (NM_000133.3:c.230T>G) which has not been reported previously in hemophilia B database. Single nucleotide transversion of Thymine to Guanine occurs at nucleotide position 230, leading to amino acids substitution from Valine to Glycine at codon 77 in Gla domain. This amino acid substitution affects the protein structure and function in the Gla domain of FIX protein. Seven prediction tools were shown highly consistent result in predicting this novel mutation.

Conclusion: In this study, all point mutations were found in the coding sequence especially exon 2, exon 5 and exon 8 and distributed among Gla domain, EGF2 domain and SP domains. Novel mutation c.230T>G occurred at exon 2 of *F9* gene which has damaging impact to decrease the stability of protein structure and dysfunction in Gla domain of FIX protein.

Keywords: Hemophilia B; Novel; Mutation; *F9* gene; FIX protein

Introduction

Hemophilia B is also well-known as Christmas disease. It is an X-linked recessive bleeding disorder caused by deficiency of coagulation FIX, resulting reduced antigen level in plasma or dysfunctional FIX protein [1]. FIX protein encoded by *F9* gene comprises of 7 introns and 8 exons. *F9* gene is located on the long arm of chromosome X at band Xq27.1-q27.2. 461-residue pre-pro-protein is translated from 2802bp mRNA and subsequently cleaved to the 415-residue mature protein when undergoes a serial of posttranslational modifications [2, 3]. FIX protein compose of c-carboxyglutamic acid-rich (Gla) domain, 2 epidermal growth factor-like domains (EGF1 and EGF2) and serine protease (SP) domain. The prevalence of hemophilia B is around 1 in 30,000 live births. Hemophilia B is less common than hemophilia A (1 in 25,000 males versus 1 in 5,000 males worldwide) [4].

Recently, more than 3000 pathogenic mutations and neutral polymorphisms have been identified in *F9* gene [5, 6]. Missense mutation is the most common mutation in hemophilia B in which about more than 58.4% and 15.4% mutations are frame shifts caused by duplication, deletion or insertion. Nonsense mutation and splice site mutation is 8.3% and 9.4%, respectively [7]. Approximately half of all mutations occurred in exon 8, which is the largest exon in *F9* gene. About 2% mutations are found in promoter region [8]. In this preliminary report, we analyzed 9 hemophilia B patients from 6 families using PCR and direct sequencing to screen 8 exons of *F9* gene. The aim of this study is to identify novel and recurrent mutations in hemophilia B patients.

Methodology

DNA sample

A total of 6 families were studied from 2014 to 2015. Twenty three DNA samples obtained from National Blood Centre, consist of 9 patients and 14 family members. Informed consents were obtained from the subjects. The severity of the disease was based on the coagulation factor levels. Five patients were severe (<1%), 2 patients were moderate (1%-5%) and 2 patients were mild (>5%). The concentration and the quality of the DNA were determined using a ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Polymerase chain reaction (PCR) and direct sequencing

Eight sets of primers were synthesized to amplify all exons of *F9* gene. The sequences of the primers were shown in Table 1, which were published previously [9-11] except EX6F/R and EX7F/R. Both sets of primers (EX6F/R and EX7F/R) were designed to amplify exon 6 and exon 7, respectively. All primers were complimentary with the flanking intronic regions to cover the whole coding region of *F9* gene. Briefly, 2µl genomic DNA (20ng) was added into the total volume of 23 µl PCR mixture (New England Biolabs, MA, USA) containing 5µl Q5 Reaction Buffer (5X), 0.5µl dNTPs (10mM), 1.25µl Forward Primer (10µM), 1.25 µl Reverse Primer (10 µM), 0.25 µl Q5 Hot Start High-Fidelity DNA Polymerase and 14.75 µl Nuclease-Free Water. PCR amplification was performed using Veriti® Thermal Cycler ABI (Applied Biosystems Inc, CA, USA) for 35 cycles of 10 seconds at 98°C, 30 seconds at 62°C, and 60 seconds at 72°C. Subsequently, the PCR products were separated by Bioanalyzer (Agilent, CA, USA). The PCR products were sent for sequencing service with 8 sets of primers previously used for generation of the PCR fragments.

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Primer	Primer sequence	Exon	Size of PCR product
EX1F	5'AGACTCAAATCAGCCACAGT3'	1	361
EX1R	5'TTTCTATATCTAAAAGGCAAGCATACTC3'		
EX2-3F	5'AAAACAAAGACTTTCTTAAGAGAT3'	2 & 3	507
EX2-3R	5'CATATGTTTCATATATTAGCTAGAG3'		
EX4F	5'CATCCCAATGAGTATCTACAGG3'	4	244
EX4R	5'TTCAGAGGGAAACTTTGAACCATGAG3'		
EX5F	5'TCTGTCGACCCCAATGTATATTTGACCCATAC3'	5	298
EX5R	5'TTTGTCGACGCTGAAGTTTCAGATACAGA3'		
EX6F	5'TGTAATACATGTTCCATTTGCC3'	6	506
EX6R	5'CCTGCTATGCTGTTTCCTTAAT3'		
EX7F	5'TTCTGCCAGCACCTAGAA3'	7	374
EX7R	5'CGACGTGGGTTCTGAAATTA3'		
EX8AF	5'TTTGTCGACGCCAATTAGGTCAGTGGTCC3'	8	387
EX8AR	5'GGAACTCTAAGGTACTGAAG3'		
EX8BF	5'GGATCTGGCTATGTAAGTGGCTGG3'	8	418
EX8BR	5'TGGGAAAGTGATTAGTTAGTGAGAGGCCCTG3'		

Table 1: Summary of primer sequences was used for mutation screening.

Mutation analysis

The DNA sequences were aligned with reference sequence of *F9* gene NG_007994 and NM_000133.3 obtained from National Center for Biotechnology Information (NCBI) database. CLC Main Workbench Version 7.0.2 and BioEdit Version 7.2.4 softwares were used to identify mutation occurring in the *F9* gene. The mutations were named based on Human Genome Variation Society (HGVS) nomenclature standards and referred to University of College London (UCL) Factor IX Mutation Database, Hemobase and Disease Control and Prevention (CDC) Hemophilia B Mutation Project (CHBMP) F9 mutation list to confirm either the existing mutation or novel mutation.

Prediction of novel mutation

Prediction of novel mutation was performed using online prediction tools, including MUpro [12], PhD-SNP [13], SNPs&GO [14], SIFT [15], PolyPhen-2 [16], SNAP [17] and PROVEAN Protein Batch Human [18]. PhD-SNP and SNPs&GO were used to predict amino acid substitution may be related to diseases from protein sequence changes. MUpro tool was used to predict the effect of amino acid substitution on the stability of protein structure. For the structural and functional site of protein, we used PROVEAN, PolyPhen-2 and SNAP tools to predict the impact of a novel mutation on the function of protein in FIX protein and SIFT was used to predict the pathogenicity of novel mutation. The reference FASTA sequence of FIX protein (NP_000124.1) was obtained from the NCBI database and submitted with the amino acid change to the online prediction tools for the analysis.

Result

A total of 9 hemophilia B patients were screened for all exons (exon 1- exon 8) of *F9* gene by PCR amplification and direct sequencing. Summary of mutation in 9 hemophilia B patients from 6 families was shown in Table 2. Six point mutations were identified in this study including 4 missense mutations at nucleotide position 173, 230, 415, 1136 and 2 nonsense mutations at nucleotide position 880 and 1135. No deletion, duplication or insertion was found. One of the six point mutations is a novel mutation (NM_000133.3:c.230T>G) which has not been reported previously in hemophilia B database (UCL Factor IX Mutation Database, Hemobase and CHBMP *F9* Mutation List). Nonsense mutation c.880C>T is frequently reported in hemophilia B database and maybe a hot spot for hemophilia B disease. In this study, all point mutations were found in the coding sequence especially exon 2, exon 5 and exon 8 and distributed among Gla domain, EGF2 domain and SP domains. However, no mutations were distributed in EGF1 domain.

Online prediction tools were classified novel mutation c.230T>G as "disease-related polymorphism", "disease associated variation", "decrease the stability of protein structure", "deleterious", "probably damaging", "effect", "damaging" by PhD-SNP, SNPs&GO, MUpro, PROVEAN Protein Batch Human, PolyPhen-2, SNAP and SIFT, respectively.

Discussion

Missense mutation (NM_000133.3:c.230T>G) is a novel mutation at nucleotide position 230, which has not been reported previously in hemophilia B database. This homozygous missense mutation was found in patient 18/15 and 20/15. Chromatogram of patient 18/15 was shown in Figure 1. Their mother 17/15 is a carrier with heterozygous missense mutation. Single nucleotide transversion of Thymine to Guanine in DNA sequence, leading to amino acids substitution from Valine to Glycine at codon 77 in the Gla domain. Seven online prediction tools were shown high accuracy level of prediction and the results are highly consistent in predicting of this novel mutation (Table 3). This novel mutation has damaging impact to decrease the stability of protein structure and dysfunction in the Gla domain of FIX protein.

Missense mutation c.415G>A was found in 3 siblings (patient 5/15, 10/15, 15/15) with severe hemophilia B in this study. This mutation has been reported previously in UCL Factor IX Mutation Database, in which Guanine was substituted to Adenine at nucleotide position 415. This substitution of nucleotide caused amino acid change from Glycine to Serine at codon 139 in EGF2 domain. In FIX protein, EGF domain is an active site of FIX protein, which can interact with its co-factor, substrate and interplay with other protein [19]. Deficiency of this EGF2 domain may decrease the ability of protein function.

Patient 10/14 and 11/15 with severe hemophilia B have nonsense mutation c.880C>T and c.1135C>T, respectively. These homozygous nonsense mutations have been reported in hemophilia B database previously. The substitutions of single nucleotide C>T at nucleotide position 880 and 1135, may lead to change codon of Arginine (CGA) to stop codon (TGA) at codon 294 and 379. The premature translation stop and truncated protein may generate an abnormal FIX protein with 293 amino acids and 378 amino acids, respectively when compared

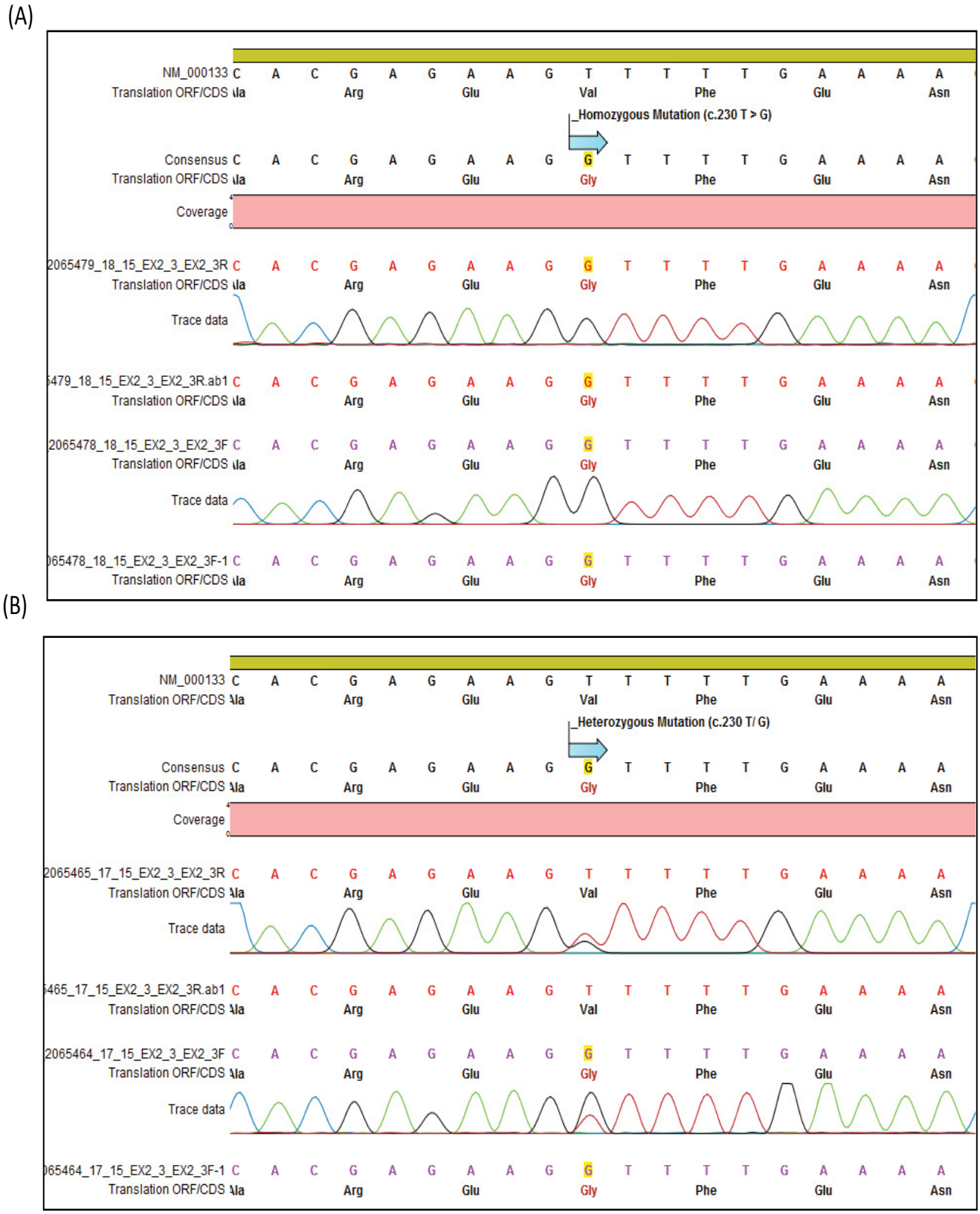

Figure 1: Chromatograms show (A) a novel homozygous missense mutation (c.230T>G) in patient 18/15 and (B) heterozygous missense mutation (c.230T/G) in carrier 17/15.

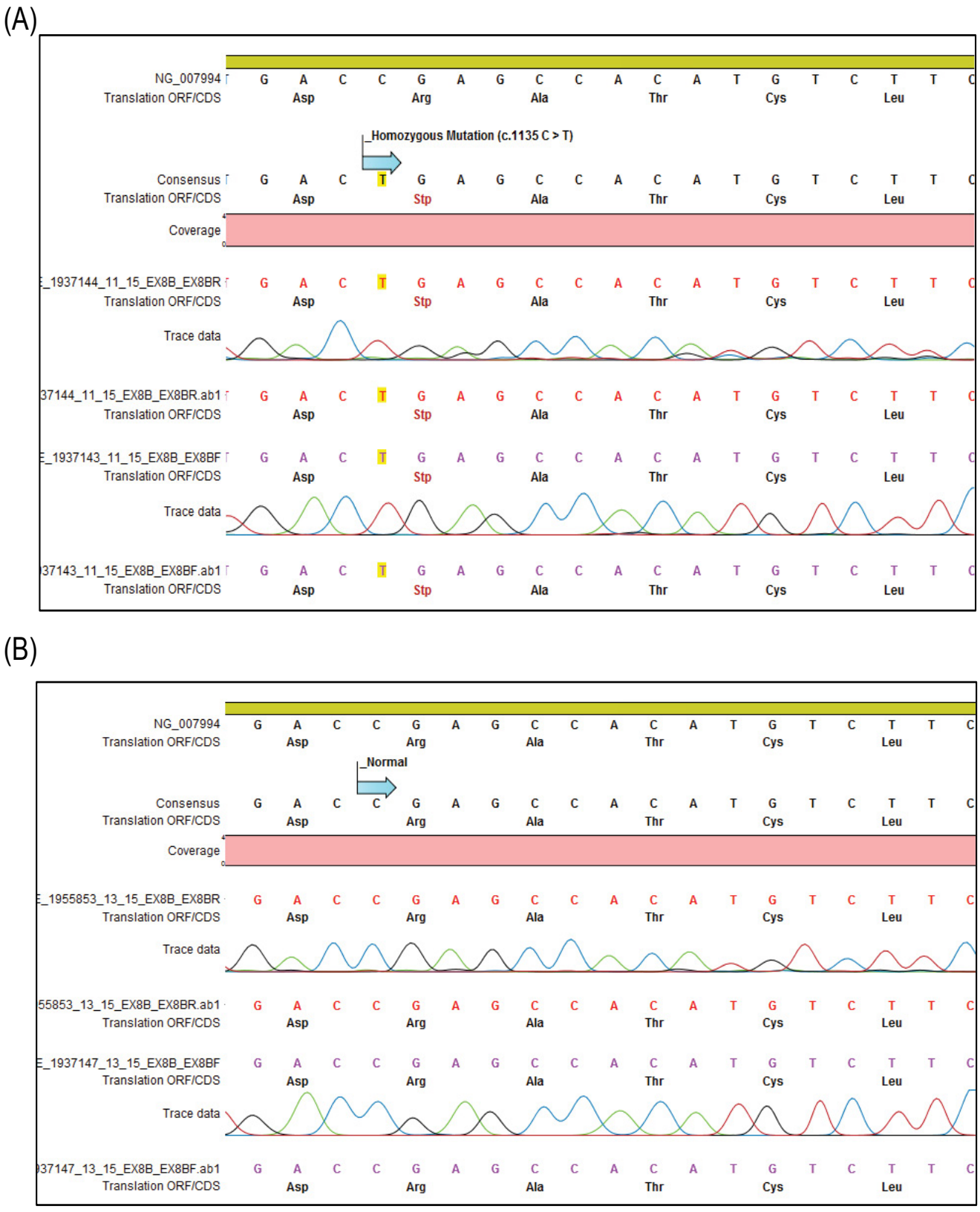

Figure 2: Chromatograms show (A) a somatic homozygous nonsense mutation (c.1135C>T) was found in patient 11/15 and (B) his mother 13/15 was shown normal chromatogram without nonsense mutation at nucleotide position 1135.

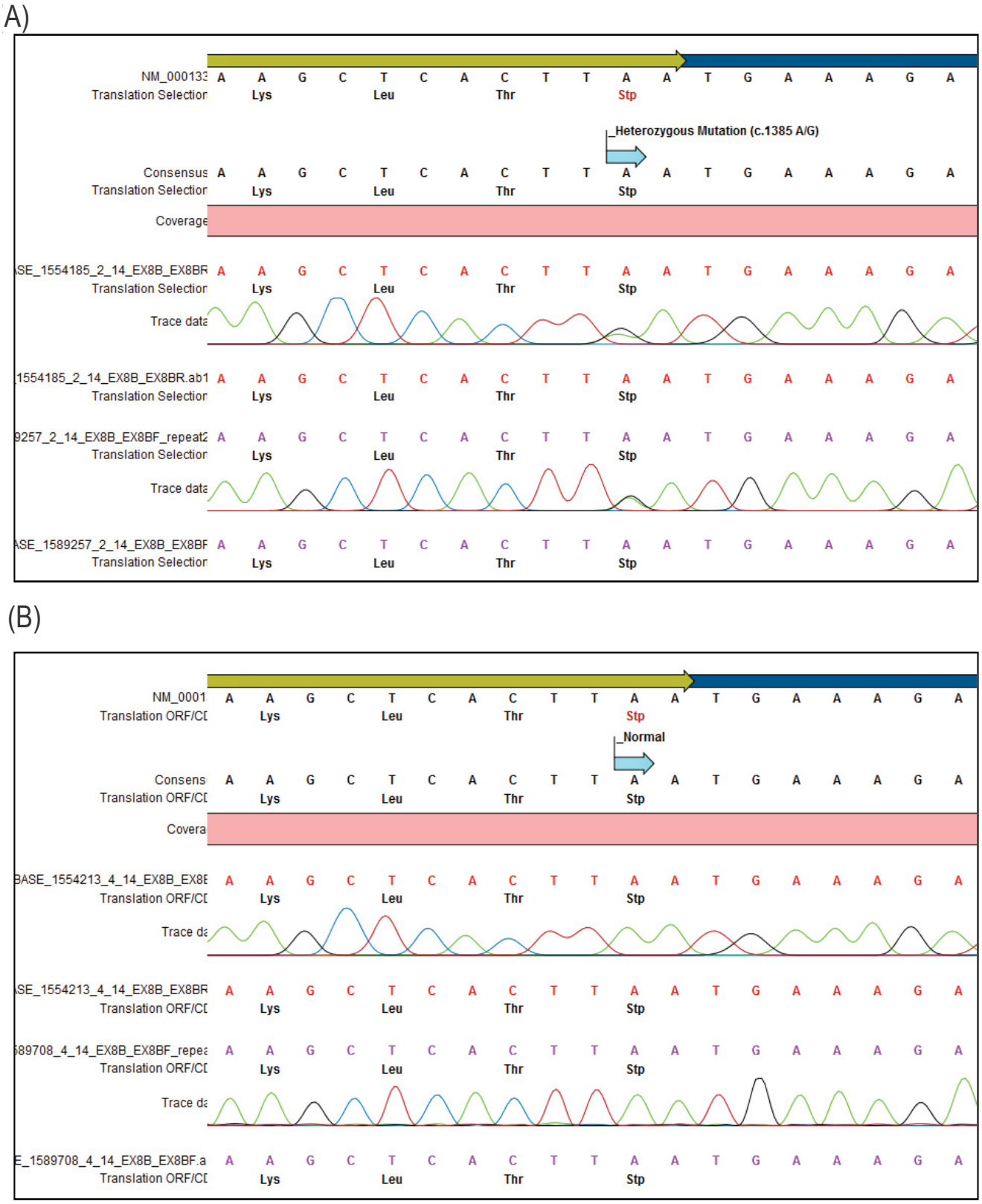

Figure 3: Chromatograms show (A) a heterozygous silent mutation (c.1385 A/G) was found in carrier 2/14 and (B) patient 4/14 was shown normal chromatogram without silent mutation at nucleotide position 1385.

Family	Relationship	Sample	Age	Mutation	Exon	Type	Amino Acid Change	Effect	Domain	Severity	FIX Level (%)	Novel Mutation
1	Mother	2/14	36	c.1136G/A	8	Heterozygous	-	-	-	-	-	-
				c. 1385A/G	8	Heterozygous	-	-	-	-	-	-
	Sister (Twin1)	3/14	7	c.1136G/A	8	Heterozygous	-	-	-	-	-	-
	Sister (Twin 2)	1/14	7	c.1136G/A	8	Heterozygous	-	-	-	-	-	-
	Patient	4/14	12	c.1136G>A	8	Homozygous	p.Arg379Gln	missense	SP	Moderate	1.6	No
2	Mother	8/14	29	c.173G/A	2	Heterozygous	-	-	-	-	-	-
	Sister	5/14	6	c.173G/A	2	Heterozygous	-	-	-	-	-	-
	Patient	6/14	4	c.173G>A	2	Homozygous	p.Gly58Glu	missense	Gla	Moderate	1.6	No
3	Mother	11/14	33	c.880C/T	8	Heterozygous	-	-	-	-	-	-
	Sister	9/14	11	c.880C/T	8	Heterozygous	-	-	-	-	-	-
	Patient	10/14	14	c.880C>T	8	Homozygous	p.Arg294*	nonsense	SP	Severe	<1.0	No
4	Mother	6/15	51	c.415G/A	5	Heterozygous	-	-	-	-	-	-
	Patient	5/15	10	c.415G>A	5	Homozygous	p.Gly139Ser	missense	EGF2	Severe	<1.0	No
	Patient (Twin 1)	10/15	27	c.415G>A	5	Homozygous	p.Gly139Ser	missense	EGF2	Severe	<1.0	No
	Patient (Twin 2)	15/15	27	c.415G>A	5	Homozygous	p.Gly139Ser	missense	EGF2	Severe	<1.0	No
5	Mother	13/15	43	Wild Type	-	-	-	-	-	-	-	-
	Brother	12/15	17	Wild Type	-	-	-	-	-	-	-	-
	Sister	14/15	22	Wild Type	-	-	-	-	-	-	-	-
	Patient	11/15	26	c.1135C>T	8	Homozygous	p.Arg379*	nonsense	SP	Severe	1	No
6	Mother	17/15	54	c.230T/G	2	Heterozygous	-	-	-	-	-	-
	Brother	16/15	32	Wild Type	-	-	-	-	-	-	-	-
	Brother	19/15	29	Wild Type	-	-	-	-	-	-	-	-
	Patient	18/15	33	c.230T>G	2	Homozygous	p.Val77Gly	missense	Gla	Mild	19	Yes
	Patient	20/15	26	c.230T>G	2	Homozygous	p.Val77Gly	missense	Gla	Mild	38	Yes

*SP=serine protease domain, Gla= c-carboxyglutamic acid-rich domain, EGF2=epidermal growth factor-like 2 domain
**Arg=Arginine, Gln=Glutamine, Gly=Glycine, Glu=Glutamic acid, Ser=Serine, Val=Valine

Table 2: Summary of mutation in 9 hemophilia B patients from 6 families

Program	Prediction result	Score/Reliability index	
PhD-SNP	Disease-related polymorphism	6	Reliability Index
SNPs&GO	Disease associated variation	3	Reliability Index
MUpro	Decrease the stability of protein structure	-1	Score
PROVEAN Protein Batch Human	Deleterious	-5.24	Score
PolyPhen-2	Probably damaging	1	Score
SNAP2	Effect	64	Score
SIFT	Damaging	0	Score

Table 3: The result of online prediction tools.

with normal protein with 461 amino acids. The incomplete protein sequence may decrease or destroy the structure and function of FIX protein.

Somatic mutation very rarely occurred in male when compared with germ-line mutation. Patient 11/15 was diagnosed hemophilia B disease with somatic nonsense mutation c.1135C>T at nucleotide position 1135 was shown in Figure 2. However, his mother 13/15, sister 14/15 and brother 12/15 were shown normal chromatograms without any homozygous or heterozygous mutation at nucleotide position 1135. The main factor causing somatic mutation in male is poorly understood.

Missense mutation c.1136G>A was found in Patient 4/14 with moderate hemophilia B and this missense mutation has been reported previously in hemophilia B database. The substitution of single nucleotide from Guanine to Adenine at nucleotide position 1136, leading to amino acids substitution from Arginine to Glutamine at codon 379 in SP domain. His mother 2/14 is a carrier with heterozygous missense mutation c.1136G/A. Additional heterozygous silent mutation c.1385A/G was also found at nucleotide position 1385 in one of her alleles. Chromatogram of silent mutation c.1385A/G of carrier 2/14 was shown in Figure 3. However, this silent mutation was not inherited by her son 4/14. Although substitution of nucleotide occurred in mutant allele from Adenine to Guanine, there is no significant change in stop codon (TAA → TGA).

Conclusion

Missense mutation is the most common mutation found in hemophilia B. Some mutations may or may not lead to effect the the structure and function of FIX protein based on the position of mutation in *F9* gene. Novel mutation c.230T>G is a missense mutation occurred at exon 2 of *F9* gene which has damaging impact to decrease the stability of the protein structure and function in Gla domain of FIX protein.

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Disclosures

The authors stated that they had no interests which might be perceived as posing a conflict or bias.

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