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Original Article

Mutational Profiles of *F8* and *F9* in a Cohort of Haemophilia A and Haemophilia B Patients in the Multi-ethnic Malaysian Population

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Abstract. ***Background.*** **Haemophilia A (HA) and Haemophilia B (HB) are X-linked blood disorders that are caused by various mutations in the factor VIII (F8) and factor IX (F9) genes respectively. Identification of mutations is essential as some of the mutations are associated with the development of inhibitors. This study is the first comprehensive study of the *F8* mutational profile in Malaysia.**
Materials and methods. **We analysed 100 unrelated HA and 15 unrelated HB patients for genetic alterations in the *F8* and *F9* genes by using the long-range PCR, DNA sequencing, and the multiplex-ligation-dependent probe amplification assays. The prediction software was used to confirm the effects of these mutations on factor VIII and IX proteins.**
Results. **44 (53%) of the severe HA patients were positive for *F8* intron 22 inversion, and three (3.6%) were positive for intron one inversion. There were 22 novel mutations in *F8*, including missense (8), frameshift (9), splice site (3), large deletion (1) and nonsense (1) mutations. In HB patients, four novel mutations were identified including the splice site (1), small deletion (1), large deletion (1) and missense (1) mutation.**
Discussion. **The mutational spectrum of *F8* in Malaysian patients is heterogeneous, with a slightly higher frequency of intron 22 inversion in these severe HA patients when compared to other Asian populations. Identification of these mutational profiles in *F8* and *F9* genes among Malaysian patients will provide a useful reference for the early detection and diagnosis of HA and HB in the Malaysian population.**

Keywords: Factor VIII, Factor IX, Genetic mutation, Haemophilia A, Haemophilia B.

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Introduction. Haemophilia is an inherited X-linked blood disorder which causes prolonged bleeding time after injuries or trauma.[1] Haemophilia A (HA) and Haemophilia B (HB) are due to the deficiency of coagulation factor VIII (gene, *F8*) and factor IX (gene, *F9*) respectively.[2] Upon activation, factor VIII and factor IX form an active complex (tenase complex) which activates

factor X and the following factors in the coagulation pathway.[1] Thus, a deficiency or dysfunction of any of these factors can impair clot formation and consequently causes bleeding diathesis.

In HA cases, the most recurrent genetic mutations are the inversion of intron 22 (IVS22), which accounts for about 45% of severe patients,[3] and intron 1 (IVS1) which accounts for 2-5% of severe patients.[4] As for HB patients, the most common mutations identified in the *F9* gene are the missense mutations (74%).[5] Apart from these mutations, there is a wide range of different genetic alterations spread throughout *F8* and *F9* genes, including single nucleotide substitutions, small and large deletions.[5-7] Until now, about 1968 unique variants of *F8* are listed in the factor VIII database,[6,7] and 1094 unique variants of *F9* in the factor IX database.[5]

The current standard of treatment of HA and HB is primary prophylaxis, with regular infusion of factor VIII or factor IX respectively to prevent joint bleeding and damage.[8] However, the development of inhibitors in these patients is a severe complication of this infusion therapy.[9] Such inhibitory response happens in 25-30% of HA patients[10,11] and 1-4% of HB patients,[12,13] and these incidences may be higher depending on the ethnicity. [14,15] A systematic review showed that factor VIII inhibitory response was strongly associated with large deletions and nonsense mutations compared to IVS22 mutation,[16] therefore suggesting a strong genetic predisposition, hence the importance of identifying such mutations before commencing the infusion treatment. In Malaysia, despite the prevalence of HA and HB are around 5.9/100000 males and 1.0/100000 males respectively,[17] there have been only small studies on the mutational status of *F8* (only in exon 14)[18] and in *F9*[19,20] genes. Therefore, this study aimed to investigate comprehensively the mutational spectrums of *F8* and *F9* genes in a representative cohort of Malaysian patients corresponding to their disease severity as well as the inhibitory response.

Materials and Methods. *Sample collection.* This study was approved by the Universiti Kebangsaan Malaysia Ethics Committee and the ethics committee of the Ministry of Health of Malaysia. Written informed consent taken from all patients with confirmed non-familial HA (n=100) and HB (n=15) who were being followed-up at the National Blood Centre, Kuala Lumpur. Detailed clinical history along with pedigree data were taken, and the disease severity classification was as the following: 1) mild HA (FVIII/FIX:C:>5-40%), 2) moderate HA (FVIII/FIX:C:1-5%), and 3) severe HA (FVIII/FIX:C:<1%).[21] Venous blood (10mL) was collected in EDTA Vacutainer collection tubes (BD, New Jersey, USA) and proceeded to DNA extraction using the salting-out extraction method.[22] DNA quality and concentration were determined by using NanoDrop Spectrophotometry and gel electrophoresis according to the manufacturer's instruction.

Detection of intron 22 inversion (IVS22) in F8. Detection of IVS22 in *F8* performed by using the Long-Range PCR kit (QIAGEN, Hilden, USA) according to previously published methods[23,24] with slight modifications. Primers P, Q, A & B (Life Technologies, Wien, Austria) were utilised to amplify the region of interests.[23,24] Briefly, a total of 25 µl PCR reaction which contained 20ng genomic DNA, LD-PCR reaction master-mix (Qiagen LD-PCR kit, Hilden, USA), 7.5% DMSO, 10 mM of 7-deaza-dGTP and 10 pmol of primers P, Q, A & B in each single-tube PCR reaction. Conditions for the PCR reaction: initial denaturation at 95 °C for 2 min and 15 s; followed by 30 cycles of denaturation at 95 °C for 12 s, annealing at 65 °C for 30 s and elongation at 68 °C for 12 min for the first ten cycles. The remaining 20 cycles had 20 s addition to each cycle step. Confirmation of the PCR products was visualised using agarose gel electrophoresis.

Detection of intron 1 inversion (IVS1) in F8. Detection of IVS1 in *F8* done by using the PCR Core kit (Roche Diagnostics, Indiana, USA) according to the previously published methods and primers.[4] The PCR products were visualised using agarose gel electrophoresis.

Detections of other mutations in F8 and F9. F8 coding regions (26 exons) including the intron/exon boundaries and the promoter regions were amplified using 26 sets of previously published primers and methods.[25] The entire *F9* coding region (8 exons) including the intron/exon boundaries, the promoter region, and the polyadenylation site was amplified using eight sets

of previously published primers and methods.[26,27] The confirmation of the PCR products was performed using gel electrophoresis and sequenced using the BigDye Terminator v3.1 sequencing kit (Applied Biosystems, California, USA) on an ABI 3130xl Genetic Analyzer (Applied Biosystems, California, USA) according to the manufacturer's instructions.

Detection of large deletions in F8 and F9. Samples that did not show any exon amplification (but flanking exon amplification) or did not show any mutations were suspected of having large deletions. Detection of large deleted regions in *F8* and *F9* was performed using the multiplex-ligation-independent probe amplification (MLPA) kits, namely the SALSA MLPA P178 F8 and SALSA MLPA P207-C1 F9 probe mix kits (MRC-Holland, Amsterdam, Netherlands), according to the manufacturer's instructions. Amplified PCR products were separated by ABI 3130xl Genetic Analyzer (Applied Biosystems, California, USA) with LIZ-500 (Applied Biosystems, California, USA) as the size standard. The data was analysed using Coffalyser.Net (MRC-Holland, Amsterdam, Netherlands) according to the provided guidelines. Probe ratios <0.7 were considered as deletions and probe ratios >1.3 as duplications. Negative controls were the donor DNA samples from healthy males.

Molecular genetic analysis and nomenclature. F8 and *F9* nucleotide numbering (c.) is designated according to coding bases from A (nucleotide+1) from the initiation codon for methionine (ATG) at position -171 (*F8*:ref. NM_000132.3) and (ATG) at position -29 (*F9*:ref. NM_000133.3) respectively. While the protein numbering (p.) follows the amino acid sequences that assign the first residue methionine as +1 in each factor VIII and IX sequences (FVIII: NP_000123.1 and FIX: NP_000124.1 respectively) according to the Human Genome Variation Society guidelines. [28] Sequence variants were aligned with the corresponding wild-type sequences using BLAST (NCBI) and compared to the HA and HB mutation databases (Factor 8,[6,7] Factor 9,[5] Human Gene Mutation Database and CDC Haemophilia A Mutation Project database[29]). Novel variants further analysed for their effects on the factor VIII and IX protein by using multiple software, including Sorting Intolerant From Tolerant (SIFT) and Polymorphism Phenotyping (PolyPhen2),[30,31] PROVEAN (Protein Variation Effect Analyzer)[32] and Mutation Taster2.[33] A SIFT score ranges from deleterious (< or equal to 0.05) to tolerated SNP (> 0.05). For PolyPhen2, the score ranges from 0.0 (tolerated) to 1.0 (deleterious). The PROVEAN score of an equal to or less than a predefined threshold of -2.5 value indicates for a "deleterious" effect. The visualisation of affected amino acid was performed on a crystal structure of the protein from the Protein Data Bank database for factor VIII protein (PDB-ID:2R7E)[34] and IX protein (PDB-ID:2WPI)[35] using Pymol, version 1.8.6.1 that is freely available online.

Results. *Demographic and clinical data.* Among the 100 HA patients in the study, 83 were severe (FVIII:C:<1%), nine were moderate (FVIII:C:1-5%), and eight were mild (FVIII:C:>5-40%) whereas, for the 15 HB patients, nine were severe (FIX:C:<1%), and six were moderate (FIX:C:1-5%) (**Table 1**). Fourteen of the severe HA patients developed inhibitors against factor VIII, while none of the HB patients had factor IX inhibitor. Majority of the patients were Malays (HA:54%, HB:73%), and followed by Chinese (HA:37%, HB:13%), Indians (HA:8%, HB:13%) and other (HA:1%) (**Table 1**).

F8 mutations. Out of 83 severe HA patients, 44 (53%) of them were positive for intron 22 inversion (IVS22), and three (3.6%) were positive

Table 1. Demographic data on Haemophilia A (HA) and Haemophilia B (HB) non-familial patients in a representative cohort of the Malaysian population.
Descriptive summary of the HA and HB non-familial patients included in the study. Data were expressed as patient count (n) and frequency from total patients (%).

Description	HA, n (%)	HB, n (%)
Total Patients	100	15
Ethnicity		
Malay	54 (54)	11 (73)
Chinese	37 (37)	2 (13)
Indian	8 (8)	2 (13)
Others	1 (1)	0 (0)
Disease Severity		
Severe (C :< 1%)	83 (83)	9 (60)
Moderate (C: 1-5%)	9 (9)	6 (40)
Mild (C :> 5-40%)	8 (8)	0 (0)
Inhibitory Response		
Yes	14 (14)	0 (0)
No	86 (86)	15 (100)

Table 2. The *F8* mutational spectrum in Malaysian Haemophilia A (HA) patients without intron 22 and 1 inversions. The summary of the genetic alterations in the *F8* gene in HA patients that were negative for intron 22 and intron 1 inversions. Nucleotide numbering (c.) is according to coding bases from A (nucleotide +1) the initiation methionine (ATG) at position -171 (*F8* mRNA gene bank ref. NM_000132.3) and protein numbering (p.) follows amino acid sequences that assign the first residue Methionine as +1 in factor VIII protein sequence (NP_000123.1) according to Human Genome Variation Society guidelines.[28]

Patients	Ethnicity	F8 Exon	F8 domain	Nucleotide Changes	Amino Acid Changes	Novelty	Disease Severity	Mutation Effects
HA5/HA36	Malay/Malay	14	B	c.3610InsA	p.N1204Kfs*2	Novel	Severe	Frameshift
HA9	Malay	4	A1	c.553DelAA	p.K185Rfs*14	Novel	Severe	Frameshift
HA11	Malay	Exon7/ Intron7	A1	c.1007A>C c.1008-1009 DelTG/GT	-	Novel	Severe	Donor splice site
HA17	Chinese	14	B	c.49464947 DelGT	p.G1649Efs*3	Novel	Severe	Frameshift
HA18	Malay	9	A2	c.1354G>T	p.D452Y	Novel	Mild	Missense
HA24	Other	14	B	c.3625C>T	p.Q1209*	Novel	Severe	Nonsense
HA25	Chinese	8	A1	c.1016T>G	p.M339R	Novel	Severe	Missense
HA45#	Malay	14	B	c.3175DelA	p.V1060*fs	Novel	Severe	Frameshift
HA52	Malay	14	B	c.4820InsA	p.T1609Nfs*3	Novel	Severe	Frameshift
HA54	Indian	Intron6	-	c.787+1G>T	-	Novel	Severe	Donor splice site
HA57	Indian	14	B	c.2444DelAG	p.P817Yfs*9	Novel	Severe	Frameshift
HA61	Malay	14	B	c.2696DelG	p.S899Ifs*6	Novel	Moderate	Frameshift
HA62	Malay	14	B	c.3762DelT	p.N1254Kfs*2	Novel	Severe	Frameshift
HA67	Malay	26	C2	c.6986C>G	p.P2329R	Novel	Severe	Missense
HA70	Chinese	19	A3	c.6085A>T	p.M2029L	Novel	Mild	Missense
HA76	Malay	14	A2	c.2159G>T	p.G720V	Novel	Severe	Missense
HA77	Malay	17	A3	c.5609T>C	p.L1870P	Novel	Moderate	Missense
HA80	Malay	21	C1	c.6272A>C	p.K2091T	Novel	Mild	Missense
HA86	Chinese	25	C2	c.6857A>T	p.D2286V	Novel	Severe	Missense
HA87	Chinese	23	C1	c.6355DelC	p.Q2119Sfs*24	Novel	Severe	Frameshift
HA91	Malay	Intron22	-	c.6429+2T>A	-	Novel	Moderate	+2 Donor splice site
HA93	Malay	8 – 12	A1-a1-A2	-	-	Novel	Severe	Large deletion

HA1	Malay	4 – 6	A1	-	-	Reported	Severe	Large deletion
HA2	Chinese	11	A2	c.1696C>T	p.L566F	Reported	Moderate	Missense
HA6/HA23/ HA27/HA40	Chinese/Malay/ Malay/Chinese	14	B	c.3629InsA	p.I1213Nfs*28	Reported	Severe	Frameshift
HA8/HA44/ HA50	Malay/Malay/ Malay	Intron18	-	c.5998-1G>A	-	Reported	Severe	-1 Acceptor splice site
HA12	Malay	4	A1	c.524A>C	p.Y175S	Reported	Mild	Missense
HA26	Chinese	14	B	c.4156C>T	p.Q1386*	Reported	Severe	Nonsense
HA32	Chinese	3	A1	c.274 G>C	p.G92R	Reported	Severe	Missense
HA33	Malay	22	C1	c.6317A>C	p.Q2106P	Reported	Severe	Missense
HA38/HA65	Malay/Malay	24	C2	c.6682C>T	p.R2228*	Reported	Severe	Nonsense
HA41	Malay	8 – 9	A1-a1-A2	-	-	Reported	Severe	Large deletion
HA42	Chinese	3	A1	c.274G>A	p.G92S	Reported	Mild	Missense
HA45#	Malay	14	B	c.2383A>G	p.R795G	Reported	Severe	Missense
HA48	Chinese	Intron6	-	c.787+1G>A	-	Reported	Severe	Donor splice site
HA55	Malay	18	A3	c.5941G>A	p.V1981M	Reported	Mild	Missense
HA58	Chinese	8	a1	c.1171C>T	p.R391C	Reported	Moderate	Missense
HA66	Malay	9	A2	c.1443G>A	p.L481L	Reported	Mild	Splice site end of Exon 9
HA68	Indian	Intron16	-	c.5586+2T>G	-	Reported	Severe	Donor splice site
HA71	Chinese	18	A3	c.5879G>A	p.R1960Q	Reported	Mild	Missense
HA73	Malay	12	A2	c.1812G>C	p.W604C	Reported	Severe	Missense
HA78	Malay	14	B	c.3637DelA	p.I1213Ffs*5	Reported	Severe	Frameshift
HA85	Malay	17	A3	c.5689-5690 DelCT	p.L1897Vfs*6	Reported	Severe	Frameshift

HA, Haemophilia A, #, indicates the same sample having double mutations.

for intron 1 inversion (IVS1). Among those 44 IVS22 positive patients, 18 of them had sporadic occurrence while 26 were familial based on the family history. For those remaining HA patients without IVS22/1 mutations, a total of 22 novel mutations were identified (**Table 2**) consisting of missense (8), frameshift (9), splice site (3), large deletion (1) and nonsense (1) mutations. Additionally, 21 of previously reported *F8* mutations were also detected (**Table 2**) consisting large deletions (2), missense (10), nonsense (2), splice site (4) and frameshift (3) mutations. Excluding the IVS22 and IVS1 mutations, 41.7% of these identified mutations mainly occurred at the exon 14 of *F8*. In three severe HA patients, three large deletions were detected. Patient HA93 has a novel deletion which spans from exon 8 to exon 12 corresponding to A1-a1-A2 domains of factor VIII. Whereas, patient HA1 and HA41 have large deletions that span from exon 4 to exon 6 (corresponding to the A1 domain of factor VIII) and span from exon 8 to exon 9 (corresponding to A1-a1-A2 domains of factor VIII) respectively (**Table 2**). Unfortunately, we were unable to detect any mutation in four of the severe HA patients (HA4, HA15, HA60, and HA64).

To further evaluate the impact of the novel missense mutations identified, we performed prediction analysis on the effect of these mutations on the factor VIII protein using multiple prediction software (**Table 3**). Except for one missense mutation in a mild HA patient, all other novel missense mutations were predicted to have damaging effects (**Table 3**). We also visualised the amino acid location of these novel missense mutations in factor VIII (**Figure 1**). For example, the HA67 patient who has a severe disease was detected to have a missense mutation of c.6986C>G that results in the substitution of proline to arginine at position 2329, and this mutation was predicted to be damaging. In the wildtype position, the large cyclic hydrophobic residue of Pro2329 lies within the C2 domain and forms hydrogen bonds with the neighbouring polar residue of glutamine (**Figure 1B**). Substitution of this hydrophobic proline to the positively charged arginine would disrupt this hydrogen bond. Patient HA77 who has a moderate disease was detected to have a missense mutation of c.5609T>C, which was also predicted to be damaging. For this mutation, the wildtype residue of Leu1870 forms a hydrogen bond with the polar Ser1819 residue through its hydroxyl group in the A3 domain (**Figure 1C**). Substitution of Leu1870 to cyclic proline would alter the hydroxyl group interaction therefore consistent with the predicted damaging score. Patient HA70 who has a mild disease also has a non-damaging missense mutation of c.6085A>T. For this mutation, the wildtype residue of Met2029 forms a hydrogen bond with the positively charged histidine residue in the A3 domain near to the core (**Figure 1D**). Substitution of this methionine to leucine that is similar in structure and polarity would minimally disrupt this bond, and thus consistent with the predicted score.

F9 mutations. From the 15 HB patients, there were four novel mutations identified (**Table 4**), namely the splice site mutation (1), small deletion (1) and large deletion (1) and missense (1) mutations. A novel large deletion spans from exon 1 to exon 4, corresponding to signal-propeptide-GLA-EGF1

Table 3. Summary of prediction values for eight novel missense mutations identified in *F8* gene. Prediction of the novel missense mutation effect was performed on factor VIII protein and biological function using multiple software. SIFT score ≤ 0.05 indicates the damaging/deleterious effect and SIFT score above than 0.05 indicates a tolerated effect. A PolyPhen2 score ranges from 0.0 (tolerated) to 1.0 (deleterious), while for PROVEAN score that is equal to or less than a predefined threshold of -2.5, the variant is predicted to have a "deleterious" effect.

Patients	Nucleotide Changes	Amino Acid Changes	Disease Severity	SIFT Score	PolyPhen2 Score	PROVEAN Score	Missense Prediction
HA18	c.1354 G > T	D452Y	Mild	<0.05	1.00	-7.71	Damaging
HA25	c.1016 T > G	M339R	Severe	<0.05	1.00	-5.35	Damaging
HA67	c.6986 C > G	P2329R	Severe	<0.05	1.00	-7.85	Damaging
HA70	c.6085 A > T	M2029L	Mild	0.05	0.106	-2.47	Neutral/Benign
HA76	c.2159 G > T	G720V	Severe	<0.05	1.00	-7.26	Damaging
HA77	c.5609 T > C	L1870P	Moderate	<0.05	1.00	-6.32	Damaging
HA80	c.6272 A > C	K2091T	Mild	0.01	1.00	-2.92	Damaging
HA86	c.6857 A > T	D2286V	Severe	<0.05	0.999	-6.23	Damaging

HA, Haemophilia A.

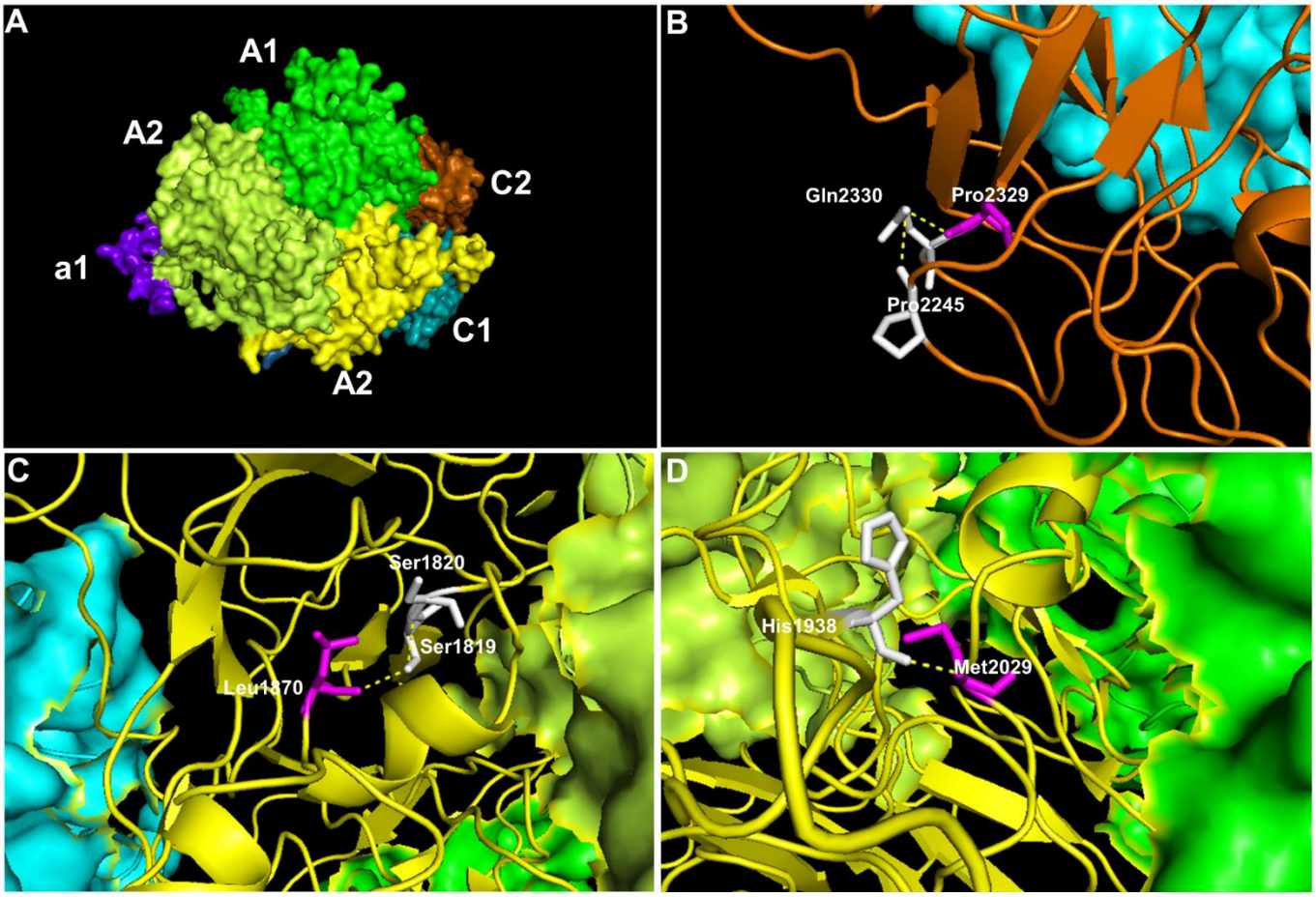

Figure 1. The representative model of factor VIII protein showing the affected amino acids by missense mutations. The visualisation of affected amino acids by missense mutations based on factor VIII protein (PDB:2R7E). A) The localisation of the domains in the factor VIII, B) Position of Pro2329 in the C2 domain C) Leu1870 in the A3 domain and D) Met2029 in the A3 domain. The visualisation of the whole structure of factor VIII as a surface model with colour coding that represents A1 domain (green), a1 domain (purple), A2 domain (lime), A3 domain (yellow), C1 domain (cyan) and C2 domain (orange). Except when the domain is affected, the region is visualised as a ribbon model. In this ribbon model, the affected residue (magenta), the neighbouring amino acids (white), and the hydrogen bonds (yellow dotted lines) are highlighted.

Table 4. The *F9* mutational spectrum in Malaysian Haemophilia B (HB) patients. The summary of the genetic alterations in the *F9* gene from our Malaysian HB patients. Nucleotide numbering (c.) is according to coding bases from A (nucleotide +1) the initiation methionine (ATG) at position -29 (*F9* mRNA gene bank ref. NM_000133.3) and protein numbering (p.) follows amino acid sequences that assign the first residue Methionine as +1 in factor IX protein sequence (NP_000124.1) according to Human Genome Variation Society guidelines.[28]

Patient	Ethnicity	F9 Exon	F9 domain	Nucleotide Changes	Amino Acid Changes	Novelty	Disease Severity	Mutation Effects
HB2	Malay	Intron2	-	c.253-17_253-13DelTCTTT	-	Novel	Severe	Acceptor splice site
HB4	Malay	7	Serine Protease	c.803G>A	p.C268Y	Novel	Severe	Missense
HB12	Indian	1	Signal Peptide	c.39DelC	p.L14Sfs*7	Novel	Moderate	Frameshift
HB13	Malay	1 – 4	Signal-Pro-peptide-GLA-EGF1	-	-	Novel	Severe	Large deletion
HB1	Indian	Intron2	-	c.252+1G>A	-	Reported	Severe	Donor splice site
HB3	Malay	8	Serine Protease	c.1237G>A	p.G413R	Reported	Severe	Missense
HB5	Malay	8	Serine Protease	c.1135C>T	p.R379*	Reported	Moderate	Nonsense
HB7/ HB14	Malay/ Malay	2	Pro-peptide	c.128G>A	p.R43Q	Reported	Moderate/ Severe	Missense
HB8	Malay	Intron1	-	c.88+5G>C	-	Reported	Moderate	Donor splice site
HB9	Malay	7	Serine Protease	c.800A>G	p.H267R	Reported	Severe	Missense
HB10	Malay	5	EGF2	c.415G>A	p.G139S	Reported	Severe	Missense
HB11	Chinese	4	EGF1	c.383G>A	p.C128Y	Reported	Moderate	Missense
HB15	Malay	2	GLA	c.223C>T	p.R75*	Reported	Severe	Nonsense
HB16	Chinese	2	GLA	c.159_160DelAG	p.E54Vfs*7	Reported	Moderate	Frameshift

HB, Haemophilia B.

domains of factor IX, was detected in patient HB13 who has a severe disease. Additionally, there were ten previously reported mutations in the *F9* identified among the patients (**Table 4**) consists of the splice site (2), missense (5), nonsense (2) and frameshift (1) mutations. A novel missense mutation (c.803G>A) in the patient HB4 was predicted to be damaging (SIFT score= <0.05, PolyPhen2 score = 1.00, PROVEAN score= -10.52). This missense mutation (c.803G>A) resulted in the substitution of cysteine to tyrosine residue at position 268. In this serine-peptidase catalytic domain, a wildtype residue of Cys268 forms strong disulphide hydrogen bonds with small hydrophobic Ala266 residue, contributing to the helical structure and folding of the factor IX (**Figure 2B**). Substitution of this Cys268 residue with tyrosine would affect the structure of the protein.

Genotype-Phenotype relationship in HA and HB patients. From those 44 severe HA patients with the IVS22 mutation, five of them developed inhibitors against factor VIII, while two of the three severe HA patients with IVS1 mutation also had factor VIII inhibitors (**Table 5**). Among those remaining patients negative for IVS22/1 mutations, seven patients had inhibitory presence with different types of mutations, namely patient HA38 (nonsense mutation), patient HA73 (missense mutation) and patient HA4 (undetected mutation), patients HA85 and HA87 (small deletions) and patients, HA41 and HA93 (large deletions). In contrast, none of the HB patients exhibited any inhibitory response against factor IX.

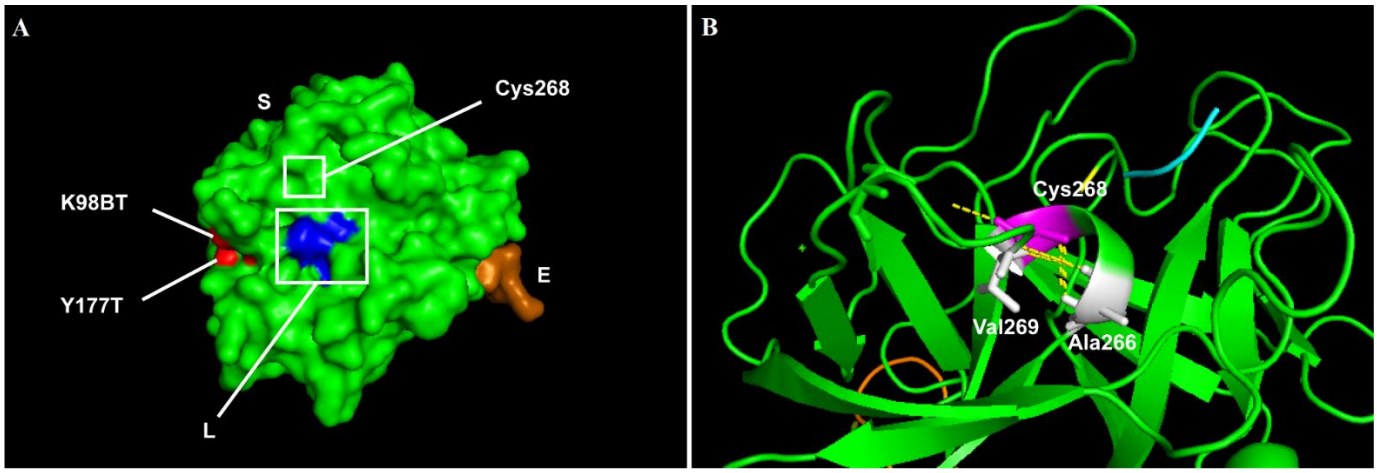

Figure 2. The representative model of factor IX protein showing the affected amino acids by missense mutations. The visualisation of affected amino acids by missense mutations based on factor IX protein (PDB:2WPL) of double-mutant. A) Visualisation of the selected structure of factor IX protein as a surface model with colour-coding that represents S chain, containing Peptidase S1 domain (green), E chain containing EGF2 domain (orange) and L chain domain (blue). Original mutants' residues from the crystal structure (2WPL) are in red. B) The affected domain of Peptidase S1 is visualised as a ribbon model, with the affected residue (Cys268, magenta), the neighbouring amino acids (white), and the hydrogen bonds (yellow dotted lines) are highlighted.

Table 5. Factor VIII inhibitory response distribution across the mutational spectrum of *F8* in a representative cohort of Malaysians. Factor VIII inhibitory response distribution across the mutation types in Haemophilia A patients (n=14) within the *F8* gene. Data were expressed as count (n) and frequency (%). Numbers of mutations are not equal to the number of patients, due to some patients share the same mutations. IVS, Intron Inversion.

Mutation type	No. of mutations n (%)	No. of patients with inhibitors, n (% of total mutations of same type)			
		Malay, n (%)	Chinese, n (%)	Indian, n (%)	Other, n (%)
IVS22	44 (44)	2 (4.54)	2 (4.54)	1 (2.27)	-
IVS1	3 (3)	1 (33.3)	-	1 (33.3)	-
Missense	18 (18)	1 (5.56)	-	-	-
Nonsense	3 (3)	1 (33.3)	-	-	-
Large deletion	3 (3)	2 (66.6)	-	-	-
Small deletion	9 (9)	1 (11.1)	1 (11.1)	-	-
Small Insertion	3 (3)	-	-	-	-
Splice donor site	7 (5)	-	-	-	-
No mutation detected	4 (4)	1 (25.0)	-	-	-

Discussion. This study is the first to report a comprehensive mutational spectrum of *F8* in 100 HA patients from Malaysia, together with the mutational profile of *F9* in 15 HB patients. Among the 83 severe and non-familial HA patients, 53% of them have IVS22 mutation which is slightly higher than other Asian populations.[36-40] We also identified 22 novel mutations in *F8* and four novel mutations in *F9*. Among those with novel mutations, we found one HA patient and one HB patient each with a novel large deletion in the *F8* and *F9* respectively, and both patients have severe disease. Unfortunately, we were unable to detect any mutation in *F8* in four HA patients with severe disease. Similarly, previous studies also reported that in a small number of HA patients, no mutation was detected despite the use of multiple techniques.[38,41,42] This is probably due to the location of the mutations which lie deep within the introns or outside the analysable region of *F8* using the current techniques.[43,44] Given the associations between *F8* and *F9* mutational status and the disease severity as well as the development of inhibitors following the treatment, our findings suggest that detection of the mutational spectrum of *F8* and *F9* can improve the disease management and treatment outcome in HA and HB patients in Malaysia.

Three of the severe HA patients have large deletions, including one novel deletion. Patient HA93 with the novel deletion affecting A1-a1-A2 domains of factor VIII showed a high inhibitor level at the age of 12 years. Likewise, patient HA41 with a large deletion of A1-a1-A2 domains also had a high inhibitor level since the age of 4 years. Large deletions and nonsense mutations identified in factor VIII are shown to associate with higher risk of inhibitory response,[16,36,45-47] particularly at the A2 and C2 domains.[48] This evidence is in agreement with our findings, as our patient HA1 who has a large deletion affecting only the A1 domain did not develop the inhibitory response. Thus, depending on which factor VIII domains that are being affected by the large deletions, a differential outcome in the inhibitory response may be observed. Therefore, further investigations are needed to evaluate the prognostic value of these large deletions in predicting the inhibitory response.

Excluding IVS22 and IVS1 mutations, 41.7% of the identified mutations in the present study mainly occurred in exon 14 that is corresponding to the B domain of factor VIII. B domain has no homology sequence to any other known genes and has been shown to participate in the intracellular processing and trafficking of factor VIII.[49] The role of the B domain in the pro-coagulant activity is minimal, as this domain is cleaved off during the activation.[50,51] Here, we reported that among our eight severe HA patients, seven novel nonsense/frameshift mutations identified were in exon 14. Previous studies have reported that only some of these nonsense/frameshift mutations were causative mutations,[52] despite that they could result in premature termination or frameshift codon. Therefore, further works should be pursued to elucidate the pathogenic impact of these mutations on factor VIII activity and production. Due to the limitation of the budget, we did not perform a functional study to assess the effects of these novel exon 14 mutations. However, four of these identified mutations are near to the 'hotspot' region at codon position 1210-1213 that is associated with a severe phenotype.[53] Further functional studies are needed to elucidate the mechanism of how these mutations can affect factor VIII activity.

Missense mutations may represent polymorphisms,[54] thus may require further evaluation. In the present study, seven novel missense mutations in *F8* were predicted to be damaging. The structural visualisations of the affected amino acids in factor VIII (**Figure 1**) were consistent with the prediction scores, therefore suggesting that these mutations are more likely to be disease-causing. For example, we identified that patient HA67 has the strongest prediction of disease-causing mutation (Pro2326Arg) in the C2 domain, and this was consistent with the severe disease phenotype exhibited by the patient. Any mutation that lies within the C2 domain is likely to be causative as the C1/2 domains are essential for the binding of factor VIII to the von Willebrand factor[55] and membrane-binding motif of tenase complex.[56,57]

In comparison to previous studies, we found 21 recurrent mutations in *F8*. Among them, a nonsense mutation (c.6682C>T) was reported in various populations,[6,7] including two cases from the Asian populations.[58,59] Another recurrent missense mutation (c.1171C>T, Arg391Cys) was also reported before with differential disease outcomes,[6,7] even though this mutation lies within the thrombin activation site.[60-62] HA disease

severity can vary upon which substitution of the amino acid at Arg391/Arg372 (mature protein) that can influence the rate of thrombin cleavage.[63] Histidine residue substitution at Arg372 position resulted in lower activation and thrombin cleavage, though no effect on factor VIII pro-coagulation activity,[63] thus consistent with the mild disease phenotype. Whereas, Arg372 substitutions to cysteine, leucine, and proline residues were reported in moderate to severe HA patients,[6,7] due to impairments in thrombin cleavage and activity.[64,65] Interestingly, our severe patient HA45 has double mutations in *F8*, namely one novel frameshift deletion (c.3175DelA) and a previously reported missense mutation (c.2383A>G) in one Taiwanese woman with a severe disease phenotype.[66] As this recurrent missense mutation (c.2383A>G) lies within the B domain, therefore, it may not be a disease-causing mutation. However, a presence of frameshift deletion would affect the factor VIII synthesis and function.

We identified four novel mutations in *F9*, including in the severe patient HB13 who had a novel large deletion affecting the signal-pro-peptide-GLA-EGF1 domains of factor IX. This finding is consistent with the previous findings that 90% of the large deletions identified were in severe HB patients[48,51,60] and associated with higher risk of inhibitor development. [67] One HB2 patient with severe disease has a novel small 5bp deletion (c.253-17_253-13delTCTTT) at the acceptor splice site in intron 2. This novel small deletion is similar to a previously reported 5bp deletion (c.253-18_253-14delTTCTT) in two Malaysian siblings with moderate disease.[19] Despite a difference of a single nucleotide position, the two siblings[19] and our patient exhibited differential disease outcomes, in which our 5bp deletion is more detrimental due to being nearer to the intron-exon boundary. As this novel 5bp deletion may interfere with the acceptor-binding site and causing an exon skipping event,[68] therefore it could explain such differential disease phenotypes. We also found a novel frameshift mutation in the signal-peptide domain (patient HB12) in which this deletion of C nucleotide is consistent with previous findings that any mutation lies within the early pre-pro leader sequences of factor IX is detrimental.[69] In our severe HB4 patient, the novel missense mutation (c.803G>A) is likely a disease-causing mutation as it lays within the serine-protease domain and is also predicted to disrupt the helix structure of factor IX (**Figure 2B**), consistent with the vital role of the serine-protease domain in factor IX activity.[33]

In comparison to previous studies of *F9*, we found ten previously reported mutations. Both nonsense mutations (c.1135C>T and c.223C>T) have been reported in various populations,[5] including two Malaysian patients (c.1135C>T only).[19] Similarly, a frameshift mutation (c.159_160DelAG), the missense mutations (c.415G>A and c.128G>A) and a splice site mutation c.252+1G>A were also reported before in Malaysian patients.[19] As for the remaining recurrent missense mutations, these have been reported in non-Malaysian populations. The missense mutation of c.383G>A was reported before in German[70] and Indian patients[71] though, the latter had a severe disease phenotype. The recurrence splice site mutation, c.88+5G>A was reported in a Chinese patient with the same moderate disease.[72] Two recurrence missense mutations (c.1237G>A and c.800A>G) in our patients are possibly the disease-causing missense mutations because they are within the serine-peptidase domain.[73] Intriguingly, a missense mutation (c.800A>G) in our severe HB9 patient was reported before with differential disease phenotypes across two different populations namely, in a French patient with a moderate phenotype[74] and an Indian patient with a severe phenotype. [75]

Conclusions. This study is the first to comprehensively analyse the mutational spectrum of *F8* in HA patients in Malaysia. The 53% prevalence of the IVS22 mutation in our severe HA patients is slightly higher than other Asian populations. A total of 22 and four novel mutations were identified in *F8* and *F9* respectively, thus suggesting a high heterogeneity of molecular changes in factor VIII and IX in our local patients. How these mutations can affect the disease severity and the inhibitor development, is worth exploring further to provide a better understanding of the genotype-phenotype association in our patients. These mutational profiles of our Malaysian HA and HB patients can provide a useful reference database in the detection of carrier status and the diagnosis of HA and HB in the Malaysian population.

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