 Int J Hum Genet, 6(1): 27-32 (2006)

Genetics of Bleeding Disorders

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KEYWORDS Bleeding disorders; hemophilia; platelet; mutation

ABSTRACT Genetic bleeding disorders form an important presentation among various genetic disorders occurring in children. A prevalence of 6 per 100,000 population has been reported in India. Bleeding disorders constitute a heterogenous group of disorders with varying clinical presentations. The common bleeding disorders are Hemophilia A and B, von Willebrand disease, and inherited thrombocytopenias. In addition, there are a number of other defects of coagulation pathway and platelet function, about which only limited knowledge is available. This review article attempts to organize the information available regarding the clinical presentations, molecular defects and methods available for molecular diagnosis of these disorders.

Homeostasis maintains blood flow and the integrity of blood vessels. It involves coordinated action of more than 100 proteins. This complex, highly regulated system has evolved in higher organisms and consists of extensive interactions between the endothelial lining of the blood vessel, blood platelets and an intricate cascade of coagulation factors (Dahlbäck 2000)

The diseases caused due to inherited defects in the hemostatic system can be broadly classified as:

1. Platelet number (thrombocytopenia) or function (thrombasthenia) defects
2. Coagulation factor defects

The details of various disorders, inheritance, related clinical features, and investigations are shown in Table 1.

EPIDEMIOLOGY OF BLEEDING DISORDERS IN INDIA

There are a number of studies reporting on the prevalence of different types of bleeding disorders in India (Mehta and Agarwal 1981; Mohanty et al. 2002). A prevalence of 6 per 100,000 population has been reported with about 50,000 patients affected and 1300 new patients born every year in India (Mehta and Agarwal 1981; Chandy et al. 1993; Pandey 2003). Shanthala Devi et al. (1999) reported a study of 430 cases of bleeding disorders, of which 266 were due to inherited coagulopathies, 21 were due to qualitative platelet defect and 17 due to acquired coagulation defect. A study from western part of India reported on 630 patients, diagnosed to have hereditary bleeding diathesis. Amongst these, 598 (95%) patients had a coagulation disorder and 32 (5%) patients had a platelet function abnormality. In a group of coagulation disorders, hemophilia A (70.5%) was the most common disorder followed by hemophilia B (14%) and von Willebrand disease (10.8%). However Glanzman's thrombasthenia (84.3%) was found to be the most common platelet function disorder followed by Bernard-Soulier syndrome (12.5%) (Manisha 2002). Kashyap et al. (1996) reported a series of rare inherited defects of coagulation which included 8 of factor X deficiency, 7 of factor XIII, 4 each with factor VII and fibrinogen deficiency and 1 patient with factor V deficiency.

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Molecular Pathophsiology of Bleeding Disorders

Since the cloning and characterization of the first coagulation factor gene in 1982, remarkable progress has been made in the use of molecular genetic strategies to assist in diagnosis of bleeding disorders.

Hemophilia A

Hemophilia A is caused due to factor VIII deficiency. The factor VIII gene is on the long arm of the X chromosome at Xq28 spans 186 kb, and consists of 26 exons. It codes a 9 kb mRNA

Table 1: Inheritance pattern, chromosomal localization, clinical features and available investigations of inherited bleeding disorders

S. No.	*Disorder*	*Inheritance/ Chromosomal locus*	*Clinical features and complications*	*Investigations*
1	Inherited Thrombocytopenia		Sudden onset of small spots of hemorrhage on the skin; Bleeding into mucous membranes; Blood in vomit or stools; Bleeding during surgery; Bleeding of intracranial vessels (rare)	Platelet count (decreased) APTT (normal) PT (normal) TT (normal) Fibrinogen levels (normal)
2	Thrombasthenia (Impaired platelet function) Glanzmann, Bernard-Soulier disease	Autosomal recessive	Bleeding of varying severity; Mucocutaneous bleeding or excessive hemorrhage following surgery	Platelet adhesion, aggregation tests
3	Hemophilia A (Factor VIII deficiency)	Sex-linked recessive Xq28	Bruising; Spontaneous bleeding; Bleeding into joints; Gastrointestinal and urinary tract hemorrhage; Prolonged bleeding with surgery or trauma	APTT (increased), Factor VIII activity decreased, vWF levels (normal)
5	Hemophilia B Christmas Disease (Factor IX deficiency)	Sex-linked recessive Xq27.1-27.2	Similar to factor VIII deficiency	APTT (increased), Factor IX activity decreased, vWF levels (normal)
6	Von Willebrand Disease (Most common disorder of platelet function, many variants (Type I-III, Pseudo, etc.) manifestations mild to severe	Autosomal dominant, recessive, X -linked	Nose bleeds; Bruising; Gastrointestinal tract hemorrhage; Prolonged bleeding from cuts, tooth extraction, and surgery	APTT (may be elevated) von Willebrand antigen test, Factor VIII activity, Ristocetin-cofactor activity (decreased in Type I andII; absent in Type III) Platelet adhesion and aggregation (abnormal)
7	(Factor I deficiency) Afibrinogenemia/ Hypofibrinogenemia/ Dysfibrinogenemia	Autosomal recessive 4q23-34	Probable predisposition to thrombosis; Individuals with hypofibrinogenemia may suffer from little, moderate or severe bleeding	APTT (prolonged),PT (prolonged),TT (prolonged),Fibrinogen levels (maybe abnormal),Platelet count (normal)
8	(Factor II deficiency) Hypoprothrombinemia/ Dysprothrombinemia	Autosomal recessive 11p11-q12	Umbilical cord bleeding at birth; Bleeding after trauma or surgery; Easy bruising	PT (prolonged), Factor II assay, APTT (prolonged)
9	Owren's disease/ Parahemophilia (Factor-V deficiency)	Autosomal recessive 1q21-25	Bleeding into the skin;Nose bleeds; Bleeding of the gums; Prolonged/excessive bleeding with minor injuries, surgery or trauma	BT (may be prolonged), PT (prolonged), APTT (prolonged), Factor V decreased
10	Factor-VII (Proconvertin)	Autosomal recessive 13q34	Bleeding of mucous membranes; Excessive bruising; Bleeding into muscles and/or joints; Hemorrhage or neurological problems related to CNS bleeding	PT (prolonged),PTT (normal),Factor VII assay

Table 1: Contd....

S. No.	*Disorder*	*Inheritance/ Chromosomal locus*	*Clinical features and complications*	*Investigations*
11	Factor X (Stuart Prower Factor) deficiency	Autosomal recessive 13q32	Mucous membrane bleeding; Bleeding into joints; Muscle bleeding;Gastrointestinal bleeding	PT (may be prolonged), APTT (prolonged), Factor X assay(decreased activity)
12	Hemophilia C/Plasmathro-mboplasticantecedent / Rosenthal Syndrome (FactorXI Deficiency)	Autosomal recessive 4q35	Prolonged/excessive bleeding with surgery or trauma; Bruising; Blood in the urine; Hemorrhage (usually after an injury orsurgery); Delayed bleeding	APTT (prolonged), Factor XI assay(decreased activity)
13	Factor XII (Hageman Factor) deficiency	Autosomal recessive 5q33	Generally no complications; May predispose to thrombosis	APTT (prolonged),PTT (prolonged),Factor XII assay
14	Factor XIII (Fibrin Stabilizing Factor) deficiency	Autosomal recessive A-subunit- 6p24-25 B-subunit- 1q31-32	Bleeding from umbilical stump after birth; Prolonged bleeding from trauma; Delayed Bleeding; Spontaneous	Factor XIII assay, APTT (normal),PT (normal),TT (normal),Fibrinogen levels

of which 7.1 kb encodes the protein. There are two special characteristics of this gene. The large exon 14 that covers about 40% of the coding region encodes a functionally less important part of the protein. Exon 22 contains two more genes, F8A and F8B, whose function is not known (Kashyap and Choudhry 2000; Paula et al. 2003).

A number of mutation screening methods have been used for detecting mutations, which include denaturing gradient gel electrophoresis (DGGE), single strand conformational polymorphism (SSCP), conformational sensitive gel electrophoresis (CSGE) and chemical cleavage mismatch (CMC). Most of these studies resulted in detection rate up to 90% (Oldenburg and Schwaab 2001).

Of all the mutations detected, inversion 22 accounts for 45% of the mutations in severe hemophilia A. This mutation occurs by a novel mechanism. The inversion occurs due to homologous recombination between F8A gene and exon 22 of factor VIII gene. This mutation disrupts the expression of factor VIII gene and the C-terminus of the protein encoded by exons 23-26. In addition to this mutation, two more hotspots have been identified. About 40% of point mutations occur at one of the 70 CpG sites in the gene and about 25% of all small deletions affect one of two adenine runs within exon 14. The list of mutations known is available at http: //europium.csc.mrc.ac.uk.

Hemophilia B

The factor IX gene (FIX) consists of eight exons, spans about 34 kb and is located on long arm of X chromosome at Xq27. The mRNA is 2.8kb and contains1.4 kb coding sequence and 1.4 kb poly (A) tail. Mutations have been described in all parts of the gene except the poly (A) tail. They include missense (68%) and nonsense (14%) mutations, deletions (3%). About 40% of all point mutations affect the CpG sites (Goodeve and Peake 2002) http:// www.umds.ac.uk/molgen/haemBdatabase.htm.

Von Willebrand Disease

The vWF gene is located on chromosome 12 and spans 52 exons. It codes for 8.7 kb mRNA . A pseudogene of vWF representing 97% homology to exons 23-34 has been identified and mapped to chromosome 22. This is important because it may interfere with the results of the diagnostic tools. A large number of mutations including deletions, missense mutations, nonsense mutations and frameshift mutations have been reported. A database of mutations is available at http://mmg2.im.med.umich.edu/v/VWF/.

Rare Clotting Factor Deficiencies

Hemophilia A, B and von Willebrand disease are classic bleeding disorders. Their high frequency compared to other factor deficiencies can be explained on basis of X- linked inheritance and heterogeneity of von Willebrand disease phenotype. Other bleeding disorders are rare as they are transmitted as autosomal recessive traits and hence homozygosity is required to produce disease. Molecular basis of many of these disorders has been elucidated but they are of low clinical significance due to rarity of occurrence.

Inherited Platelet Disorders

The mutational spectrum in Bernard-Soulier syndrome is heterogenous, including homozygous and compound heterozygous deletions, frameshift nonsense and missense mutations. Mutations have been identified in genes encoding GpIbá, GpIbâ and GpIX. In Glanzmann's thrombasthenia a similar pattern of mutations have been identified in genes for GpIIb and GpIIIa.

Mutation Detection Methods

Biological assay of factor varies according to the type of defects. For prenatal diagnosis and carrier detection, the molecular methods are based on:

1. Polymorphism Linkage Analysis: Direct detection of mutation may not be feasible when the size of gene is large as in case of factor VIII gene. Linkage analysis using polymorphic markers can be used especially when there is family history of disease. The polymorphic markers like microsatellite repeats are different in different populations. Hence knowledge regarding ethnic background of the kindred is important (Lillicarp 1999). Different polymorphic markers routinely analyzed are shown in Table 2 and 3 along with the primer sequence and the size of PCR product after digestion. There are certain limiting factors for linkage analysis like

- Isolated case of hemophilia
- Lack of heterozygous (informative) markers
- Unwillingness to provide family samples
- Incorrect paternity
- Recombination possibility between the marker and the gene

2. Factor VIII Inversion Mutation Detection: The inversion mutation in factor VIII accounts for 45% of mutations in severe hemophilia A. Hence this is the first step in mutation detection in a case of hemophilia A. The mutation detection is done by Southern blot using sequences within intron 22 of the factor VIII gene.

3. Direct Mutation Detection: The rationale for direct mutation analysis in hemophilia A is in case of isolated case of hemophilia, potential germ line mosaicism, and prediction of inhibitor risk. Certain factor VIII genotypes have been shown to be associated with high risk of

Table 2: Primer sequences used for detection of common polymorphisms in linkage analysis of Hemophilia A. (→ Forward primer, ← Reverse primer)

Primer Sequence	*Restriction enzyme*	*PCR Product size(bp)*	
		Before	*After digestion*
5'-TAA AAG CTT TAA ATG GTC TAG GC3' →	*Bcl* I	142	99+43
5'TTC GAA TTC TGA AAT TAT CTT GTT C3' ¬			
5'AAG GTC CTC GAG GGC GAG CAT3' →	Hind III	717	469+248
5'AAG GTC GGA TCC GTC CAG AAG 3'¬			469+167+ 81
5'CTGGAGAATCTAAGAGGATAGAGGACAACATTTACC3' →	*Xba*I	6.6kb	6.1kb+0.5kb
5'AGTACTTCTCCAGGGTCTGGGCGTGCTC3/ ¬			1.3kb+4.8kb+0.5kb

Table 3: Primer sequences used for detecting common polymorphisms in linkage analysis of Hemophilia A. (→ Forward primer, ← Reverse primer)

Primer Sequence	*Restriction enzyme*	*PCR Product size(bp)*	
		Before	*After digestion*
5'ACA GGC ACC TGC CAT CAC TT 3'→	HhaI	230	150+80
5'AGA TTT CAA GCT ACC AAC AT 3'¬			
5'GGG ACC ACT GTC GTA TAA TGT GG 3'→	DdeI	369	319
5'CTG GAG GAT AGA TGT CTC TAT CTG 3'¬			

acquiring a factor VIII inhibitor. The strategies used for mutation detection include either mutation screening followed by sequencing of abnormal region or direct sequencing strategy. The screening techniques used include denaturing gradient gel electrophoresis (DGGE), single strand conformational polymorphism (SSCP), conformational sensitive gel electrophoresis (CSGE) and chemical cleavage mismatch (CMC). Automated sequencing is used more and more as the cost and efficiency of direct sequencing has improved. This approach is more suited for factor IX mutation detection as the gene size is small. Recent technique of oligonucleotide microarray analysis is now routinely being used for detection of mutation. A list of mutations detected in Factor VIII gene till 2004 is shown in Table 4.

Table 4: Summary of mutations reported in Hemophilia A (http://europium.csc.mrc.ac.uk.)

	Point mutations				
Exon	*Mis-sense*	*Non-sense*	*Spli-cing*	*Dele-tions*	*Inser-tions*
1	14	1	4	2	0
2	5	1	5	6	2
3	24	0	3	4	0
4	24	5	2	1	1
5	11	1	7	3	1
6	6	0	3	4	2
7	32	4	0	6	0
8	21	4	1	8	1
9	22	3	1	5	1
10	10	1	0	4	0
11	27	1	3	1	1
12	20	5	2	0	1
13	23	3	2	4	1
14	37	39	3	63	31
15	13	1	4	2	0
16	18	4	2	5	0
17	24	3	1	5	4
18	26	4	1	3	3
19	14	1	5	3	1
20	5	1	0	0	1
21	5	4	0	0	1
22	16	5	5	3	1
23	25	1	4	6	0
24	10	3	3	3	2
25	11	2	1	4	2
26	19	3	0	7	0
Total	462	100	62	152	57

ANTENATAL DIAGNOSIS AND CARRIER TESTING

Antenatal diagnosis for hemophilia is possible to prevent the birth of an affected male child. This requires invasive procedures like prenatal sex determination for selection of only the male fetuses. Chorionic villus biopsy is the method of choice to obtain fetal DNA for further analysis. It is usually carried out after 11 weeks of gestation via transabdominal or transvaginal route. Fetal blood sampling can also be done at 15-19 weeks. If couple visits antenatal clinic at late period of gestation or if DNA based diagnosis is confusing then the coagulation factor is assayed in fetal blood after confirmation that the blood is wholly fetal and not contaminated by maternal blood.

Carrier status determination is important in female relatives of affected patient so that they can be offered prenatal diagnosis. Carrier status can be determined either by linkage analysis or direct sequencing.

There are few studies reported in literature on molecular genetics of bleeding disorders in India (Verma et al. 2003). Pandey et al. (2001) reported 95% detection rate in families with hemophilia A using restriction fragment length polymorphism and intragenic short tandem repeats CA repeats). The same authors published further data on linkage analysis as well as direct detection of inversion mutation in intron 22 of the gene. The observed heterozygosity for RFLP markers HindIII, BclI and XbaI was 0.63, 0.60 and 0.48 while that of STR markers introns 13 and 22 were 0.60 and 0.40 respectively. Six and four alleles were identified for introns 13 and 22 and the most frequent allele was 13(CA) 26 and 22(AG) n (GT) 26 with an allele frequency of 0.53 and 0.62 respectively (Pandey et al. 2002). A similar study was reported by Jayandharan et al. (2004) and they found that XbaI site polymorphism was the single most informative marker followed by HindIII, intron 13 CA repeats, intron 22 CA repeats, DXS52, and intron 7 G—>A polymorphism. The combined use of these markers was informative in 92% of hemophilia A families. PCR and multiplex Long-Distance Subcycling-PCR to detect Intron 1 inversion in 3 (3.75%) and intron 22 inversion was carried out in 35(43.75%) patients. Of the severe hemophiliacs, 47.5% had either of these inversions.

Chowdhury et al. (2003) published data on prenatal diagnosis for hemophilia A in 41 families and concluded that linkage analysis, with the combined use of these five PCR-based polymorphic markers, gives good informativeness of 87.8% in the Indian population.

There are few reports of similar studies in

cases of hemophilia-b with reported identification of 22 independent mutations including five novel mutations in 24 unrelated patients of hemophilia B (Mukherjee et al. 2004). Shetty et al. (2003) did direct detection of factor IX gene deletions in Indian hemophiliacs by multiplex PCR.

Advances in molecular biology have greatly increased our understanding of the hemostasis network. As more and more refined techniques are developed, mutation detection will become more and more easy and specific.

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