

# Role of Molecular Genetics in Hemophilia: From Diagnosis to Therapy

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## Abstract

### Keywords

- ▶ hemophilia
- ▶ genetic diagnosis
- ▶ phenotype
- ▶ variation
- ▶ inhibitor
- ▶ gene therapy

Despite significant advancements, state-of-the-art care remains inaccessible to patients with hemophilia, especially those from developing countries. Thus, innovative approaches in the management of this condition are needed to improve their quality of life. In this context, genetic studies in hemophilia have contributed to the better understanding of its biology, the detection of carriers, and prenatal diagnosis, and even fostering newer therapeutic strategies. This article reviews the applications of molecular genetics in hemophilia, in general, and how such techniques can be useful for optimizing patient care, in particular.

Hemophilia A and hemophilia B are X-linked bleeding disorders caused by a deficiency in blood coagulation factor (F) VIII or FIX, respectively. The disease has an incidence of 1 in 5000 and 1 in 25,000 male births, respectively, with a prevalence of approximately half-a-million people worldwide. No ethnic or geographic predisposition has been defined.<sup>1</sup> Both *F8* and *F9* genes map to the long arm of X chromosome at Xq28 and Xq27, separated by 35cM.<sup>2–4</sup> While the *F8* gene has 26 exons spanning 186 kb,<sup>5</sup> the *F9* gene is relatively smaller (34 kb), and has 8 exons<sup>3</sup> (▶ **Fig. 1**). Mutations in both these genes (*F8*, *n*~ 2179; *F9*, *n*~ 1097) including a variety of deletions, insertions, missense, nonsense, and splice-site mutations, apart from the common intron 1 and intron 22 inversions in the *F8* gene, have been reported to cause the clinical phenotype (HGMD®, Human Gene Mutation Database. <http://www.hgmd.cf.ac.uk/ac/gene.php?gene=F8>; <http://www.hgmd.cf.ac.uk/ac/gene.php?gene=F9>).

Molecular genetic diagnosis of this condition remains an important and integral part of its evaluation. Apart from helping our understanding of the functional biology of these two genes, this information is useful for genotype–phenotype correlations as well as understanding the basis of inhibitor development or for newer approaches of hemophilia therapy such as development of newer clotting factor concentrates and gene therapy. This article reviews the applications of molecular genetics in hemophilia, in general, and how such

techniques can be useful for optimizing patient care, in particular.

## Inheritance of Hemophilia

There is a 50% chance that a carrier mother will transmit the defective X-linked gene to the male or female child. All female offspring born to a hemophilic father are obligatory carriers (▶ **Fig. 2**). To identify the females at risk of being a carrier, it is important to understand the inheritance. Sporadic cases result from de novo mutations. Apart from assessing levels of FVIII coagulant (FVIII:C), molecular genetic analysis is required to reliably determine carrier status. However, one needs to consider the potential risk of somatic mosaicism in families with sporadic hemophilia (~10%), as it causes uncertainty about the recurrence risk in parents who appear to be noncarriers. In this situation, conventional mutation detection procedures may fail to detect the underlying genetic defect if the proportion of mutated alleles is <5% of wild-type allele background.<sup>6</sup>

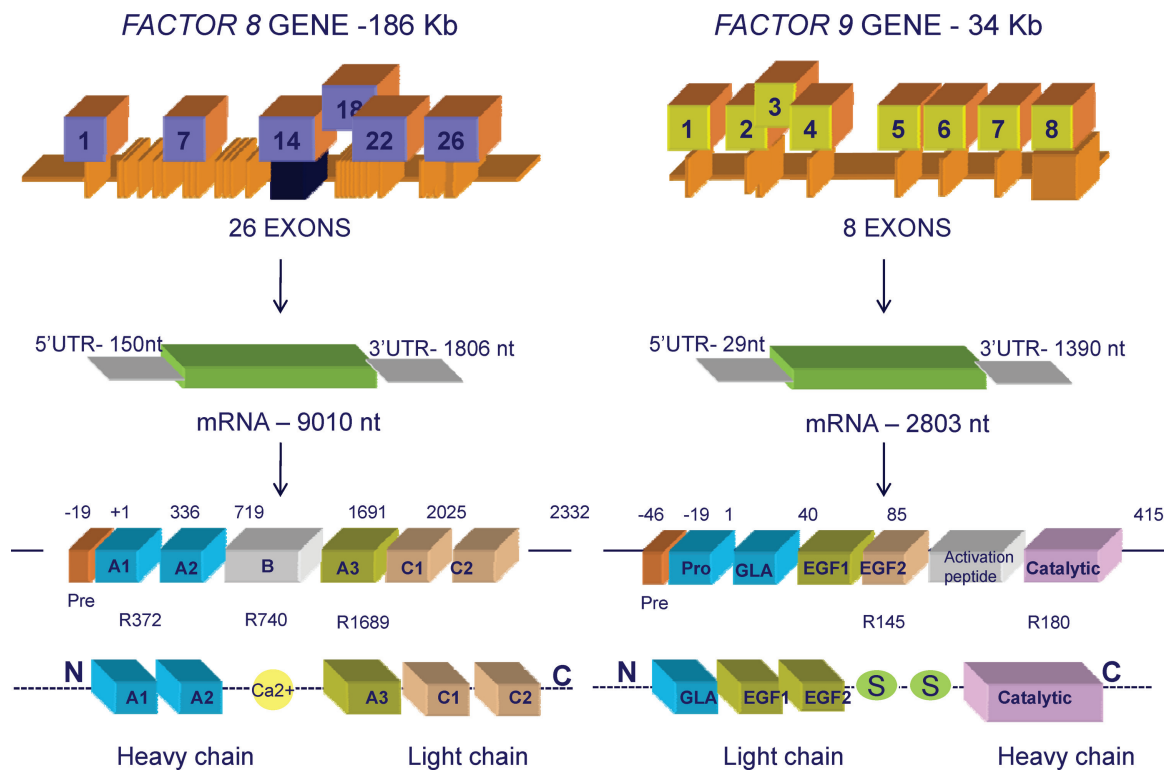
## Approach to Genetic Diagnosis

There are two different approaches to the genetic evaluation of hemophilia. Analysis of single-nucleotide polymorphism (SNP) or microsatellite variable number tandem repeat

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**Figure 1** Organization of human *factor 8* and *factor 9* genes. *Factor 8* gene is 186 kilobases (kb) in length and encodes a messenger RNA of ~9 kb. The newly synthesized factor VIII protein molecule is composed of a presequence of 19 amino acids and a mature peptide of 2332 amino acids. The mature multidomain factor VIII protein contains triplicated A domains, duplicated C domains, and a single B domain. The arginine residues, which are the sites for proteolytic activation, are R372, R740, R1689. Activated factor VIII is a heterotrimer in which the dimeric N-terminal heavy chain is held together with the monomeric C-terminal light chain by a metal ion bridge ( $\text{Ca}^{2+}$ ). *Factor 9* is 1/6th the size of *factor 8* gene, ~ 34 kb and encoding a transcript of ~1.4 kb. The mature factor IX protein consists of a pre- and pro-sequence and a mature peptide of 415 amino acids (total length, 461 amino acids). Activated factor IX has an N-terminal light chain and a C-terminal heavy chain held together by a disulphide bridge between cysteine residues 132 and 279. GLA, "GLA" domain, in which 12 glutamic acid residues undergo post-translational gamma-carboxylation by a vitamin K-dependent carboxylase; EGF, epidermal growth factor-like domain; activation peptide released after proteolytic activation at arginine 145 and arginine 180; catalytic, the serine protease domain responsible for cleavage of factor X to Xa.

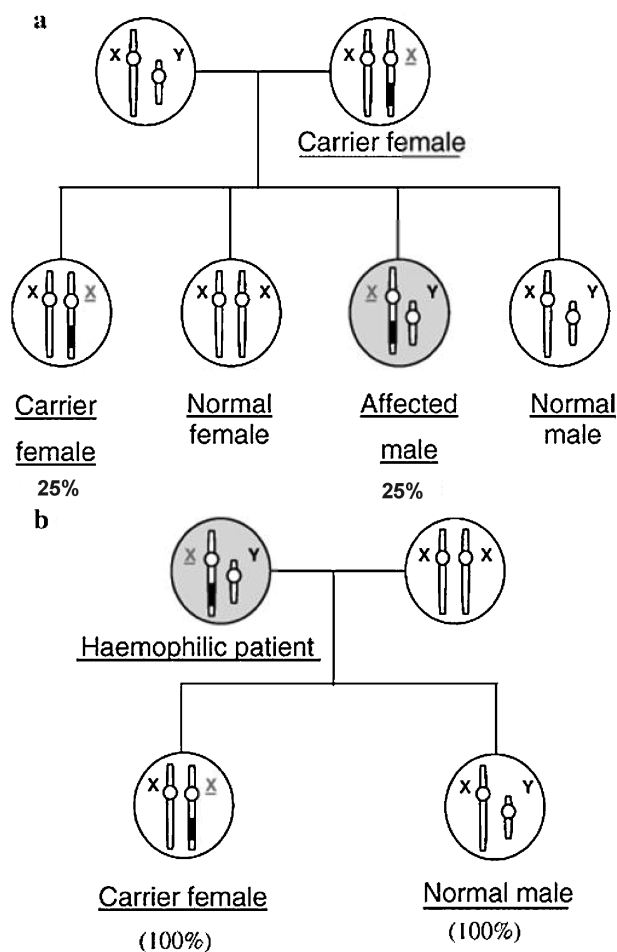
(VNTR) markers in the *F8* or *F9* gene to track the defective X chromosome in the family (linkage analysis) or identification of the disease causing mutation in the defective *F8* or *F9* gene (direct mutation detection) are employed.<sup>7,8</sup> Before embarking on genetic diagnosis, it is imperative that detailed clinical evaluation and factor assays be available. Postgenetic test counseling for the family is also an important part of genetic testing to help the family make an informed choice for their childbirth. For linkage analysis, DNA samples are required from the affected patient(s) and the parents to understand the inheritance pattern. For direct mutation detection, DNA samples are required from the proband and any other affected patient in the family, if available, to improve the accuracy of diagnosis.

### Linkage Analysis

SNP are commonly detected by polymerase chain reaction (PCR) amplification of the target site followed by restriction fragment length polymorphism<sup>7</sup> whereas VNTR are detected by conventional polyacrylamide gel electrophoresis<sup>9</sup> or by fluorescent PCR and capillary electrophoresis.<sup>10</sup> The key requirement for linkage analysis is the heterozygosity of the polymorphic marker in the mother of the index case.

This requires a strategy for sequential analysis of different polymorphisms in *F8* or *F9* genes depending on heterozygosity rates in the population.

Although the principle on which linkage analysis is applied to hemophilia A and hemophilia B is similar, the severity of hemophilia A in the pedigree influences the diagnostic strategy employed. Many laboratories<sup>11-13</sup> in developing countries use linkage analysis following long PCR detection of two common mutations in the *F8* gene, the intron 1 or intron 22 inversions caused by a homologous recombination with one of their two extragenic copies.<sup>14,15</sup> These inversions constitute the molecular basis for hemophilia A in ~45 to 50% of patients with severe disease.<sup>16</sup> In inversion negative cases and in patients with moderate or mild hemophilia A, several polymorphisms in the *F8* gene may be tracked (→ Fig. 3).<sup>16</sup> Some of these polymorphisms such as *HindIII/BclI* are in linkage disequilibrium thereby reducing the overall informativity of this approach. However, the use of two VNTR together with *HindIII* and *XbaI* biallelic polymorphisms allows gene tracking in up to 80% families.<sup>8</sup> In studies performed in Indian population, *XbaI* has been identified as the most informative marker (70%) for linkage analysis followed by *HindIII*



**Figure 2** X-linked inheritance in hemophilia: The inheritance is shown in families where either mother is a carrier (A) or the father is a hemophilic patient (B).

(60%), intron 13 CA repeats (57%), intron 22 CA repeats (50%), *DXS52* VNTR (23%), and intron 7 G→A polymorphism (7%) in *F8* gene. The combined use of these markers is informative in 92% of hemophilia A families.<sup>10</sup> Based on these data a comprehensive algorithm for linkage analysis of hemophilia A has been proposed (►Fig. 4). For linkage analysis in hemophilia B, the polymorphisms studied in the *F9* gene are detailed in ►Fig. 3.<sup>16</sup> By combining three markers, namely, *Ddel*/intron 1, *XmnI*/intron3, and *HhaI*/3'UTR in *F9* gene a cumulative informativeness of 80% may be achieved.<sup>16</sup>

The feasibility of this approach is restricted by several factors. These include the requirement of multiple members including one affected member from the same family and the significant chance (~1%) of an erroneous result from potential recombination between the mutant gene and polymorphic site. The approach may be diagnostic in only ~85 to 90% of families. In view of considerable ethnic and geographical variation in the allele frequencies of these polymorphisms,<sup>7</sup> it is necessary to establish the informativeness of these polymorphisms in different populations.<sup>8,17</sup> Varying repeat lengths attributed to DNA polymerase slippage could occur between generations within a family.<sup>18</sup> Therefore microsat-

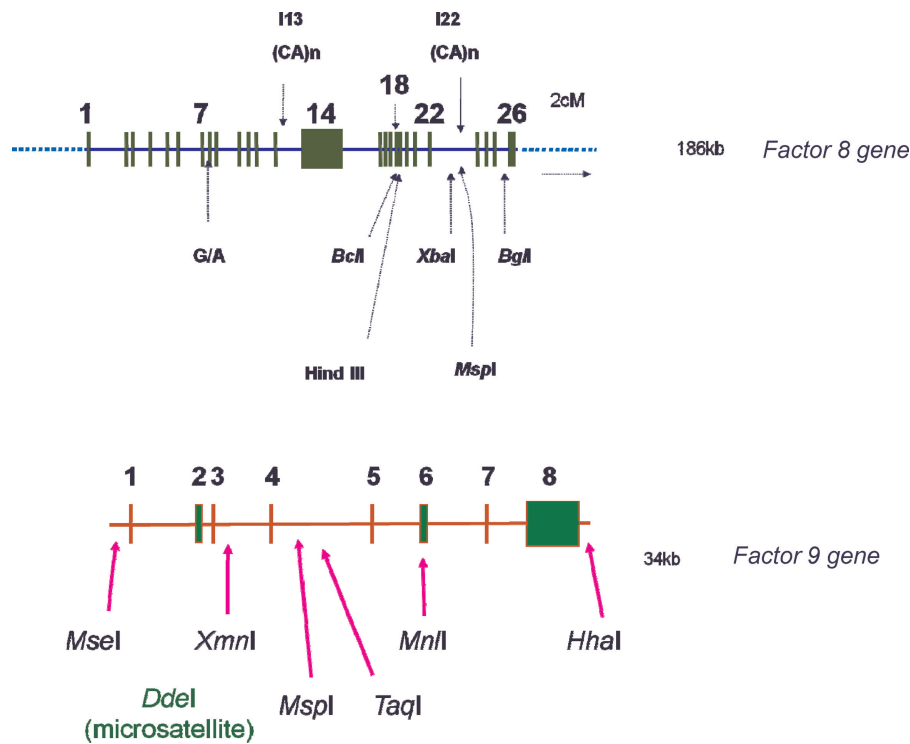
ellite data should be interpreted with caution in a linkage study. Despite its drawbacks, linkage analysis is widely used in developing countries.

### Direct Mutation Detection

Direct detection of disease causing mutation has a near 100% accuracy and is informative in over 95% of families with hemophilia A and hemophilia B.<sup>17</sup> It is equally efficient and sensitive in detecting mutations in both familial and sporadic hemophilia, even in the absence of a proband. In ~45 to 50% cases with severe hemophilia A, two common inversions at intron 1 or intron 22 are detected,<sup>16</sup> and therefore are first screened by PCR-based protocols either before linkage analysis or the point mutation screening in most laboratories.

The strategy employed for point mutation screening includes amplification of the *F8* or *F9* gene (exonic and their flanking intronic regions, the 5'UTR and 3'UTR) by PCR followed by detection of mutations by various screening methods or/and DNA sequencing. For the *F9* gene, this is easier as it has only 8 exons, the largest of which is less than 2 kb. In contrast, the large size and complexity of the *F8* gene necessitates amplifications of genomic DNA in over 30 fragments to cover the target regions.<sup>19</sup> Various mutation screening techniques can be used to screen PCR products of *F8* or *F9* genes, such as single-strand conformation polymorphism,<sup>20</sup> denaturing gradient gel electrophoresis,<sup>21</sup> conformation-sensitive gel electrophoresis (CSGE),<sup>19</sup> and denaturing high pressure liquid chromatography<sup>22</sup> with sensitivities ranging from 80 to 98%. Abnormal PCR product profiles are sequenced to identify the nucleotide change. Modification of these mutation screening methods such as multiplexing of amplification reactions and CSGE (~13 vs. 33 PCR reactions for larger genes such as *F8*) has been described (►Fig. 5),<sup>23,24</sup> which has significantly reduced the cost and time for direct mutation screening in hemophilia and also in other disorders of hemostasis.<sup>25</sup> However, with the declining cost of DNA sequencing reagents the adoption of direct nucleotide sequence analysis is becoming a viable option even for service laboratories.<sup>26</sup> Indeed, because of limited number of tests that are involved in genetic testing, it can be easier to set up reliable genetic testing service as opposed to tests of hemostasis.

One also needs to be cautious with the use and interpretation of mutation data. Despite the utility and superiority of direct mutation detection, a disease-causing mutation is not identified in the *F8* gene in ~5% of cases with hemophilia A,<sup>27</sup> affecting genetic diagnoses in these families. The genotype-phenotype correlation of novel mutations identified is challenging, but can be generally predicted (►Fig. 6). It must be, however, noted that establishing the causality of a novel missense mutation relies mostly on a series of candidate explanations based on their effect on the structure of FVIII/FIX proteins and location, their evolutionary conservation between species as well as in related proteins and their absence in the general population.<sup>28</sup> Some patients can also have two independent causative mutations (K1439fs; R1966Q) as described in a patient with familial hemophilia



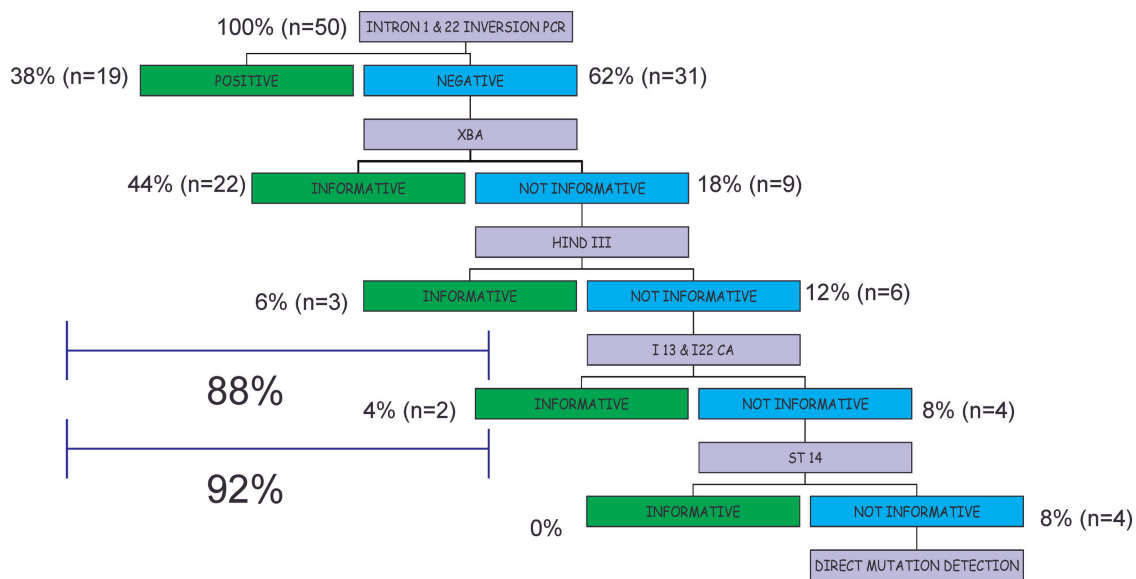
**Figure 3** Commonly used polymorphic markers in *factor 8* (A) or *factor 9* (B) genes for linkage analysis. *Factor 8* gene intron 7 G/A, intron 13 (CA)n, intron 18 BclI, intron 19 HindIII, intron 22 XbaI, intron 22 MspI, intron 22 (CA)n, intron 25 BglI are shown. *Factor 9* gene 5' MseI, intron 1 Ddel, intron 3 XmnI, intron 4 TaqI, intron 4 MspI, exon 6 MnlI, and 3' HhaI are shown.

A with one being a de novo mutation.<sup>29</sup> Such instances increase the likelihood of erroneous data reporting. Recent studies have also demonstrated that missense mutations within B domain of FVIII other than those at glycosylation or protease cleavage sites may not be causative of hemophilia.<sup>30</sup> This illustrates the importance of expression studies for ascribing causality of novel missense substitutions, a tech-

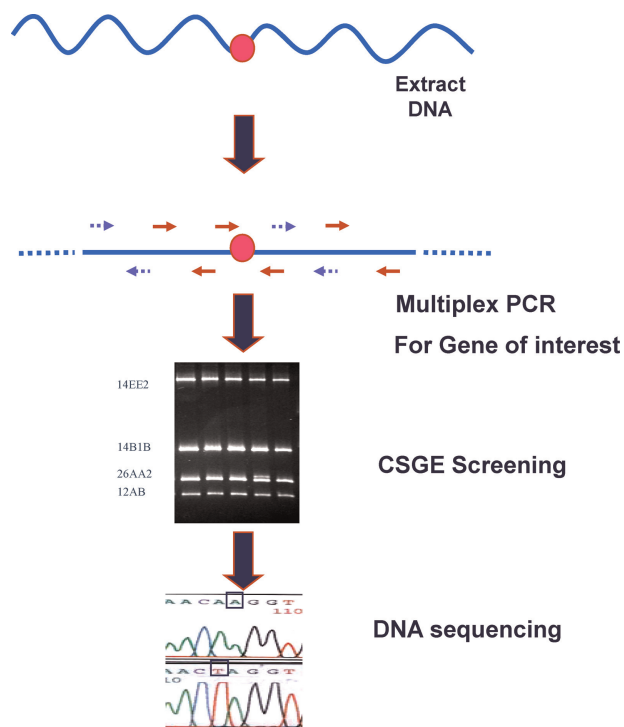
nology that is impractical to follow in the setting of a service laboratory.

**Advances in Molecular Screening**

DNA microarray-based approach for the screening of mutations in hemophilia A<sup>31</sup> and hemophilia B,<sup>32</sup> and real-time PCR (Light Cycler™, Roche Applied Science, Indianapolis, IN)



**Figure 4** Algorithm for linkage analysis of hemophilia A in India (Adapted from Jayandharan et al, 2004.<sup>10</sup>) Families are first screened for the common intron 22 and intron 1 inversion mutation in *factor 8* gene. In inversion negative families, sequential analysis of the polymorphic markers is performed. Using this strategy, genetic diagnosis can be offered to ~90% of families.



**Figure 5** Direct mutation screening by multiplex polymerase chain reaction (PCR) and conformation sensitive gel electrophoresis. Following its isolation from peripheral blood, genomic DNA from patients and normal control are amplified for *factor 8* or *factor 9* gene coding and flanking intronic regions by a multiplex PCR. For multiplex PCR, amplifications with identical annealing temperatures but producing different fragment sizes were grouped together (*F8* = 13 groups, *F9* = 4 groups). These amplicons are then screened by conformation sensitive gel electrophoresis (CSGE) a heteroduplex based mutation screening method that relies on the differential migration of DNA heteroduplexes in comparison with homoduplexes during polyacrylamide gel electrophoresis under mildly denaturing conditions. Finally, PCR fragments displaying heteroduplexes are screened by DNA sequencing to confirm the nature of nucleotide change. A representative gel picture for multiplex PCR group 3 of *factor 8* gene is shown in this figure.

for determining carrier status in families with gross deletions in *F8* gene have been described.<sup>33</sup> These choices reflect the fact that there are many options for post-PCR product analysis. However, all these need further evaluation and validation before clinical application.

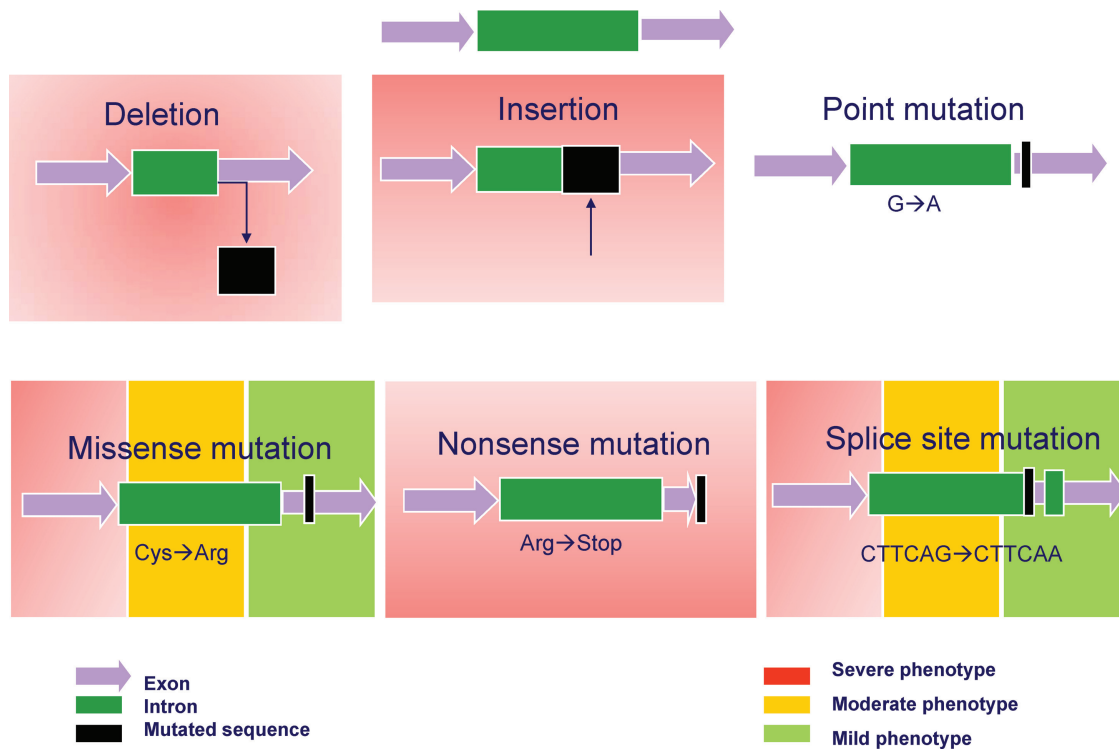
### Quality Assurance in Genetic Testing

With a steady growth in the number of laboratories that offer genetic tests for hemostatic disorders worldwide and in the absence of international frameworks to regulate them, laboratories rely largely on various internal quality control and external quality assessment and proficiency testing programs to maintain the quality and integrity of their reporting data.<sup>34</sup> In countries such as in the United Kingdom, proficiency testing for the diagnosis of hemophilia is offered by the National External Quality Assessment Scheme (UK-NEQAS) and similar programs also exist in most developed countries from North America, Western Europe as well as in

Australia.<sup>35–38</sup> Participation in such programs is mandatory for laboratory certification in these countries. However, except for data from a few laboratories, a significant gap exists in knowledge about the practices of molecular genetic testing laboratories across the world. To address this, we initiated a questionnaire survey among laboratories in both the developing and developed countries including participants from Argentina, Australia, Belgium, Brazil, Canada, China, Germany, India, Italy, Japan, Netherlands, Thailand, and United Kingdom ( $n = 19$ ).<sup>39</sup> Our data showed considerable differences in the personnel standards in these laboratories while surprisingly, the type and number of tests offered did not vary considerably among them (►Table 1). However, examination of the quality assurance practices in the surveyed laboratories showed wide variability in laboratory practices during the preanalytical, analytical, and postanalytical stages of genetic testing.<sup>39</sup> Only 43% of laboratories in developing countries (vs. 100% in developed countries) participated in any kind of proficiency testing program (►Fig. 7). We have initiated an External Quality Assessment Scheme (EQAS) for molecular genetic analysis of hematological disorders for laboratories in India since 2006.<sup>39</sup> Two surveys are conducted each year. Nine laboratories currently participate in the program for thrombophilia (100%), hemophilia A (44%), and hemophilia B (55%) modules. Eight External Quality Assessment (EQA) cycles have been completed and their details are provided in ►Table 2. For genetic testing of hemophilia, linkage analysis was most commonly used with only one laboratory performed direct mutation analysis using CSGE and DNA sequencing. All laboratories performing hemophilia B genetic testing used CSGE and DNA sequencing. Response rate for this EQA schemes are between 70 and 80%. In the last two cycles, a reporting accuracy of over 90% was noted for thrombophilia mutations while it was ~70% for the hemophilia genetic testing. A performance report is provided to all participants. Our experience suggests that EQAS for genetic tests can be effectively established in developing countries and efforts should be made to increase the awareness and benefits of voluntary participation in such programs.

### Genetic Basis for the Phenotypic Heterogeneity in Severe Hemophilia

Patients conventionally classified as having severe hemophilia (<1% of normal clotting activity) usually have 15 to 35 spontaneous joint and muscle bleeds per year without any treatment.<sup>40–42</sup> The vast majority (60–70%) of patients with hemophilia falls into this group. However, within this group, there is considerable heterogeneity in clinical presentation. A subset of these patients (10 to 15%) with severe hemophilia have clinically mild disease.<sup>41–44</sup> Variations in the bleeding frequency, age at first bleeding, and extent of joint damage have all been reported in patients with severe hemophilia by many groups.<sup>41–47</sup> Though such phenotypic heterogeneity is intriguing, only a few studies have attempted to address its basis. Factors, such as varying levels of FVIII:C activity (below 1%),<sup>48,49</sup> pharmacokinetics of the replaced clotting factor concentrate,<sup>50</sup> the type of mutation, and the concomitant



**Figure 6** Genotype-phenotype correlation in hemophilia. Deletions or insertions in the *factor 8* or *factor 9* gene lead to a shift in frame of the translation and generally cause severe hemophilia. Exceptions to these are recurrent deletions in a polyrun of A nucleotides such as in the *factor 8* gene. They result mostly in severe and occasionally moderate disease. Point mutations caused by a single nucleotide change can result in a nonsense substitution predicting abrupt premature termination of translation. Other type of point mutations can result in missense substitution (e.g., Cys→Arg) or affect splice junctions of intron-exon boundaries and their disease severity depends on the location and any particular function of the amino acid affected.

presence of prothrombotic factors<sup>51–53</sup> have been reported to impact the phenotype of severe hemophilia (► **Table 3**).<sup>54</sup>

Our clinical observations among minimally treated patients with severe hemophilia has revealed two types of heterogeneity as described earlier.<sup>55</sup> The first relates to the frequency of bleeding as is commonly recognized and it is likely that the balance of hemostasis factors determines this (the hemostasis component). The second relates to the degree of synovial reaction and the extent of damage to joint cartilage among those who bleed frequently (the vascular and inflammatory component). There are patients who develop serious arthropathy even with a moderate number of bleeds while there are others who maintained good joints in spite of many bleeds (► **Fig. 8**). We therefore hypothesized that differences in the overall hemostatic potential and the vascular inflammatory responses impact this variation. Subsequently, our data showed that apart from the primary disease causing mutations in *F8* or *F9* genes, an Arg353Gln functional polymorphism in *F7* gene and the coinheritance of interferon gamma + 874G>A polymorphism may contribute to this phenotypic variation.<sup>56,57</sup> However, the data need to be substantiated in larger and diverse groups of patients in a multicenter setting, especially when the classification of milder phenotype of severe hemophilia is not uniform across the literature.<sup>55</sup> Apart from such studies, it is also crucial to identify other molecular and cellular determinants that contribute to hemophilic arthropathy, to reveal targets for inter-

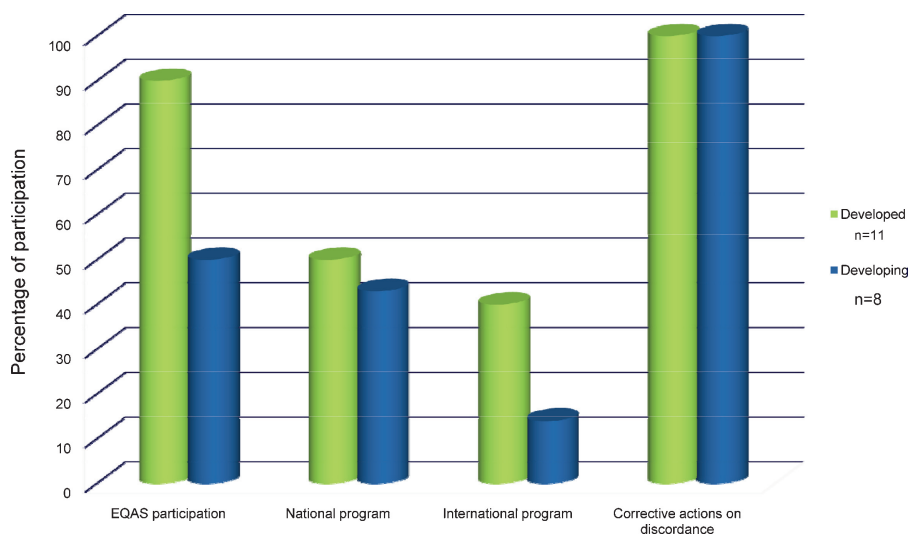
vention and to design potential treatment strategies to prevent or delay the onset of blood-induced arthropathy.

## Inhibitor Development

The development of inhibitors to FVIII/FIX represents a major therapeutic problem in the treatment of hemophilia. Fortunately, and for reasons that are largely unexplained, the incidence of inhibitors to FIX, 3 to 5%, is significantly less than those to FVIII where figures of 30% are now well substantiated.<sup>58–60</sup> The propensity for inhibitor development has at least two genetic components, one of which relates to the type of clotting factor gene mutation and the other(s) that likely involves elements of the immune system. In case of hemophilia A and hemophilia B, patients who carry a severe molecular defect (large deletions, inversions, and nonsense mutations) that result in the complete absence of the coagulant protein seem to have a higher propensity to develop inhibitors compared with those with mild molecular defects such as missense or splice site mutations, where some residual FVIII/FIX antigen is present.<sup>60–62</sup> This is supported by the reported inhibitor prevalence of 21 to 88% in hemophilia A and 6 to 60% in hemophilia B patients with severe defects as opposed <10% prevalence in patients with mild molecular defects.<sup>63</sup> Exceptions to this are patients with missense mutations where inhibitors develop when conformational changes within the immunogenic domains of the

**Table 1** Data on Personnel Standards and the Type and Number of Genetic Tests Done for Hemophilia in Various Laboratories across the World

Category		Developed (n = 11)	Developing (n = 8)	p Value
Setting	Hospital-based	91%	100%	
	Research-based	9%	–	
	Urban	100%	88%	
Laboratory Director	M.D.	36%	75%	ns
	Ph.D.	36%	12.5%	
	M.S.	9%	12.5%	
	M.D. + Ph.D.	18%	–	
	Experience (y)	20 (7–40)	14 (11–28)	0.028
Laboratory personnel	Number	4 (2–82)	4 (2–7)	ns
	Supervisor (experience, y)	20 (10–30)	10 (5–17)	0.01
	Technician (collective experience, y)	22 (2–110)	12 (5–20)	ns
Genetic service provided	Proportion of laboratories offering tests			p Value
	Developed (n = 11)	Developing (n = 8)	Total	
Hemophilia A	100%	88%	94%	
Linkage analysis	10%	–	6%	
Direct mutation screening and/or DNA sequencing	70%	43%	59%	
Both linkage and direct mutation detection	20%	57%	35%	
No. of cases/year	32 (10–118)	33 (11–60)		0.856
Hemophilia B	73%	88%	81%	
Linkage analysis	0%	17%	7%	
Direct mutation screening and/or DNA sequencing	89%	67%	80%	
Both linkage and direct mutation detection	11%	17%	13%	
No. of cases/year	9 (2–35)	7 (4–19)		0.791

ns =  $p > 0.05$ .**Figure 7** Proficiency testing characteristics of laboratories (n = 19) surveyed in our questionnaire survey.

**Table 2** Details of Samples Distributed for the Molecular Genetics EQAS for Hemostasis Disorders in India

Survey Year	Hemophilia A	Hemophilia B	Factor V R506Q	PT 20210G>A	MTFHR 677C>T
2006	Intron 22 inversion	Negative for mutation	RR	G/A	C/C
2007	Intron 22 inversion	Tyr115Cys	RR	G/G	T/T
2008A	Informative for Intron 13 CAn markers	Lys409X	RR	G/A	C/T
2008B	Informative for <i>XbaI</i> marker	Glu245Lys	RQ	G/G	–
2009A	Informative for <i>BclI</i> , <i>HindIII</i> , Intron 13, 22 CAn marker	Arg-4Gln	RR	G/G	T/T
2009B	Informative for intron 13 CAn/ <i>XbaI</i> marker	Val196Phe	RQ	G/G	C/T
2010A	Informative for intron 22 CAn / Glu1704X	Arg248X	RR	G/G	C/C
2010B	Informative for <i>XbaI</i> , <i>HindIII</i> Glu1875X	Gly207Glu	RR	G/G	C/T

FVIII protein occur.<sup>64–66</sup> Thus, the risk stratification proposed earlier by Giannelli et al based on genetic risk factors may be of increasing relevance.<sup>67</sup>

If the molecular defect is unknown, the risk of an inhibitor is 3%; if the mutation results in a single amino acid substitution, the risk is further reduced to near zero; while for frameshift, premature stop codon and splice site mutations, the risk of inhibitor development is 20%. Finally, for gross deletions and gene rearrangements, which constitute 50% of the mutations in inhibitor patients, the risk is even higher. However, the discordance for inhibitor development seen in patients or siblings with identical gene mutations suggests that other genetic factors could play a modifier role.<sup>68</sup> More recently several polymorphisms in the genes encoding immunoregulatory cytokines and molecules such as interleukin-10, cytotoxic T-lymphocyte antigen-4, tumor necrosis factor- $\alpha$ , and specific *F8* haplotypes have shown to be associated with the development of inhibitors in patients with hemophilia A.<sup>69–72</sup>

From these data it is clear that the immunological response leading to inhibitor formation is complex and several cellular-, cytokine-, and immunogen-related parameters can have a bearing on this response.<sup>73</sup> Of these, only the impact of genotype on inhibitor formation is well established.<sup>74,75</sup> Not all patients with such disruptive mutations and associated cytokine polymorphisms develop inhibitors, suggesting that other cellular and environmental factors contribute to its development. Understanding these factors will be crucial to reduce the risk of inhibitor formation in the high-risk patient.

## Gene Therapy

Gene therapy for hemophilia offers a promising curative option, especially considering that a modest increase in FVIII or FIX levels above 1% can prevent spontaneous bleeding and substantially enhance the quality of life in patients with hemophilia.<sup>76</sup> Although a variety of physical and chemical methods have been developed for introducing the defective gene into target cells, viruses have generally been proven to

be much more efficient for this purpose. Among the currently available viral vectors, the adeno-associated virus (AAV)-based gene delivery is known to be potentially safer than retroviral and adenoviral vectors.<sup>77–80</sup> AAV serotype 2 (AAV2) is the prototype vector that has been extensively studied.<sup>81</sup> Indeed, AAV2 has become a preferred choice by many investigators for in vivo viral gene transfer, and due to its wide tissue tropism, it has been tested in over 20 clinical trials to treat a wide variety of monogenic diseases<sup>82,83</sup> with therapeutic success achieved in the retinal degenerative disorder, Leber congenital amaurosis.<sup>84,85</sup>

## Gene Transfer Studies for Hemophilia A

The prevalence of hemophilia A is approximately six times more than hemophilia B, so it is quite pertinent to have curative gene therapy options for hemophilia A. The *F8* cDNA is 7.3 kb in size, which is much larger than 1.4 kb for *F9* cDNA.<sup>16</sup> Due to its large molecular weight and the need for stabilization with von Willebrand factor, *F8* transgene expression has largely been pursued in the context of hepatic gene transfer.<sup>86</sup> Although circulating at low concentrations in humans (normal plasma levels are 100 to 200 ng/mL instead of 5  $\mu$ g/mL in the case of FIX), FVIII has been more difficult to express at therapeutic levels.<sup>86</sup> Recombinant single-stranded (ss) AAV vectors can package  $\sim$ 4.6 kb, therefore, the 4.3 kb-B domain-deleted (BDD) *F8* remains a preferred source for gene transfer in hemophilia A.<sup>87</sup>

Sarkar et al<sup>88</sup> reported partial correction of hemophilia A mice using a ssAAV2 vector expressing BDD-murine *F8*. They found that despite long-term phenotypic correction, plasma FVIII activity peaked to only  $\sim$ 8% and declined to 2 to 3% at 9 months, attributing these modest levels to the use of a short promoter lacking regulatory elements necessary for greater *F8* expression.<sup>89</sup> Subsequently, the same group evaluated alternate AAV serotypes 5, 7, and 8 in murine models of hemophilia A, where AAV8 serotype achieved a near 100% correction of plasma FVIII activity irrespective of the route of administration.<sup>90</sup> Jiang et al<sup>91</sup> demonstrated the efficacy and



**Table 3** Factors contributing to variation in clinical phenotype in patients with severe hemophilia

Reference	51	118	119	48	120	121	53	122	56	123	124	125	126	127	128	129	130	50, 132, 133	131
<b>Clinical features</b>																			
Age at first joint bleed																		*	
<b>Genetic factors</b>									*						*				
F8/F9 mutation																			
FVL 1691G>A	*	*		*			*	*			*	*	*	*			*		
PT 20210G>A		*		*				*				*				*			
MTHFR 677C>T		*					*	*				*					*		
F7 Arg353Gln									*										
TF 5'UTR I/D				*															
EPCR 23bp I/D																	*		
Fibrinogen $\beta$ Arg448Lys																	*		
PAI-1 4G/5G																	*		
HFE C282Y						*											*		
<b>Plasmatic factors</b>																			
Procoagulant										*								*	*
Anticoagulant	*						*	*				*	*				*	*	*
Fibrinolytic								*									*	*	*
FVIII half-life			*															*	*
<b>Cellular factors</b>					*														

\* studied

FVL-Factor V 1691G>A mutation

PT 20210G>A - prothrombin G20210A mutation.

MTHFR 677C>T mutation - methylenetetrahydrofolate reductase

TF 5'UTR I/D- Tissue factor 5' untranslated region insertion or deletion polymorphism

EPCR: Endothelial protein C receptor, exon 3, 23bp insertion/deletion polymorphism

HFE C282Y- hemochromatosis gene cysteine → tyrosine polymorphism at codon 282.

Cellular factors denote the total number of circulating endothelial cells and progenitors studied by this group.



**Figure 8** Radiological pictures of joints with and without intra-articular damage. The figure shows the radiological picture of knee joints in patients with severe hemophilia A. On the panel A, we see a severely damaged knee joint. This patient had 37 bleeds in the preceding year and all his joints were affected. His Pettersson score was 27 and the clinical score was 10. On the panel B, the radiological picture shows a normal joint architecture. This patient had no bleeds and none of his joints were affected. This is a classic example of a milder form of severe hemophilia. Even among those patients who bleed frequently (Panels C, D), some have minimal synovial reaction, but loss of articular space and flexion deformity (Panel C) while others have hypertrophic synovium but their articular space is preserved with full range of motion (Panel D).

safety of AAV-canine *F8* vectors of serotypes 2, 5, 6, and 8 in achieving long-term liver-specific *FVIII* expression, in both hemophilia A mice and dogs. Generally, a very high dose of  $>10^{12}$  vg/kg (AAV2 serotype) vector is required to obtain *FVIII* activity of 2 to 4% of normal in hemophilia A mice and dogs. This underscores the need to develop additional strategies for a successful *F8* gene transfer before it can be tested in humans.

Apart from use of AAV8 serotype-based vectors, several other strategies are being tested to optimize AAV-*F8* delivery in preclinical models. One of the promising approaches is the coadministration of a self-complementary (sc)AAV vector containing protein phosphatase 5 gene (scAAV-PP5) as a helper virus, which improves the transgene expression (>fivefold) from a conventional ssAAV vector by interfering with the transcriptional block induced by a host cell protein, FKBP52.<sup>92,93</sup> In addition, the use of exogenous agents such as proteasome inhibitors to achieve improved *FVIII* expression and immune evasion,<sup>94</sup> the optimization of *F8* transgene cassette by the use of a transsplicing vector<sup>95</sup> have also been proposed.

### Gene Transfer Studies for Hemophilia B

For several reasons, such as the small size of the *F9* cDNA and the relatively easy end-point laboratory measurements of FIX activity, hemophilia B has been a long-standing target of

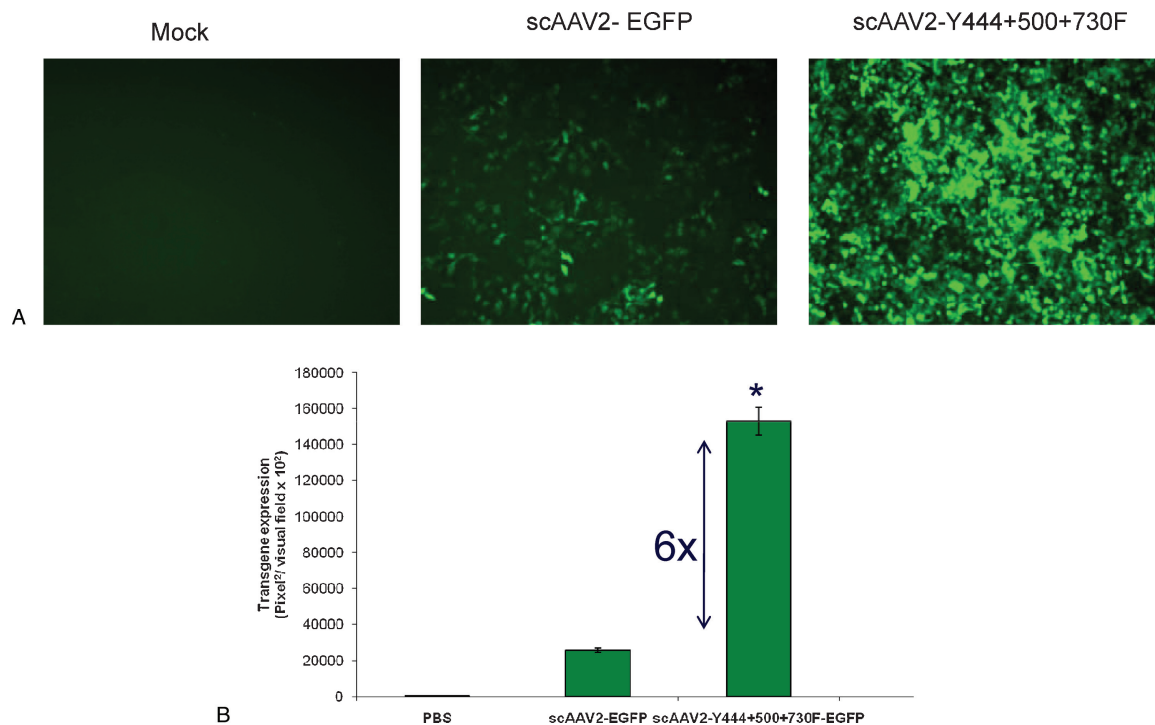
interest in the development of AAV-based gene transfer therapeutics.<sup>96</sup> Substantial multiyear correction of hemophilia B has been documented in animal models using muscle/liver-directed gene transfer.<sup>97–103</sup> Due to the stability of expression of most foreign transgene products in murine tissues coupled with absence of prior exposure of AAV in these models, AAV was considered as minimally immunogenic for many years.<sup>103,104</sup> But, it has been difficult to attain sustained expression of FIX in human clinical trials with AAV vectors.

In the first clinical trial for hemophilia B<sup>105,106</sup> (NCT00076557, <http://clinicaltrials.gov>), muscle-directed gene transfer of some 2 to  $6 \times 10^{11}$  vg/kg of AAV-F9 resulted in only a modest increase (<2%) in FIX levels in the eight patients treated on a dose-escalation model. This study also established for the first time the safety of the gene transfer protocol. Subsequently, in a Phase I/II clinical trial for hepatic FIX gene transfer (NCT00515710, <http://clinicaltrials.gov>), a subject with severe hemophilia B (<1% FIX activity) obtained a therapeutic level of expression (~10% of normal FIX levels), exactly as the canine data had predicted for this vector dose ( $2 \times 10^{12}$  vg/kg).<sup>107</sup> However, expression gradually declined to baseline between 1 and 2 months after gene transfer concomitant with a transient rise in liver enzyme levels. No antibodies against FIX were formed. Subsequent studies revealed a CD8<sup>+</sup> T-cell response to AAV2 capsid and suggested that MHC I presentation of input capsid to reactivated memory T cells led to elimination of transduced hepatocytes.<sup>108–110</sup>

In a more recent hemophilia B clinical trial (NCT00979238, <http://clinicaltrials.gov>),<sup>111</sup> the use of a scAAV8 vector to deliver an optimized *F9* transgene cassette in four hemophilia B patients has shown promise, with a sustained FIX expression of 2 to 4% at significantly lower vector doses (2 to  $6 \times 10^{10}$  vg/kg). However, in two patients who received  $6 \times 10^{10}$  vg/kg of scAAV8-F9 vector, a dose-dependent activation of capsid-specific T cells against AAV8 vectors have been noted but has not had an effect on FIX transgene expression, so far.

Although responses to AAV-FIX hepatic gene transfer in humans have not been fully reconciled with results from animal studies, a theme has emerged from clinical trials, that is, immune response and toxicity correlates with high vector doses. Transaminitis in the AAV-FIX liver gene transfer was only observed at AAV2 doses of  $\geq 5 \times 10^{11}$  vg/kg.<sup>107</sup> More recently, in an attempt to block CD8<sup>+</sup> T-cell responses against AAV1 capsid in muscle-directed gene transfer in patients with lipoprotein lipase deficiency, immune suppression with cyclosporine and mycophenolate mofetil was effective at lower vector doses ( $3 \times 10^{11}$  vg/kg) but failed to prevent IFN- $\gamma$  CD8<sup>+</sup> T-cell responses against capsid at high doses ( $1 \times 10^{12}$  vg/kg).<sup>112</sup> These data suggest that combination strategies to attenuate capsid- or transgene-specific immune responses either by developing novel AAV vectors or AAV-specific transient immunosuppression protocol would be required to achieve long-term liver-directed gene transfer of FIX. Recent developments in the field to achieve this have been encouraging.

The generation of modified AAV vectors containing mutations of the surface-exposed tyrosine residues (**Fig. 9**) has



**Figure 9** Representative image of AAV2-mediated transduction of HeLa cells with multiple tyrosine-mutant (Y444 + 500 + 730F) capsid scAAV2-EGFP vectors. (A) Cells were either mock-infected or infected by wild-type and triple mutant scAAV2-EGFP vectors (a kind gift from Dr. Arun Srivastava, University of Florida, Gainesville, FL) at a multiplicity of infection of 2000 vg/cell. Transgene expression was detected by fluorescence microscopy 48 hour postinfection. Original magnification 100 $\times$ . (B) Quantitative analyses of tyrosine mutant -AAV2 transduction efficiency in HeLa cells shows a sixfold increase in EGFP expression. \* $p < 0.05$  versus WT scAAV2-EGFP.

been shown to protect vector particles from proteasome degradation.<sup>113</sup> This tyrosine mutant vector after administration of a vector dose that only results in subtherapeutic and transient expression with wild-type AAV2 encapsidated vector has resulted in a long-term therapeutic and tolerogenic expression of human *F9* in a murine model of hemophilia B.<sup>114</sup> In addition, it has been recently identified that the host cell nuclear factor kappa B is the major regulator of innate/adaptive immune response against AAV vectors and specific inhibition of this activation can result in sustained transgene expression from AAV vectors.<sup>115</sup> However, these and other novel approaches<sup>116,117</sup> need to be rigorously scrutinized in higher animal models before they can be tested in humans.

## Conclusions

Genetic studies in hemophilia have given patients and their treating physicians better options for the management of this condition. However, there is also scope and promise for further research in this field to achieve a better outcome. Despite applying sensitive methods for mutation detection by PCR-based analysis of genomic DNA, a causative mutation is not identified in the *F8* gene in ~2 to 5% patients with severe hemophilia A. Newer strategies are needed for such cases. Epistatic factors that affect the clinical severity and inhibitor development in this condition need further definition to develop strategies for risk prediction. Further refinements in gene therapy vectors are needed to translate the success seen in preclinical models into patients with hemophilia.

## Note

A recent published report from Dr Nathwani's group has demonstrated that peripheral-vein infusion of scAAV8 vectors expressing human FIX improved the bleeding phenotype in patients with hemophilia B. Importantly, the authors have circumvented the host immune-mediated clearance of AAV-transduced hepatocytes with a short course of glucocorticoids.<sup>111,134</sup> These strategies offer renewed hope for the success of gene therapy in patients with hemophilia.

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