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Genetic testing in bleeding disorders

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Summary

The aim of molecular genetic analysis in families with haemophilia is to identify the causative mutation in an affected male as this provides valuable information for the patient and his relatives. For the patient, mutation identification may highlight inhibitor development risk or discrepancy between different factor VIII assays. For female relatives, knowledge of the familial mutation can facilitate carrier status determination and prenatal diagnosis. Recent advances in understanding mutations responsible for haemophilia and methods for their detection are presented. For reporting of such mutations, participation in external quality assessment ensures that essential patient and mutation details are routinely included and that pertinent information is incorporated in the interpretation.

Keywords

external quality assessment; haemophilia A; haemophilia B; genetic analysis; intrachromosomal inversion; missing mutations

Introduction

In families with haemophilia, identification of the underlying mutation(s) in an affected male followed by its analysis in female relatives “at risk” is the method of choice for clarification of carrier status and for prenatal diagnosis. In other inherited bleeding

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disorders, genetic analysis can help with the diagnosis when the phenotype is unclear and can provide differential diagnosis between similar disorders. Establishing the underlying mutation may also enable prediction of the risk of inhibitor development.

Haemophilia A (HA) and haemophilia B (HB) are X-linked recessively inherited coagulopathies that manifest in hemizygous males with worldwide frequencies of 1:5,000 and 1:25,000, respectively. Although heterozygous female carriers only rarely express symptoms, haemophilia carrier diagnosis provides valuable information for genetic counselling. This article describes advances in understanding of the genetics of haemophilia, particularly those made by laboratories in Argentina and Germany and it then discusses the requirement for and utility of external quality assessment (EQA) for bleeding disorder genetic analysis.

Haemophilia genetic analysis; the Argentinian experience. De Brasi

Since 1995, the Argentinian Molecular Genetics of Haemophilia Laboratory has pursued two intertwined objectives: molecular diagnosis including establishing new approaches to investigate *F8*/*F9* DNA markers and mutations and to study the genotype-phenotype relationship in an Argentinian series of haemophilia patients and carriers.

In 1993, the most common recurrent mutation in haemophilia A, the *F8* intron 22 inversion (Inv22) was described, which is implicated in 35–50% of severe-HA cases regardless of ethnic/geographic origin. Using Southern blotting, molecular diagnosis of Inv22 has been available in Argentina since 1995. Shortly after the second recurrent inversion affecting *F8;* intron 1 (Inv1) was described, our series was reported along with a review of the literature estimating that Inv1 causes less than 3% of severe-HA in Argentina [1]. Inv22 originates from homologous recombination between a 9.5 kb sequence located within *F8* intron 22 (*int22h*-1) to one of two oppositely oriented extragenic copies of *int22h* (*int22h*-2 and *int22h*-3) located by the Xq-telomere. Similarly, Inv1 originates from homologous recombination between intra- and extragenic 900bp homologs. Inv22 and Inv1 are occasionally associated with DNA gain/loss or altered DNA sequence, making their genotyping challenging. Liu et al developed a rapid analysis of Inv22 based on long distance-PCR (LD-PCR) [2]. Our variant of inverse-PCR (inverse shifting-PCR, IS-PCR) that avoids PCR amplification through the *int22h* region was devised in 2004. In this technique, genomic DNA is digested with *Bcl*I restriction enzyme, and self-ligated producing *Bcl*I-DNA circles that provide the target sequence for conventional PCR analysis [3]. The finished sequence of the human X-chromosome indicated that *int22h*-2 and *int22h*-3 are inversely oriented to one another and it became clear that only one of these sequences generates inversions through head-to-head pairing with *int22h*-1. The other copy may generate deletions (Del22) or duplications (Dup22) but not inversions by recombining with equally oriented *int22h*-1. To support experimental evidence that Inv22 type I results from recombination between *int22h*-1 and *int22h*-3 and type II between *int22h*-1 and *int22h*-2, Bagnall et al hypothesized a non-deleterious 68kb inversion mediated by large inverted repeats (50kb) exchanging *int22h*-2/*int22h*-3 locations [4]. To distinguish these genomic variants including haemophilia-causing Inv22 and Del22, and non-causing Dup22, Bagnall et al [5] developed a LD-PCR-based approach. Our laboratory modified the

previous IS-PCR-based approach, which now enables genotyping of Inv1 and Inv22 from the same template [6] and is applicable to *chorionic villus* extracted-DNA for prenatal diagnosis [7]. El-Hattab et al found that hemizygous Dup22 and Del22 associate with intellectual disability and *in utero* male lethality, respectively [8]. The extreme severity of Del22 in males resulting from loss of several genes suggests that reliable Del22 genotyping should be supported by detecting both of the specific juxtaposed sequences of Del22, and the specific DNA loss associated with the ~0.5Mb deletion [9].

Non inversion HA- and HB-causative mutations include large deletions of an exon or more that are detected by a consistent absence of contiguous exon-specific PCR products. These mutations can be characterised by PCR amplification across deletion junctions, and include both those caused by non-homologous and by homeologous recombination, e.g. that between equally oriented AluSx sequences in introns 4 and 10 of *F8* [10]. For genotyping small *F8* and *F9* mutations, high-resolution conformation sensitive gel electrophoresis (CSGE) on 37 and 8 amplimers respectively, followed by Sanger sequencing of the selected exon(s) showing anomalous CSGE-patterns detects mutations in the majority of subjects. These procedures allowed characterisation of insertions/deletions of 1–10bp (indels) mostly associated with frameshifts, and nucleotide substitutions predicting missense, nonsense or RNA splicing defects [11, 12]. Once a proband's sequence variant has been determined, the genotype-phenotype correlation can be investigated following the Clinical Molecular Genetics Society Practice Guideline for Unclassified Variants [13] along with 3D-structural modelling [14].

In conclusion, the characterisation of causative haemophilia mutations is essential to provide the best information for carrier and prenatal diagnosis, for genetic counselling and to predict phenotypic characteristics, such as genotype-specific inhibitor risks.

Missing mutations in Hemophilia A. El Maarri, Pezeshkpoor & Oldenburg

In almost all HA patients, the deficiency of factor VIII (FVIII) activity can be traced to mutations in *F8*. With advances in molecular diagnostic techniques and particularly in sequencing technology in the last decade, it has become possible to sequence all *F8* exons in all patients, for an affordable cost even in small clinics. Therefore, it was expected that the molecular defect in *F8* would be detected in every HA patient. However, it became clear that this was not the case. At that point, different centers started to characterize these patients and document their clinical phenotypes.

For such "mutation-negative" cases, the first step in the investigation is to verify the HA phenotype. This question can been addressed in two ways; firstly, to verify that only FVIII levels are decreased in these patients; secondly, to exclude combined FV/FVIII deficiency that may be caused by mutations in *LMAN1* or *MCFD2* that may alter the secretion pathways of both FVIII and factor V. In addition, defects in *VWF* should be excluded, as any sub-optimal binding of FVIII to its plasma carrier (VWF) would lead to reduced FVIII activity as observed in von Willebrand disease type 2N. Finally, the two *F8* inversions and deletions, duplications and exonic mutations are excluded by established tests [5, 6]. Only

after all the above possibilities are excluded is further detailed analysis described below recommended.

The first molecular clue to identify the genetic defects in mutation-negative patients was described in 2008 [15]. Large duplications were identified in some of these patients [16]. Such duplications of entire exons escape detection when individual exons are sequenced. Therefore these duplications are only efficiently detected by multiplex ligation-dependent probe amplification (MLPA) [15], or possibly by array comparative genomic hybridization.

In 2011, Castaman et al identified intronic mutations lying deep in *F8* introns causing abnormal *F8* splicing leading to a decrease in the levels of normally spliced *F8* mRNA [17]. They identified these mutations based on their effect on ectopic *F8* mRNA only after sequencing the neighboring genomic regions. Recently we developed a detailed protocol for detecting the molecular defects in “mutation negative" patients [18, 19]. A systematic stepwise investigation to detect all possible changes in the *F8* locus is proposed. The first step is to exclude gross rearrangements caused by gross duplications, recombinations or inversions. Such rearrangements could leave the exons intact but in the wrong order. Such rearrangements can be excluded by the long-range (LR) amplification of overlapping amplicons that cover the whole *F8* genomic locus. Using this strategy, one patient with a rearranged genomic structure due to recombination between inverted repeats was identified [20]. The second step is to search for abnormal splicing by RT-PCR that covers all exon-exon boundaries. Once abnormal splicing is detected then the involved intronic regions surrounding the breakpoints are sequenced to identify the intronic mutations involved [17]. If no mutation is detected then a third step is to sequence all the LR-PCR products using a massively parallel sequencing approach (next generation sequencing). The advantage of this approach is the rapid identification of all variants in the locus at once [19]. Novel variants can then be further investigated for their effect on splicing (that may have been missed by previous RT-PCR) or for enhancer/silencer effect by functional assays. By undertaking these steps, mutations are expected to be identified in a proportion of previous “mutation negative” cases.

Quality assurance in genetic testing; David Perry on behalf of UK NEQAS BC

In contrast to phenotypic data, the results of genotypic assays are unequivocal with no borderline values. Accordingly, there is an acceptance of the accuracy of such data by referring physicians. However, several studies have shown that mutation detection in common with any analytical test has an intrinsic error rate [21, 22]. A failure to correctly identify a mutation or to interpret its significance can have major implications for an individual and their family members.

In the UK, participation in a recognised EQA scheme is a requirement for laboratory accreditation and a number of such schemes exist, coordinated through UK National External Quality Assessment Service (NEQAS). The only EQA scheme for the genetics of the heritable bleeding disorders in the EU is that administered by UK NEQAS for Blood Coagulation (UK NEQAS BC).

In 1998, UK NEQAS BC established a pilot scheme to assess the performance of laboratories in genetic testing [23]. In 2003, a Special Advisory Group (SAG) on Haemophilia Molecular Genetics for UK NEQAS BC was established, with the remit of developing a robust EQA scheme for both UK and international participants. The scheme was designed to address three fundamental aspects of genetic testing: 1. The correct identification of the patient and their reason for referral; 2. The correct identification of the causative genetic mutation(s); 3. The interpretation and reporting of genetic data in the context of the any relevant clinical and family data.

Between 2003–2013, 18 exercises were undertaken (Table 1), the most recent was circulated in June 2013 (Exercise 22). The disorders and underlying genetic mutations evaluated by UK NEQAS have been chosen to reflect the routine workload in molecular genetics laboratories. Ten exercises have involved analysis of the *F8* gene of which three were for the Inv22, one for Inv1 and the remainder various sequence variations. Four exercises involved analysis of *F9*, two for a promoter mutation (not associated with HB Leiden) and two for missense mutations. Finally, three exercises involved analysis of missense mutations within *VWF*.

A formalised template for scoring reports was introduced in 2003. This template was employed to introduce a degree of objectivity to a subjective assessment process. The template is based upon recommendations of the UK Clinical Molecular Genetics Society (CMGS) best practice guidelines on report writing [24] with a maximum score of 2 marks for each of three sections; namely clerical accuracy, genotyping and interpretation. In each category, information considered “essential” or “recommended” has a different weighting and this weighting is established in advance of the laboratory report assessment. A score of <1 in any one category constitutes a “fail” in that exercise. Reports are scored independently by four experienced individuals and a consensus subsequently reached. Laboratories that are registered with the scheme who either fail to submit a report or do so outside the allocated turnaround time of 6 weeks (chosen to reflect UKHCDO recommendations) will also fail. A fail in any exercise generates a letter from the Director of UK NEQAS BC with the offer of assistance. Each participating laboratory is assigned a unique identification number that allows the continuing performance of each lab to be reviewed. The identification of participating labs is unknown to the reviewers.

All participating laboratories use the mutation nomenclature system proposed by the Human Gene Variation Society (HGVS) [25] that requires all sequence variations to be defined in relation to a specified reference sequence and the “A” nucleotide of the ATG-translation initiation codon to be numbered as +1 with the protein sequence representing the primary translation product numbered from the initiator methionine and therefore, includes signal peptide sequence. For some genes and proteins, this requires renumbering and makes reference to previously described mutations challenging. Laboratories are, therefore, encouraged to include legacy nomenclature as a number of published mutations including some of those listed in the on-line locus specific mutation databases remain in the “legacy” format.

Of the 18 exercises circulated between 2004 and 2013, 13 involved the use of whole blood and five DNA derived from immortalised cell lines. Whole blood samples distributed internationally yield sufficient quantity and quality of DNA for analysis even when transport delays of several days occur.

The majority of laboratories in each exercise achieve full marks, and failing is unusual. Reasons for failing an exercise include clerical inaccuracies (e.g., a failure to include unique identifiers for each individual(s)); genotyping errors (e.g. incorrectly numbering the mutation or predicted amino acid substitution; failing to identify a mutation that was present; identifying a second mutation that was not present), and finally interpretation errors. Many of the errors that have led to a fail were based upon incorrect interpretation, e.g. failure to answer the clinical question; incorrectly assigning carrier status (or not) to an “at-risk” female; failing to establish the significance of a novel mutation and failing to consider the possibility of mosaicism.

The aim of EQA schemes is to highlight problems and deficiencies in laboratory procedures. This EQA scheme has led to a more uniform inclusion of information into reports and a standardised use of mutation nomenclature. There are currently 27 laboratories registered for this scheme: 24 in the EU of which 12 are in the UK and three in non-EU countries. The scheme has received very positive feedback from participants and is seen as a fundamental part of good laboratory practice.

Summary

The article has demonstrated the continuing development of molecular genetic analysis of hemophilia directed towards identifying the causative mutation in virtually all patients and for mutations identified, that participation in an EQA scheme promotes reporting and interpretation of the effect of these mutations to a recognized international standard.

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Table 1

A summary of the exercises circulated between 2004 and 2012

Exercise Number	Year	Gene	Mutation	Material
4	2004	Paper Exercise – *F8* Intron 22 Inversion		N/A
5	2004	*F8*	Intron 1 inversion	Whole blood
6	2005	*F8*	Exon 14 2bp deletion	Whole blood
7	2005	*F8*	Intron 22 inversion	Whole blood
8	2006	*F8*	Exon 19 missense mutation	Cell line DNA
9	2006	*F9*	Promoter mutation	Whole blood
10	2007	*VWF*	Exon 28 missense mutation	Whole blood
11	2007	*F8*	Exon 25 missense mutation	Whole blood
12	2008	*F8*	Exon 19 missense mutation	Cell line DNA
13	2008	*F9*	Promoter mutation	Whole blood
14	2009	*F8*	Exon 8 missense mutation	Whole blood
15	2009	*VWF*	Exon 28 missense mutation	Whole blood
16	2010	*F8*	Intron 22 inversion	Cell line DNA
17	2010	*VWF*	Exon 46 missense mutation	Whole blood
18	2011	*F9*	Exon 8 missense mutation	Whole Blood
19	2011	*F8*	Intron 22 inversion	Cell line DNA
20	2012	*F8*	Exon 14 nt duplication	Whole Blood
21	2012	*F9*	Missense mutation	Whole Blood
22	2013	*F8*	Intron 1 inversion	Cell line DNA