Molecular Genetic Diagnosis of the Inherited Bleeding Disorders: Are We Close to the Perfect Test?

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This issue includes a list of reviewers who have evaluated manuscripts submitted to the journal over the past year. This will now be an annual feature and is the journal's acknowledgement of our expert reviewers' effort and time.

In this issue, Khaniani et al. [1] from Tabriz in Iran describe their experiences with the genetic diagnosis of haemophilia A in patients and female carriers. Using an array of molecular techniques including inverse-shifting PCR, long-range PCR, eight multiplex PCRs and Sanger sequencing, the authors achieved a mutation detection rate of 48 %, i.e. in 24 out of 50 haemophilia A patients. The mutations detected were not unlike those reported from India, although yields have been higher in Indian studies [2]: 22 of the Iranian cases had the intron 22 inversion, while one each had intron 1 inversion and a point mutation in exon 14. The study highlights the challenges and limitations of conventional testing strategies for the diagnosis of inherited bleeding disorders and the geographical variability.

Conventional strategies rely on step-wise performance of tests of increasing complexity. While this scientifically valid approach saves costs, it causes delays affecting not just the patients but also in prenatal diagnosis. Individual tests too have specific limitations. Hemostatic screening tests (PT, aPTT and TT) are non-specific and insensitive to mild factor deficiencies [3]. Tests of intermediate complexity like factor assays, platelet aggregometry and flow cytometry are dependent on pre-analytical variables [4]. More definitive tests like multimer-analysis, platelet

nucleotide release and vWF-collagen/FVIII binding assays are technically complex with limited availability in only selective referral centers.

Cheap and rapid molecular diagnostics, if available, would therefore be extremely valuable in bleeding disorders. However, as the Khaniani paper [1] illustrates, the heterogeneity of molecular lesions in even the commoner diseases like haemophilia A and B, von Willebrand disease and the commoner platelet function defects make it technically complex to use them as first-line diagnostic tests. Current applications of molecular testing in these disorders are therefore largely restricted to carrier detection and antenatal diagnosis [5]. Less commonly, molecular tools may be applied for the distinction of phenocopies (i.e. type 2 vWD from platelet-type vWD, or moderate haemophilia A from type 2N vWD) or sometimes to obtain clinically relevant genetic information, like haemophilia mutation-based inhibitor risk profiling [2] or to confirm *F9* promoter mutations causing haemophilia B Leyden [5] for prognostication.

Sanger sequencing is now a convenient diagnostic tool for many monogenic disorders. Bi-directional sequencing of the entire coding region, intron/exon junctions (i.e. splice sites), and proximal promoter elements of small genes like *F9* (34 kb in size with a 2.4 kb coding region) is diagnostic in 97 % cases of haemophilia B [6]. *F8* and *vWF* genes however, are much larger in size (186 and 178 kb, respectively), have numerous exons (27 and 52, respectively) that are separated by long intronic sequences, large coding sequences (~9 kb in both) and frequently display alternative splicing making it extremely difficult to evaluate them by Sanger sequencing [5–7]. An alternative approach is to first use a screening technique like the conformation-sensitive gel electrophoresis (CSGE) or multiplex ligation-dependent probe amplification (MLPA)

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or array CGH followed by sequencing of only the implicated exons [6, 8]. However, the additional techniques obviously increase effort, time and cost.

Later generation sequencing techniques have overcome many of the shortcomings of the conventional molecular assays. Sensitivity is excellent in inherited disorders, and costs have declined over time, especially with multiplexing. Research using genome-wide linkage analysis in families with specific phenotypes led to the identification of *MCFD* or *LMAN1* in combined FV + FVIII deficiency [9] and *VKORC1* in Vitamin K-dependent coagulation factor deficiency, type 2 [10]. For clinical diagnostics, there may yet be some distance to cover. Next-generation targeted capture probes against genomic regions already known to be involved in rare heritable bleeding disorders have been evaluated. The Thrombo-Genomics project, whose high-throughput sequencing panel detects variants in 63 genes implicated in bleeding and thrombotic states could, in a recent study on 300 patients, identify 100 % of patients with a previously identified pathogenic variant, 92 % of undiagnosed patients in whom a specific phenotypic diagnosis was suspected, but only 11 % of those without a suspected diagnosis [11]. The remaining 24 % (73/300) cases without a molecular label even after NGS are planned to be submitted to the 100,000 genomes project and many may well prove to be novel disease entities.

As we identify more and more mutations in the heritable bleeding disorders, it has been suggested that a possible cost-effective laboratory workflow could be the use of an upfront NGS panel including 50–100 specific genes with subsequent software interrogation of only the genes relevant to the symptoms and phenotypes of individual patients [8]. This would help in disorders like the Hermansky–Pudlack syndrome or FXIII deficiency with more than one gene requiring analysis (9 and 2 respectively in the examples cited) and also in cases with a phenotypic overlap between disorders, for e.g. in patients with a mild mucocutaneous bleeding phenotype but normal screening coagulogram [5, 8, 11]. In conclusion therefore, it seems safe to

predict that diagnostic approaches to the molecular diagnosis of inherited bleeding diatheses are likely to undergo a sea-change over the next decade.

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