

A Modern Approach to the Molecular Diagnosis of Inherited Bleeding Disorders

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Abstract

Diagnosis of inherited bleeding disorders (IBDs) requires careful evaluation of patients' clinical features and assessment of bleeding with the appropriate tools. Definitive diagnosis can be achieved by platelet functional assays for some disorders, but, in most cases, these tests are not sufficiently sensitive or specific. Moreover, tests are hindered by the need for relatively large samples of fresh blood. Identifying the underlying molecular defect not only facilitates a definitive diagnosis of an IBD, but may also help with the clinical prognosis, and enable genetic counseling. Until recently, molecular diagnosis has relied on Sanger sequencing of single or small numbers of candidate genes that are already known to cause some inherited platelet disorders. High-throughput sequencing (HTS) technologies have revolutionized molecular diagnosis of human disease, since they allow simultaneous, rapid and affordable investigation of multiple genes. HTS is being widely implemented and is rapidly improving the molecular characterization of IBDs in routine clinical practice.

Keywords: Inherited platelet disorders; Inherited rare bleeding disorders; Hemophilia A and B; High-throughput sequencing

Introduction

Inherited bleeding disorders (IBDs) are a heterogeneous group of hemorrhagic diseases caused by variants in genes involved in megakaryopoiesis and platelet function, or in those related to coagulation factor deficiencies, such as inherited platelet disorders (IPDs), von Willebrand disease (VWD), hemophilia A (HA) and B (HB), and rare bleeding disorders (RBDs) [1,2]. Genetic diagnosis is not only essential for the accurate diagnosis of IBDs, which facilitates better clinical care, prognosis and preventive treatments, but also for identifying patients with a high risk of malignancy, and for determining carrier status. This information facilitates genetic counseling, clarifies differential diagnosis between similar phenotypes, and predicts the likelihood of inhibitor development in cases of HA [3,4]. Successful genotyping has customarily relied on Sanger sequencing (SS) of candidate genes, guided by phenotyping [4,5]. However, this approach is currently costly and time-consuming, and is not applicable to disorders whose phenotype-based diagnosis is not straightforward and for which there is no obvious candidate gene to analyze [5-7]. In particular, diagnosis of inherited platelet disorders (IPDs) is usually hampered by a lack of distinctive clinical and laboratory features, and genetic diagnosis remains a challenge even with expert analysis, such that it has been attained in fewer than half of patients [5-8]. On the other hand, molecular testing based on SS of candidate genes in clear phenotypes, such as in some IPDs (e.g., Hermansky-Pudlak syndrome [HPS], Glanzmann Thrombastenia [GT]) and RBDs, is also hindered by the wide range of exons, the large size and complex genomic organization of the genes involved, and by the heterogeneity of the pathogenic variants underlying these disorders [5-10]. Customarily, molecular diagnosis of HA starts by screening for the presence of intron 22 and intron 1 inversions (IVS-22 and IVS-1) in severe patients (by inverse PCR and PCR amplification), followed by SS in negative IVS-22 or IVS-1 severe HA patients. Similarly, the remaining HA and all HB patients are usually analyzed by single-gene sequencing [2,4], which is laborious due to the length and wide range of exons of *F8* [2,4]. Moreover, deep intronic variants have been involved in cases of mild-to-moderate HA phenotypes [11], and low factor VIII levels in HA patients without identified *F8* variants may be caused by VWF variants [12].

Literature Review

The recent advent of high-throughput sequencing (HTS) —whole-genome sequencing (WGS) and whole-exome sequencing (WES)— and targeted sequencing of pre-specified genes, has revolutionized the field of genetic diagnosis and is rapidly becoming recognized as a unique tool in clinical practice [4,5,7,9,10]. WGS determines the complete genomic information, including all the coding and non-coding sequences, although the method is relatively fast and requires samples that are simple to prepare; the large amount of data needs an extensive and complex filtered workflow to establish the disease-causing variants [13]. WES and a panel of pre-specified genes target all exons from all genes (WES) or only regions from genes of pre-defined interest (panel). These approaches require library preparation protocols, design, and enrichment assays to achieve accurate variant calling that enables the relevant causative variants to be defined [10,13]. Panels offer better base-pair coverage, running times, costs and dataset handling than other HTS applications, such as WGS and WES. In any case, HTS is emerging as a valuable tool in molecular diagnosis and is increasingly important in the first-line of diagnostic investigation of these diseases [4,5,7,9,10,13]. In the context of IBDs, the initial steps in targeted DNA sequencing were undertaken by the ThromboGenomics (TG) and UK Genotyping and Phenotyping of Platelets (UK-GAPP) consortia [5,9,14-16]. TG reported on a targeted HTS panel platform, originally comprising 63 genes (Tier 1) for the diagnosis of inherited thrombotic conditions and IBDs, while the UK-GAPP study used WES to investigate IPDs, especially inherited thrombocytopenias [5,9].

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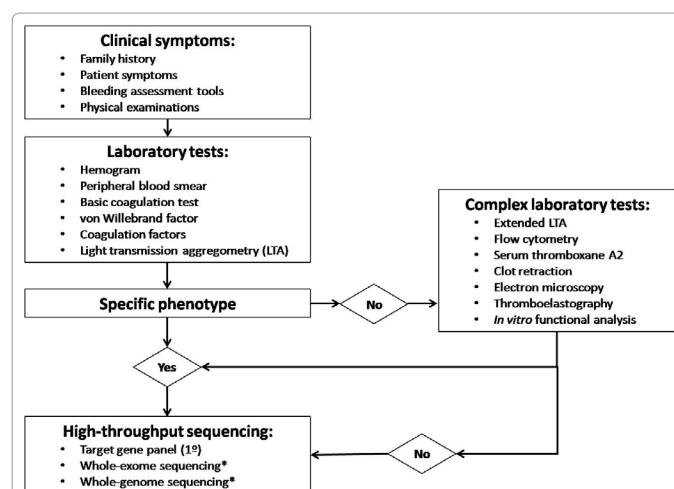
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Recently, TG incorporated 23 new genes discovered by WES or WGS, such as *SRC*, *DIAPH1*, *TPM4*, *ABCC4* and *KDSR*; in total, 3449 DNA samples were analyzed by a 79-gene TG platform and demonstrated the success of HTS approaches in providing a genetic diagnosis for patients with well-defined inherited platelet or coagulation defects [17]. Other groups have incorporated HTS technology into the molecular diagnosis of HA, sequencing the entire *F8* gene, and detecting deep intronic variants that may be the causative variant of mild HA and combined *F8/VWF* genes [11,12]; the Scandinavian group has used the WES approach to identify genes related to IPD and Ehlers-Danlos syndrome [18]. In Spain and Portugal, HTS has been successfully used for the genetic analysis of IBDs [4,7,10].

In the context of IPDs, we have recently reported the design and implementation of a multigenic HTS platform, based on a panel of 72 IPD-related genes, which has greatly aided our diagnostic process, resulting in a conclusive molecular diagnosis (70%) in the largest series of IPD patients investigated so far in the Iberian Peninsula [19]. We found 57 candidate variants in 28 genes, 70% of which had not previously been described. This approach allowed us to identify a novel microdeletion in exon 9 of the *WAS* gene in Wiskott-Aldrich syndrome in a child presenting with macrothrombocytopenia [20], two novel variants of the *ABCG5* gene that caused sitosterolemia in a 46-year-old female with life-long macrothrombocytopenia and xanthelasmas [21], and four novel variants in the *RASGRP2* gene that affected platelet CalDAG-GEFI expression and function in patients with severe bleeding diathesis [22,23]. It is important to note that finding a novel gene variant is not by itself definitive proof that it is the cause of the disease. Analysis of the association of the variant with the disease in the family, structure/function studies of the protein, and studies of mouse or zebrafish models are often necessary [19]. We designed and applied a 23-gene panel related to RBDs to identify the disease-causing variant in 20 patients with bleeding and coagulation factor deficiencies. Twenty-one pathogenic variants were found, most of which were of the missense type (18), and six were novel variants affecting the *F8*, *FGA*, *F11*, *F10* and *VWF* genes [10]. We have also incorporated and evaluated the usefulness of a molecular algorithm employing an HTS approach for sequencing the complete *F8*, *F9* and *VWF* genes for patients with factor VIII or IX deficiencies. This algorithm contemplates the detection of IVS-1 and IVS-22 by classical methods, the sequencing of the entire *F8*, *F9* and *VWF* genes by HTS, and multiplex ligation-dependent probe amplification analysis. The proposed algorithm had an overall success rate of 99% [4]. Finally, the complete coding of *VWF* by HTS technology was carried out in two multicenter studies, including 556 Spanish and 92 Portuguese patients [24,25].

Discussion

Although these reports reinforce the feasibility of introducing HTS into the mainstream laboratory for the genetic diagnosis of IBDs (Figure 1), there are several limitations [4,9,10,13,14,18]: a) the great heterogeneity of the molecular pathology underlying IPDs hampers the appropriate interpretation of the pathogenicity of candidate genetic variants and remains a major challenge, especially for novel variants and those of uncertain significance; b) HTS cannot completely replace clinical evaluation and laboratory phenotyping, because it may not be able to identify the causative variant in patients with a strongly specific phenotype; c) rearrangements, inversions and large deletions/insertions, mainly in HA, cannot be identified in many cases; d) the need to examine experimental, *in vitro* or animal models to determine the pathogenicity of the novel variants; and e) there is a clear need for a consensus guidance to report HTS results, including incidental findings [26].



Note: The diagnostic process consists of obtaining: an accurate family history of bleeding, thrombocytopenia or malignancies; information about clinical symptoms related to bleeding, systemic disease and thrombocytopenia, and from physical examinations. Bleeding assessment tools (ISTH-BATs) are recommended. Laboratory investigation includes hemogram, peripheral blood smear and basic coagulation tests, and von Willebrand antigen and activity and other coagulation factor tests. We recommend introducing an HTS gene panel at any moment in the diagnostic flowchart for patients with specific or nonspecific phenotypes in laboratory testing. *WES or WGS are most strongly indicated in patients with an unambiguous clinical suspicion of IBD but with a nonspecific laboratory phenotype, and for whom the HTS gene panel fails to identify candidate variants.

Figure 1: Incorporation of genetic analysis by HTS into the diagnostic process for inherited bleeding disorders.

Conclusion

In conclusion, the simultaneous analysis of several genes by HTS, target gene panel or WES, is a powerful tool with which to meet the increasing demand for medical accuracy in the era of precision medicine. These cost-effective technological advancements are revolutionizing the ability of medical professionals to diagnose IBDs and to improve the provision of care to patients with these disorders.

Conflict of Interest Disclosure

The authors declare no competing financial interests. This study was supported by research grants from the Gerencia Regional de Salud (GRS 1647/A/17 and GRS 1650/A/17), ISCIII & Feder (PI17/01966), CIBERER CB15/00055, Fundación Séneca (19873/GERM/15) and Sociedad Española de Trombosis y Hemostasia (SETH).

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Author Contributions

All authors contributed to the conception and design of the study, the acquisition, analysis or interpretation of the data, drafting the manuscript or critically revising it for important intellectual content. All authors approved the final version for publication.

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