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Next-generation Sequencing in Bone Marrow Failure Syndromes and Isolated Cytopenias: Experience of the Spanish Network on Bone Marrow Failure Syndromes

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Abstract

Inherited bone marrow failure syndromes (IBMFSs) are a group of congenital rare diseases characterized by bone marrow failure, congenital anomalies, high genetic heterogeneity, and predisposition to cancer. Appropriate treatment and cancer surveillance ideally depend on the identification of the mutated gene. A next-generation sequencing (NGS) panel of genes could be 1 initial genetic screening test to be carried out in a comprehensive study of IBMFSs, allowing molecular detection in affected patients. We designed 2 NGS panels of IBMFS genes: version 1 included 129 genes and version 2 involved 145 genes. The cohort included a total of 204 patients with suspected IBMFSs without molecular diagnosis. Capture-based targeted sequencing covered > 99% of the target regions of 145 genes, with more than 20 independent reads. No differences were seen between the 2 versions of the panel. The NGS tool allowed a total of 91 patients to be diagnosed, with an overall molecular diagnostic rate of 44%. Among the 167 patients with classified IBMFSs, 81 patients (48%) were diagnosed. Unclassified IBMFSs involved a total of 37 patients, of whom 9 patients (24%) were diagnosed. The preexisting diagnosis of 6 clinically classified patients (6%) was amended, implying a change of therapy for some of them. Our NGS IBMFS gene panel assay is a useful tool in the molecular diagnosis of IBMFSs and a reasonable option as the first tier genetic test in these disorders.

Introduction

Inherited bone marrow failure syndromes (IBMFSs) are a group of congenital rare diseases with high genetic heterogeneity that are mainly characterized by bone marrow failure, phenotypic findings, and cancer predisposition.[1,2] A large number of IBMFSs have been described so far, with Fanconi anemia (FA), dyskeratosis congenita (DC), Diamond-Blackfan anemia (DBA), and Shwachman-Diamond syndrome (SDS) being among the most common in this group of syndromes.[3]

The correct genetic diagnosis of IBMFSs is crucial to predict the disease course, to provide genetic counseling, and to select the most appropriate treatment, including hematopoietic stem cell transplantation (HSCT) from healthy donors. Many patients with IBMFSs remain underdiagnosed, mainly because

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they are characterized by a wide range of syndromes and, at the same time, have a high degree of phenotypic overlap. Consequently, despite having an adequate clinical orientation based on the patient's medical history and examination, it is possible that no conclusive diagnosis can be made in many of these patients.

Until recently, molecular diagnosis in clinical practice was limited to the conventional sequencing of the most frequently mutated genes, using techniques such as Sanger sequencing, based on clinical suspicion. However, nowadays, the large number of genes associated with IBMFSs makes mutation screening based on conventional techniques difficult, since gene-to-gene study strategies are expensive and slow.[4] On the other hand, this approach is not applicable to disorders whose phenotype-based diagnosis is not straightforward and for which there is no obvious candidate gene.

The recent advent of next-generation sequencing (NGS) techniques has begun to revolutionize the field of genetic diagnosis and is rapidly becoming established in clinical practice.[5] These tools allow thousands of DNA fragments to be obtained in a single reaction, making it possible to analyze a high number of genes in a short period of time.[6,7] NGS techniques include targeted gene sequencing (panels), whole-exome sequencing (WES), and whole-genome sequencing (WGS), each of them with distinct advantages and disadvantages. Compared with WES/WGS, targeted gene sequencing is a relatively inexpensive approach for identifying pathogenic mutations and has a relatively higher sequence coverage.

The use of targeted sequencing or gene panels has already been used in genetically heterogeneous diseases, including IBMFSs.[8–11] Most of these studies focused on specific syndromes, such as FA, DBA, or DC. Nevertheless, there are few published studies reporting the overall genetic diagnosis in heterogeneous groups of patients with IBMFSs. With respect to IBMFSs, NGS is emerging as a valuable tool for the molecular diagnosis of these disorders and is increasingly important as the first-line of the molecular diagnosis.[12,13]

Our hypothesis was that a panel of IBMFS genes could be used as an initial screening test to provide precise and clinically relevant molecular diagnoses in a timely manner and at a reduced cost.

Herein, we report the design and implementation of a NGS platform for the genetic diagnosis in 204 patients with suspected IBMFSs. The application of this approach has improved our diagnostic process, resulting in a molecular diagnosis in the largest series of patients with IBMFSs in Spain.

Materials and methods

Patients

A total of 214 consecutive patient samples from 47 Spanish centers were received between July 2015 and July 2018. Written informed consent for genetic investigation and research was provided by either the patients or their legal guardians. The sample collection process is described in Supplementary Figure S1 (http://links.lww.com/HS/A135).

All patients who were referred for suspected IBMFSs, according to the criteria of the reference specialist, were included. Clinical data was received from each patient. These clinical data included the clinical suspicion, description of cytopenia/s, clinical findings, family history, bone marrow investigations, and other investigations. The criteria used to define the suspicion of an IBMFS are described in Supplementary Table S1 (http://links.lww.com/HS/A135).

Clinical diagnostic suspicions included DBA, FA, DC, SDS, congenital amegakaryocytic thrombocytopenia (CAMT), hereditary thrombocytopenia (HT), severe congenital neutropenia (SCN), myelodysplastic syndrome (MDS), other cytopenia, and other IBMFSs. Depending on the clinical suspicion, the patients were divided into 2 groups: group 1 included those referred for a suspected classified IBMFS (DBA, FA, DC, SDS, HT, SCN, or MDS; Table 1) and group 2 included all those referred for unclassified IBMFSs (other cytopenia and other IBMFSs; Table 2).

NGS gene panel assay

DNA was extracted from peripheral blood following routine methods. We designed an NGS assay for a comprehensive panel of 145 IBMFS genes published as of September 2016. We devised 2 versions of the panel: version 1 (v1) included a total of 129 IBMFS genes and version 2 (v2) was updated to include 16 further genes (Supplementary Table S2, http://links.lww.com/HS/A135). Detailed panel design and bioinformatic analysis are presented in Additional file 1: Supplemental Material (http://links.lww.com/HS/A135).

Strategy used for copy number variant analysis

Copy number variation (CNV) was detected with an in-house tool (LACONv) that is based on GC-correction followed by a normalized weighted ratio of depth-of-coverage with respect to controls. The tool was optimized for targeted capture sequencing and CNV intervals were determined if "dose" value and its *z* value were above/below some threshold that was adjusted experimentally with a reference set. The CNVs of each patient were analyzed and those deletions/insertions detected were confirmed using other techniques (multiplex ligation-dependent probe amplification [MLPA] and comparative genomic hybridization [CGH] arrays).

Variant analysis and filtering strategy

The variant analysis and filtering strategy followed the guidelines of the American College of Medical Genetics and Genomics (ACMG)[14] and the publications previously reported.[12,13,15]

The variants that were previously reported to be pathogenic or disease causing in public databases (Supplementary Table S3, http://links.lww.com/HS/A135). These variants were classified according to the ACMG guidelines into 5 groups: likely benign, benign, variants of unknown significance (VUS), likely pathogenic, and pathogenic. Those variants classified as benign or possibly benign were not considered as causative. The other variants classified as VUS, likely pathogenic, and pathogenic were discussed with physicians who were experts in that particular disease area, and genetic diagnoses were made based on the mode of inheritance of each disease, type of mutation, and patient's clinical findings. All variants detected were validated by Sanger sequencing. family studies were performed to determine the segregation or "de novo" origin of the detected variants. The algorithm used to filter variants is described in Figure 1.

Results

Quality parameter analysis and variant detection

Capture-based targeted sequencing covered > 99% of the target regions of 145 genes, with more than 20 independent reads. *DDX11* and *RPS17* did not reach the quality criteria, because the capture-based targeted sequencing of these genes covered < 80%. The average read depth was 367x (0–637). In addition, 95% of the targeted regions were covered > 50x. Variants identified in v1 included in group A and those considered as probable causative variants included in group B were validated through Sanger sequencing. Among the 214 patient's cohort, 10 were positive controls (Supplementary Table S4, http://links.lww.

Table 1

Genotyping Patients With classified IBMFSs (Group 1).

Clinical Diagnosis	No. Patients Tested	No. Patients Genotyped	Mutated Genes	No. Cases With Mutations in This Gene	No. Mutations in This Gene	Novel Mutations in This Gene
Diamond-Blackfan anemia	45	31	*RPL11*	8	8	4
			RPL5	6	6	6
			RPS19	6	6	0
			RPS24	1	1	1
			RPS26	5	5	2
			RPS29	2	2	2
			RPS7	2	2	2
			Del 15q	1	1	0
Dyskeratosis congenita	12	8	*DKC1*	3	3	1
			RTEL1	3	4	2
			TERT	2	2	1
Fanconi anemia	28	16	*FANCA*	13	19	3
			FANCB	1	1	1
			FANCG	2	3	0
Hereditary thrombocytopenia	38	16	*ANKRD26*	1	1	1
			CYCS	2	2	2
			GP1BA	2	2	2
			ITGA2B	1	1	1
			ITGB3	2	2	2
			MYH9	5	5	0
			RBM8A	1	2	1
			TUBB1	1	1	1
			WAS	1	1	1
Severe congenital neutropenia	25	3	*ELANE*	1	1	0
			JAGN1	1	1	0
			TCIRG1	1	1	1
Myelodysplastic syndrome	5	3	*RPL11*	1	1	0
			TERT	1	1	0
			CEBPA	1	1	1
Shwachman-Diamond syndrome	6	1	*SBDS*	1	2	0
Congenital amegakaryocytic thrombocytopenia	5	2	*MPL*	1	1	1
			TERT	1	1	0
Other congenital anemia	3	1	*KLF1*	1	1	1

IBMFS = inherited bone marrow failure syndrome.

Table 2

Genotyping Patients With Unclassified IBMFSs (Group 2).

Clinical Diagnosis	No. Patients Tested	No. Patients Genotyped	Mutated Genes	No. Cases With Mutations in This Gene	No. Mutations in This Gene	Novel Mutations in This Gene	Final Diagnosis
Aplastic anemia	2	2	*FANCA*	1	2	1	Fanconi anemia
			TINF2	1	1	0	Dyskeratosis congenita
Bilineage cytopenia	2	2	*RECQL4*	1	2	0	Rothmund-Thomson syndrome
			MPL	1	2	2	CAMT
Bone marrow failure	21	4	*AP3B1*	1	1	1	Hermansky-Pudlak syndrome type 2
			MPL	1	1	1	CAMT
			PUS1	1	1	1	Sideroblastic anemia
			G6PC3	1	1	1	Dursun syndrome
Trilineage cytopenia	3	1	*TERT*	1	1	1	Dyskeratosis congenita

CAMT = congenital amegakaryocytic thrombocytopenia; IBMFS = inherited bone marrow failure syndrome.

com/HS/A135). All the positive control's variants were detected by the NGS assay.

We identified 104 variants in 90 patients with no previous molecular diagnosis. Using v1, we detected 58 variants in 47 patients, and using v2, we detected 46 variants in 43 patients.

Among the 104 detected variants, 56 variants had previously been reported and 48 variants were novel. The variants were classified according to the ACMG guidelines. We detected 11 pathogenic variants, 12 likely pathogenic variants, and 25 VUS. Regarding the mutation type of all variants, 50 were missense, 30 were indels, 14 were splicing variants, and 10 were nonsense.

Genetic diagnosis by targeted sequencing in the total group

Of the 204 patients with IBMFSs with no molecular diagnosis, we detected pathogenic variants in 90 patients (44%). We included a total of 104 samples in v1 and 100 samples in

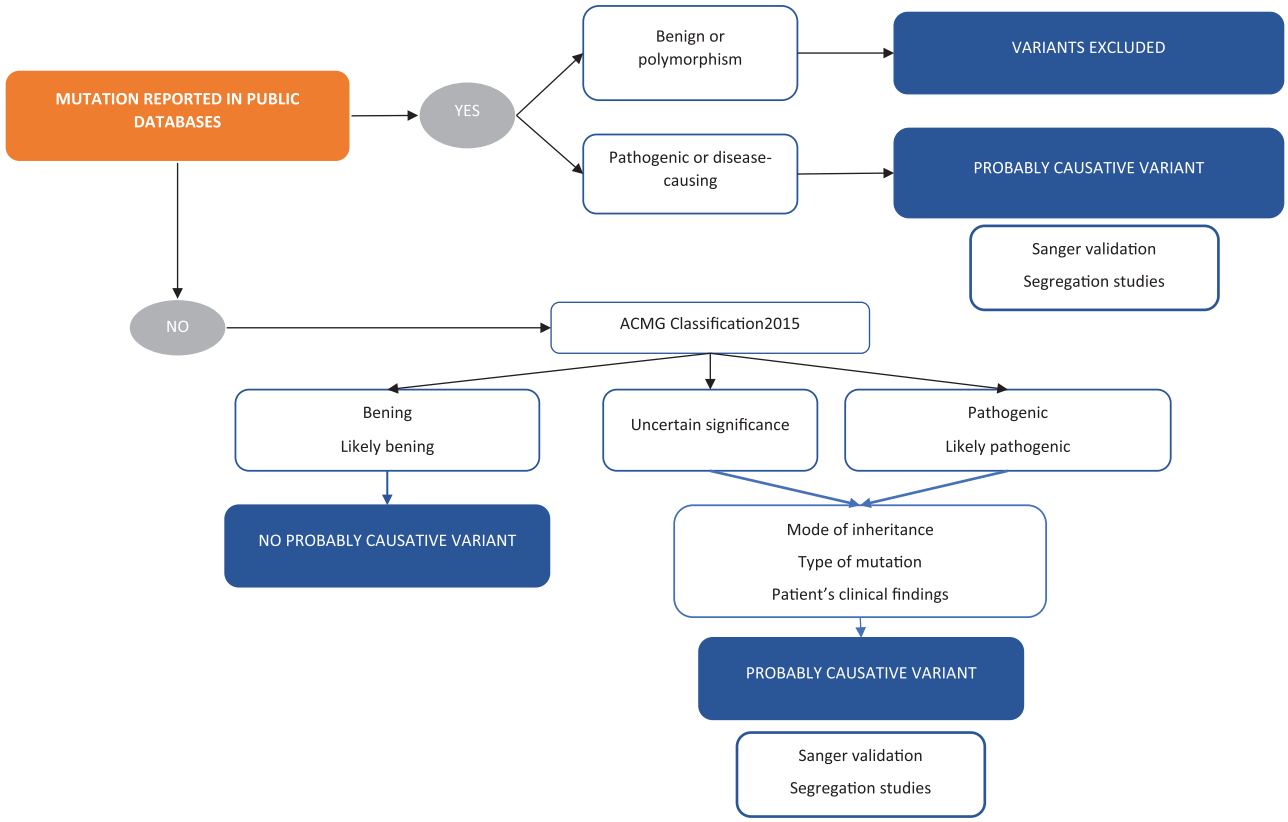

Figure 1. Algorithm summarizing analysis and filtering processes (American College of Medical Genetics and Genomics [ACMG]).

v2, with diagnostic rates of 45% (47/104) and 43% (43/100), respectively. We detected pathogenic variants in 35 genes. *FANCA* was the most frequently recognized gene with pathogenic variants, which was identified in 13 patients.

Genetic diagnosis by targeted sequencing in the classified IBMFS group (group 1)

This group included 167 patients with a suspected classified IBMFS and no molecular diagnosis (Supplementary Table S5, http://links.lww.com/HS/A135). We calculated genetic diagnostic rates through our targeted sequencing pipeline, taking into account the estimated mode of inheritance. The genetic diagnosis of patients with a clinical diagnosis of DBA (31/45, 68%), DC (8/12, 66%), MDS (3/5, 60%), and FA (16/28, 57%) was achieved in more than half of the patients. The genetic diagnostic rates for other categories were as follows: SCN (3/25, 12%), HT (16/38, 45%), SDS (1/6, 16%), CAMT (2/5, 40%), and other congenital anemias (1/3, 33%). In total, our targeted sequencing pipeline diagnosed 81 of 167 patients (48%) in the classified IBMFS group. Regarding the panel version, in the classified IBMFS group, we achieved a diagnostic rate of 56% (47/84) in the v1 panel and 47% (39/83) in the v2 panel.

In this group, we identified copy number aberrations in 2 patients. Patient 02021 had a positive chromosomal breakage test with no mutation detected by the NGS panel assay. A significant deletion was detected by the CNVs analysis from exon 9 to 43 of the *FANCA* gene (Supplementary Figures S2 and S3, http://links.lww.com/HS/A135). This deletion was confirmed by MLPA in the patient and parents. The mother carried a deletion from exon 9 to 15 of the *FANCA* gene, and her father carried a deletion from exon 16 to 43 of the same gene (Supplementary Figures S4–S6, http://links.lww.com/HS/A135).

The second case (patient 10002) involved a patient with thrombocytopenia and bilateral malformations of the radii at birth. In the NGS study, *6C>G on the *RBM8A* gene was detected, with an allelic frequency of 80%. The presence of this mutation in homozygosis was confirmed by Sanger sequencing (Supplementary Figure S7, http://links.lww.com/HS/A135). The study of CNVs performed by NGS therefore showed a significant deletion in chromosome 1 that included the *RBM8A* gene (Supplementary Figure S8, http://links.lww.com/HS/A135). After performing the single-nucleotide polymorphism microarray study, the presence of a microdeletion on chromosome 1 was demonstrated, from position 145395440 to 146089268 (Supplementary Figure S9, http://links.lww.com/HS/A135). The same study was subsequently carried out on the patient's parents using NGS, and it was observed that the *6C>G change on the *RBM8A* gene was present in the patient's father (Supplementary Figure S10, http://links.lww.com/HS/A135). The CNV study showed a deletion in chromosome 1 in the patient's mother, which included the *RBM8A* gene (Supplementary Figure S11, http://links.lww.com/HS/A135). Finally, patient 10002 was diagnosed with thrombocytopenia-absent radius syndrome associated with pathogenic variants of the *RBM8A* gene.[16]

Genetic diagnosis by targeted sequencing in the unclassified IBMFS group (group 2)

We included 37 patients with unclassified IBMFSs (Supplementary Table S6, http://links.lww.com/HS/A135). We identified 13 variants in 9 patients, with a diagnostic rate of 24% (9/37) in this group (Table 2). With regard to the panel version, we achieved a diagnostic rate of 17% (5/28) in v1 and 44% (4/9) in v2.

Of the 9 patients in whom pathogenic variants were detected, 5 patients were diagnosed with typical IBMFSs. Patients 12006

and 08001 were diagnosed with DC, having mutations in the *TINF2* and *TERT* genes, respectively. Patients 23001 and 10003 were diagnosed with CAMT, having *MPL* gene mutations in both cases. Patient 04016 was diagnosed with FA with compound heterozygosis of the *FANCA* gene.

The other 4 patients were diagnosed with other less common syndromes. Two of the successfully genotyped patients with unclassified IBMFSs had predominant neutropenia. Patient 40004 had a homozygous pathogenic variant of the *G6PC3* gene, which is associated with the development of Dursun syndrome.[17] In the other case, patient 20001, the mutation was detected in the *AP3B1* gene associated with Hermansky-Pudlak syndrome type 2.[18]

The last 2 patients diagnosed in this group were 11029 and 01015, who were diagnosed with Rothmund-Thomson syndrome and sideroblastic anemia with pathogenic variants of the *RECQL4* and *PUS1* genes, respectively.

Diagnostic amendments

The diagnosis of 6 clinically classified patients (6%) was amended after the results of the NGS gene panel assay became available.

The first example involved 2 patients clinically diagnosed with aplastic anemia (AA) based on hematological findings. In 1 of them (patient 12006, 4 years old), we found a pathogenic variant of *TINF2* (c.815G>A),[19] which produced a stop codon. In view of the results, a telomere length study was carried out and very short telomeres were detected (<p1). Accordingly, the diagnosis was amended to DC. The second patient (patient 04016) had been clinically diagnosed with AA and was unresponsive to immunosuppressive therapy. In this study, we found 2 heterozygous pathogenic changes of the *FANCA* gene (c.1115_1118del and c.2303T>C).[20,21] The chromosomal breakage test came back positive, so the patient was finally diagnosed with FA.

Patients 40001 and 38001 were eventually diagnosed with DC. Based on their clinical evolution and hematological findings, patients 40001 and 38001 were initially referred for suspected CAMT and for suspected myelodysplasia, respectively. However, in both cases, the study showed mutations associated with the development of DC on the *TERT* gene (c.2225G>A and c.1234C>T, respectively). Both mutations have been previously described in the literature.[22,23] DC was confirmed by a telomere length study of these patients, with both cases presenting with telomere shortening below the first percentile for their age.

The last 2 patients included in this group were eventually diagnosed with DBA. Patient 04006, aged 7 years, was studied for an episode of transient thrombocytopenia; however, a c.164C>T pathogenic variant was detected on the *RPS19* gene, which was associated with the development of DBA.[24] Reviewing the clinical history of patient 04006, it seems that, in early childhood, he had an episode of severe anemia but progressively recovered until reaching his present level of 10 g/dL of hemoglobin. Patient 10013, aged 23 years, had been studied for mild anemia from childhood with features of myelodysplasia in the bone marrow study performed in adulthood. This patient had a deletion on the *RPL11* gene, which has already been described as causing DBA.[25]

Analysis of undiagnosed patients

Of the 204 patients analyzed with NGS, a total of 113 patients were not diagnosed, corresponding to 55% of the total sample. Two years after the recruitment of these patients, the doctors responsible for monitoring the clinical evolution of the patients were questioned again. Among these 113 patients, it was possible to record the follow-up of 105 patients (Figure 2); 70% of these patients (75/105) presented with no significant clinical changes and no molecular diagnosis had been achieved, with IBMFSs still suspected. Of the remaining 30 patients, 3 patients proceeded to HSCT, 1 of whom died due to complications following the procedure. In 4 cases, a molecular diagnosis of IBMFS had been made using the other techniques summarized in Table 3. Three patients had had spontaneous hematological recovery without treatment, and the remaining 20 patients had been reclassified as having other noncongenital diseases. The majority of patients (12 cases) were reclassified as immune cytopenias, and the remaining patients were distributed heterogeneously: AA (3 cases), immunodeficiency (2 cases), pyruvate kinase deficiency (1 case), myeloproliferative syndrome (1 case), and mitochondrial disease (1 case).

Discussion

In recent years, the identification of the underlying molecular pathology has become the cornerstone for establishing a conclusive diagnosis of IBMFS, leading to better clinical care and follow-up of these patients. Until recently, Sanger sequencing of candidate genes has been the gold standard tool for the genetic diagnosis of IBMFS, resulting in a costly and lengthy technique.[4] In 2015, NGS techniques emerged as the herald of the revolution in the genetic diagnosis of IBMFSs.[12,13,15,26]

In this study, we have reached the molecular diagnosis of a cohort of patients from hospitals in Spain with suspected IBMFSs using a NGS assay, for which we developed a molecular diagnostic system that utilized a targeted sequencing pipeline covering 145 IBMFS genes. During the study, we have only had access to samples from peripheral blood. However, studies of family segregation have been carried out, which has supported the interpretation of the origin of the detected variants. We first performed a validation study of previously ascertained genetic variants, which confirmed the strong analytical sensitivity and reproducibility of the platform. We detected 11 known variants in 7 genes, including missense and nonsense variants and small deletions from 10 patients.

In this series of patients, we identified 104 candidate variants in 35 genes, 46% of which were absent from the main reference databases, thus emphasizing the great heterogeneity of the molecular pathology underlying IBMFSs. Appropriate interpretation of the pathogenicity of candidate genetic variants found by NGS remains a major challenge, especially for novel variants, even if present in well-established IBMFS genes. To prevent misinterpretation, the use of consensus guidelines is highly recommended, although there is significant discordance between laboratories.[14,27] Following established guidelines and studies carried out by other groups, we have designed our own variant-filtering strategy carrying out the same analysis for each variant.[12–14] Finally, of the 104 identified candidate variants, we classified 23% and 25% as pathogenic and likely pathogenic, respectively. Approximately half of the variants were classified as VUS. The high proportion of VUS supposes 1 of the major limitations of the study, since we did not have the possibility to carry out functional studies. However, through the family study and after evaluating each of the cases with the responsible specialist, we analyzed the clinical picture of the patient, the mode of inheritance of each disease, and the characteristics of the variants. Therefore, as changes are compatible with the clinical picture of the patients, we interpret that these variants could have an important role in the development of the disease. These results indicate the great complexity to interpret variants in this type of disease and, at the same time, reflect the need to deepen the knowledge of the molecular bases of IBMFSs to be able to assess their involvement in the development of the disease.

Overall, our NGS approach enabled a molecular diagnosis in 44% of patients. The diagnostic rate of our targeted sequencing platform was similar to those obtained by other groups. The

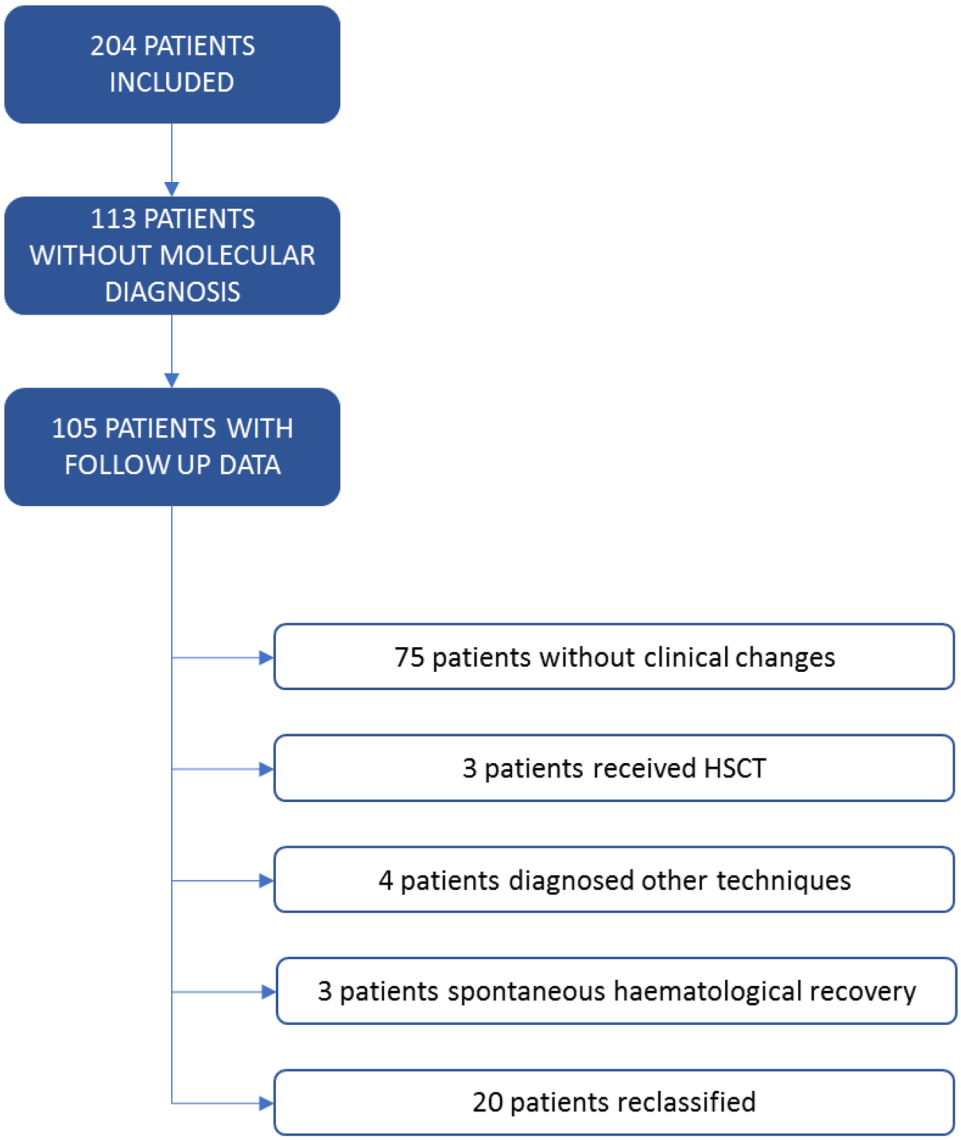

Figure 2. Description of clinical evolution in undiagnosed patients. HSCT = hematopoietic stem cell transplantation.

previous studies published by Ghemlas et al[12] and Muramatsu et al,[13] which involved similar approaches, studied patients with suspected IBMFSs using a NGS assay and analyzed the diagnostic yield. The first work published by Ghemlas et al[12] diagnosed 61 of the 158 patients studied using a panel of 72 genes, with an overall diagnostic rate of 38%. The report by the Japanese group studied diagnostic rates in a population of patients using a NGS panel and WES. With targeted sequencing, this group achieved a genetic diagnosis rate of 53 out of 121 (44%).

In our study, we analyzed the results according to the groups into which the patients were divided. The percentage of diagnoses achieved in the classified IBMFS group was 48% (81/167) compared with 24% (9/37) in the unclassified IBMFS group. Remarkably, the diagnostic rate was higher for patients presenting with a well-defined clinical phenotype indicative of a particular type of IBMFS. The Canadian group of Ghemlas et al[12] also used this division, observing a similar disparity, with a molecular diagnosis rate of 59% in the case of classified IBMFSs, compared with 18% for unclassified IBMFSs. This difference suggests that, for cases of clinically well-defined diseases with specific screening analysis (such as chromosomal breakage test, telomere length, or biochemical characteristics), the diagnostic rate is acceptable; nevertheless, for patients with more complex phenotypes, diagnosis using a NGS gene panel may be insufficient. This is supported by 1 of the first papers published in this field. Zhang et al[15] used a NGS panel of 85 genes to study a total of 71 patients affected by IBMFSs with no typical clinical characteristics. The author described the molecular basis of 8 of the 71 patients, with a diagnostic rate of 11%. These 8 patients presented pathogenic variants in the *GATA2*, *RUNX1*, *DKC1*, and *LIG4* genes but did not present the typical phenotypic characteristics associated with these genes. This illustrates the fact that the molecular diagnosis of unclassified IBMFSs using a NGS gene panel does not exceed 20%, suggesting that this group could benefit from other approaches. Data provided by Bluteau et al[28] support this idea. These authors studied a total of 179 samples from patients with suspected IBMFSs without a defined clinical phenotype, excluding those with characteristics of FA, DBA, DC, SDS, and SCN. For all the samples, WES of the DNA extracted from fibroblasts was undertaken, so all samples corresponded to the germ line. Finally, a total of 86 patients were diagnosed, with a diagnostic rate of 48%. This data exceeds the results obtained using NGS panels, which seems to indicate that WES of germline samples is an interesting

Table 3

Patients Diagnosed by Other Molecular Techniques.

Clinical Suspicion	Gene/CHR Affected	Technique	Final diagnosis
DBA	Chr 15q deletion	CGH array	DBA
SDS	*DNAJC21* mutation	WES	SDS
IBMFS	*SAMD9* mutation	WES	MDS
FA	*FANCA* deletion	MLPA	FA

CGH = comparative genomic hybridization; Chr = chromosome; DBA = Diamond-Blackfan anemia; FA = Fanconi anemia; IBMFS = inherited bone marrow failure syndrome; MDS = myelodysplastic syndrome; MLPA = multiplex ligation-dependent probe amplification; SDS = Shwachman-Diamond syndrome; WES = whole-exome sequencing.

tool to be considered for patients who do not present with typical clinical characteristics.

Regarding to specific groups, in this series, the diagnostic yield in FA group had been 57%, being lower than expected. This could be explained by patient selection. All referred patients were included in this study and, in some cases, the chromosomal breakage test had not been performed. Therefore, probably some patients were affected by another syndrome, no FA. However, 87% (14/16) patients with positive chromosomal breakage test were diagnosed using the NGS platform, being the diagnostic rate of this approach higher in patients with a positive chromosomal breakage test.

In SDS group, only 1 patient was diagnosed by NGS. The explanation could lie in the previous genetic studies performed before they were included in the study. When this NGS panel was designed, the only *SBDS* gene had been associated with the development of this disease, so of the 5 patients without molecular diagnosis, 3 of them were negative for this gene. Throughout the development of this project, new genes associated with this disease have been described, specifically *DNAJC21*[29,30] and *EFL1*[31]. In 1 of the patients in whom no result was found, WES was subsequently performed, detecting *DNAJC21* gene mutated. Therefore, we could say that those patients highly suggestive of SDS negatives for the *SBDS* gene could not benefit from approaches using NGS panels that are not updated.

In this work, 2 versions of the same panel were used, with 129 genes included in v1 and 145 genes included in v2. The percentage of molecular diagnosis achieved for the 2 versions did not differ substantially (45% in v1 versus 43% in v2), which may suggest that the number of genes included per panel was not decisive in the percentage of diagnoses achieved by this tool. One possible explanation is that the panels that include a greater number of genes are those that have been designed more recently, including recently discovered genes that have been described with a lower frequency. Since this approach is somewhat similar to screening, it is likely that, for the vast majority of patients, the mutations fall within the group of genes most frequently detected in patients with IBMFSs. However, gene panels require periodic updating to ensure they cover as many genes as possible, thus optimizing molecular diagnosis.

In most patients diagnosed, the clinical suspicion coincided with the molecular diagnosis. However, it is important to highlight the fact that 6 of the 91 patients diagnosed (6%) were reclassified, implying a change in their clinical management. In this work, the rate of diagnostic amendments is somewhat lower than that published for other series, where the rate is around 10%–26%.[12,13,32] However, even if the number is not high, it has very important clinical implications, such as avoiding unnecessary treatments or giving adequate genetic counseling. In 1 case, the clinical diagnosis of AA was amended to FA, meaning the convenience of entering into a bone marrow transplant procedure because of the high risk of progressing to MDS/AML and periodic monitoring for early detection of cancer, specifically in the head and neck, which are the most prevalent types in this disease.[28] Patients diagnosed with DC should have long-term follow-up to monitor the onset of bone marrow failure, as well as the development of pulmonary or hepatic fibrosis or the appearance of cancer. Another example was the patient under study for mild thrombocytopenia who was finally diagnosed with DBA. This finding implied the convenience of performing close monitoring of the patient due to the increased risk of cancer in patients with DBA. Therefore, in addition to the specific treatment that the development of anemia would require, it is advisable that these patients should follow an early detection program in adulthood.

In this work, there was no particular selection of the patient cohort. The selection criteria were less strict than for other groups, so it would be expected that a percentage of the patients were not affected by a congenital disease. Based on this premise, the reference specialist doctors responsible for monitoring the clinical evolution of the patients were questioned 2 years after their patients had been included in this study. After this consultation, 24 patients did not meet the clinical criteria for a IBMFS, and some experienced spontaneous hematological remission. Therefore, it seems reasonable to establish a series of minimum criteria, both clinical and analytical, before applying this type of diagnostic approach.

Using this approach, we have detected variants potentially responsible of the phenotype in only half of the patients. This could be due to the technical limitations inherent in this type of diagnostic approach, such as mutations in deep intronic areas or genes for which there was not sufficient depth of reading. Additionally, problems of bioinformatic tools determining the pathogenicity of rare variants or incomplete knowledge of the IBMFSs may have accounted for these limitations. NGS can miss large deletions or duplications; CNVs involving > 1000 bp; or large structural chromosomal variants, translocations, and aneuploidy unless they have been specifically designed for such a purpose.[29] Thus, for successful molecular diagnosis of certain cases, the NGS assay should be combined with other molecular approaches, such as CGH array, quantitative polymerase chain reaction, or MLPA.

Possibly the greatest limitation for detecting molecular alterations using a NGS panel of already defined genes is the progressive discovery of new IBMFS genes. Indeed, since the design of this panel, new genes have been discovered. In this work, there are 2 patients who have been diagnosed by WES for genes not included in this panel, such as *DNAJC21* and *SAMD9*, published in 2017.[14,30,31,33] The best option could be to use an updated panel as initial screening and subsequently select those patients who might be candidates for WES or even WGS. However, even with WES and WGS techniques, a percentage of patients are still not diagnosed.

In summary, our NGS gene panel assay constituted a useful tool for the genetic diagnosis of IBMFSs. The correct classification of IBMFSs using NGS facilitates more accurate medical management of these complex conditions. Specifically, our gene panels were able to identify the genetic alterations in a high percentage of patients and constituted a reasonable option for the genetic screening of IBMFSs prior to the application of more complex studies, including WES and WGS, which might facilitate the discovery of new genes.

Disclosures

The authors have no conflicts of interest to disclose.

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