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# Genomic Characterization of the Inherited Bone Marrow Failure Syndromes

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## Abstract

The inherited bone marrow failure syndromes (IBMFS) are a set of clinically related yet heterogeneous disorders in which at least one hematopoietic cell lineage is significantly reduced. Many of the IBMFS have notably increased cancer risks as well as other physical findings. Highly penetrant germline mutations in key pathways, such as DNA repair, telomere biology, or ribosomal biogenesis are causative of Fanconi anemia (FA), dyskeratosis congenita (DC) and Diamond-Blackfan anemia (DBA), respectively.

Next-generation sequencing (NGS) generally refers to high-throughput, large-scale sequencing technologies and is being used more frequently to understand disease etiology. In the IBMFS, NGS has facilitated the discovery of germline mutations that cause thombocytopenia absent radii syndrome, a subset of DC and DBA, and other uncharacterized, but related, disorders. Panels of large numbers of genes are being used to molecularly characterize patients with IBMFS, such as FA and DBA. NGS is also accelerating the discovery of the genetic etiology of previously unclassified IBMFS. In this review, we will highlight recent studies that have employed NGS to ascertain the genetic etiology of IBMFS, namely FA, DC, DBA and TAR and discuss the translational utility of these findings.

## INTRODUCTION

The inherited bone marrow failure syndromes (IBMFS) are a set of clinically related yet heterogeneous disorders in which at least one hematopoietic cell lineage is significantly reduced in number. Certain IBMFS, such as Fanconi anemia (FA), dyskeratosis congenita (DC), and Diamond-Blackfan anemia (DBA), are associated with increased risk of solid tumors and hematopoietic malignancies (1). The genetic etiology of the IBMFS includes germline mutations in several key biological processes (*e.g.,* DNA repair, telomere biology, or ribosomal biogenesis) (1). Highly penetrant germline mutations have been identified that can explain approximately 95% of FA and 95% of Shwachman Diamond syndrome (SDS) (2). In contrast, the genetic cause is known in only about one-half of patients with Diamond-Blackfan anemia (DBA) and about 70% of patients with dyskeratosis congenita (DC) (3).

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Prior to the advent of next generation sequencing (NGS) technology, identification of the genetic etiology of the IBMFS and other inherited disorders was conducted primarily through a combination of linkage studies and candidate gene sequencing. While successful, these approaches are limited because linkage studies require large families with multiple affected individuals; and candidate gene studies can consume significant resources by sequencing one gene at a time and success is predicated on the good fortune of picking the right gene in the right set of patients. The more expansive survey of the genome using NGS technologies has also led to rapid advances in understanding the structure of the human genome; and of mutations or single nucleotide polymorphisms (SNPs) associated with both rare and common diseases (4, 5).

NGS, also known as massively parallel sequencing or second generation sequencing refers to high-throughput, large-scale sequencing technologies (4, 6, 7). Different NGS approaches include whole genome, whole exome (*e.g.*, targeting of all known exons in the reference data base), and/or sequencing of large panels of specific genes. In addition to sequence analysis and evaluation of single nucleotide substitutions, NGS also enables characterization of copy number variants (CNVs), insertions/deletions (indels), and structural rearrangements. However, the error rate for NGS is higher than SNP genotyping and most variants require validation by another sequencing method, such as traditional Sanger sequencing. NGS of RNA transcripts (*e.g.,* RNA-Seq) allows for the quantification of transcript levels and the RNA sequence information that form the basis of what is now called the transcriptome (6). Chromosomal immunoprecipitation (ChIP) followed by DNA sequencing (ChIP-Seq) is an NGS technique that allows mapping of specific transcription factors and histone modifications to their genomic location (8). Other NGS methods include the identification and quantification of methylated DNA sites, histone-bound DNA, and protein-RNA interactions (7).

NGS has been applied to the discovery of IBMFS mutations. For example, whole exome sequencing (WES) has led to the discovery of new telomere biology genes associated with DC (e.g., mutations in the *CTC1* and *RTEL1*genes) (9-11). The discovery of *GATA1* mutations in DBA by WES has led to a novel connection between DBA and the distinct disorder of X-linked dyserythropoietic anemia and thrombocytopenia (12, 13). More recently, comparative genomic hybridization (CGH) identified *RPL15* as a novel gene causing DBA (14). Complimentary genomic approaches, including CGH, exome sequencing, and targeted sequencing of the non-deleted allele, were required for the identification of mutations that explain a subset of the thromobocytopenia absent radii syndrome (TAR) (15, 16). Even in a complex syndrome such as FA, for which the genetic cause can usually be identified, new genomics approaches combining WES, CGH, and RNA-Seq are being used to develop a more efficient and cost effective approach to new patient characterization (17-22). This review will highlight recent advances in IBMFS genetics based on NGS and consider the role of genomics approaches in future studies of these disorders.

## FANCONI ANEMIA (FA)

### Clinical features and diagnosis of FA

FA is a chromosomal instability disorder caused by germline mutations resulting in defective DNA damage response. It is associated with a myriad of clinical features, including congenital anomalies, progressive bone marrow failure (BMF), and cancer predisposition (1, 23)(Table 1). Radial bone and thumb abnormalities, short stature, skin hyperpigmentation and/or café au lait macules are the most commonly reported features, although other organ systems may also be involved (24). Importantly, less than two-thirds of FA patients present with pathognomonic physical features; BMF or cancer can be the

primary presenting sign in these individuals. The diagnostic test for FA is the detection of increased chromosomal breakage in cells cultured with a clastogen, such as diepoxybutane (DEB) or mitomycin C (MMC) (1).

BMF in FA typically occurs in the first decade of life and may initially present as a single or bilineage cytopenia. Patients may also have macrocytosis and elevated fetal hemoglobin levels (1). The severity of BMF may progress and require medical intervention such as regular blood product transfusions or hematopoietic stem cell transplantation (HSCT). Patients with severe BMF unable to undergo HSCT, due to medical or personal reasons, have been treated with androgens such as oxymetholone with an estimated 50% response rate (1). FA patients who are treated with androgens are at an increased risk of developing liver tumors (25).

Defective DNA repair confers a propensity for specific cancers in patients with FA. In addition to BMF, patients with FA are at increased risk of myelodysplastic syndrome (MDS) and acute myelogenous leukemia (AML) (26). FA also confers a higher risk of developing solid tumors, particularly squamous cell cancers (SCC) of the head, neck, skin, GI tract and genital tract. Patients with FA subtype D1, caused by biallelic mutations in *BRCA2,* have an extremely high risk of early onset AML, brain tumors, or Wilms tumor (26, 27). These risk factors make the correct diagnosis of FA essential for proper management as well as genetic counseling.

## Genetics and Pathophysiology of FA

All the known FA subtypes are caused by germline mutations in key components in the DNA repair pathway. They are inherited in an autosomal recessive (AR) pattern, except for subtype B, which is X-linked recessive (XLR). The first causative FA gene was discovered over 20 years ago, and there are now16 known FA subtypes (A, B, C, D1 (*BRCA2*), D2, E, F, G, I, J (*BRIP1, BACH1*), L, M, N (*PALB2*), O (*RAD51C*), P (*SLX4*), and Q (*ERCC4*)) (17, 28) (Figure 1). At least 95% of FA cases are caused by germline mutations in one of these 16 genes (2). In AR FA, the mutations are often biallelic and may include the combination of a single base substitution and a partial or full gene deletion (28). The heterozygote carrier frequency of FA is estimated to be 1 in 181 in the United States (29); it may be higher in populations with founder effects, such as in Ashkenazi Jewish populations that have an estimated carrier frequency of 1 in 89 for the IVS2(+4)A>T mutation in *FANCC* (30).

The genes mutated in FA encode proteins that work together to resolve DNA interstrand cross-links during cellular replication **(**Figure 1A). The FA protein core complex is a large nuclear E3 ubiquitin ligase complex consisting of FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, and FANCM. The core complex interacts with FANCD2 and FANCI, which then interact with FANCD1 (known as BRCA2), FANCN (known as PALB2), FANCJ (known as BRIP1 or BACH1), FANCP (known as SLX4) and FANCO (known as RAD51C), as well as NBS1, BRCA1, and FAN1 to preserve genome integrity (28). Germline mutations in any of the FA genes result in markedly reduced or absent protein function and deficient DNA repair.

## NGS in FA

The first gene associated with FA, *FANCA*, was discovered by linkage analysis in 1995 (31) (Figure 1A). Subsequent mutation discovery efforts utilized a complementation testing approach in which defective cells were transfected in an attempt to rescue the chromosome breakage phenotype (32). The gene encoding the protein that results in the rescue can then be sequenced to identify causative mutations (2). However, complementation testing for FA

is costly and time consuming. It requires a highly specialized technique that is not widely available. Genetic linkage and positional cloning have continued to be valuable in identifying mutations in FA-associated genes.

Although the genetic cause of FA is known in approximately 95% of patients, WES has accelerated the identification of mutations in the rare FA patients without a known genetic etiology. For example, *ERCC4,* the DNA-repair endonuclease XPF, was discovered as a cause of FA through WES in a patient with clinical FA (17). Notably *ERCC4* mutations have previously been associated with xeroderma pigmentosum type F (XPF) or XFE progeroid syndrome (33, 34) but interestingly, in this case, the patient lacked the dermatologic manifestations of XPF or XFE. Homozygous truncating mutations in *XRCC2*, a gene critical for DNA repair by homologous recombination, have also been implicated as a cause of FA after autozygome analysis failed to reveal a genetic cause in a consanguineous family (35).

Targeted NGS has the potential to be cost-effective in identifying a patient's FA-associated mutation in newly diagnosed individuals. For example, as proof of principle, Ameziane et al the sequenced germline DNA of 11 patients with known FA mutations on a custom NGS sequencing panel of eight FA genes (*FANCA, FANCB, FANCC, FANCD1, FANCE, FANCG, FANCI* and *FANCN*) (19). This approach identified not only the known mutations but a novel deletion. This study also noted that it is critical to carefully evaluate the potential confounding effect of pseudogenes, especially for *FANCD2,* and deletions in all FA-associated genes. This study also suggested that NGS is useful in uncovering somatic mosaicism by comparing different tissue types.

A multi-platform approach was recently used to successfully identify the genetic cause of FA in 27 patients (21). The combination of custom array CGH (aCGH) to detect deletions and duplications of FA genes, WES to evaluate mutations, and RNA-Seq to evaluate gene expression changes resulting from these mutations was shown to be an efficient for characterization of both the complementation group and the germline mutations.

WES has been used to successfully identify germline mutations in four patients with unknown complementation groups (18). Sanger sequencing confirmed the point mutations and small indels present in *FANCD1, FANCD2,* and *FANCJ.* Another study used WES to detect a rare, yet clinically significant germline mutation in *SLX4/FANCP* in a young adult patient clinically diagnosed with FA in early childhood and an elevated chromosome breakage with MMC (20).

## DYSKERATOSIS CONGENITA (DC)

### Clinical features and diagnosis of DC

DC is an IBMFS caused by germline defects in telomere biology and is diagnosed by the presence of the classic triad of nail dysplasia, lacy skin pigmentation, and oral leukoplakia (Table 1). Not all patients present with this diagnostic triad, but they are at high risk of trilineage BMF, pulmonary fibrosis, liver disease (cirrhosis and fibrosis), and malignancy. Additional medical problems may include avascular necrosis of the femoral or humeral heads, stenosis of the esophagus, urethra, or lacrimal ducts, and developmental delay (1, 36, 37). Patients with DC are at increased risk for many of the same cancers as patients with FA, namely, SCC of the head and neck, and anogenital region, MDS, and AML. The actuarial risk of cancer in patients with DC is 40% by 50 years of age, with an 1100-fold increased risk for tongue cancer, 2500-fold increased risk for MDS and 200-fold for AML (26, 38).

BMF can develop in up to 90% patients with DC and is often life-threatening (36, 39, 40). However, the rate of BMF in individuals who have a germline mutation in a DC-associated gene but lack clinical features at the time of evaluation (*e.g.,* silent carriers) is not known. BMF can present as single or multi-lineage cytopenia that progress to severe BMF. Hematopoietic stem cell transplant (HSCT) is the only current modality for cure of BMF associated with DC. As in FA, treatment with androgen therapy is often considered for BMF in individuals who cannot, or do not wish to undergo HSCT (36, 37). Between 50-70% of DC patients treated with androgens respond and no longer be dependent on red blood cell and platelet transfusions (41, 42).

The clinical diagnosis of DC can be challenging due to phenotypic heterogeneity, variable age of onset of the mucocutaneous triad, and presence of several clinical variants of DC. Table 1 describes the known clinical variants of DC, namely Hoyeraal Hreidarsson syndrome (HH), Revesz syndrome (RS), and Coats plus disease or cerebroretinal microangiopathy with calcification and cysts (CRMCC). The unifying feature of this complex set of clinical problems is the presence of very short telomeres, the result of germline mutations in key telomere biology genes. Telomere length less than the first percentile for age in leukocyte subsets measured by flow cytometry with *in situ* hybridization (flow FISH) is highly sensitive and specific for DC (43, 44)

## Genetics and Pathophysiology of DC

DC can be inherited in one of three forms, X-linked, autosomal dominant (AD), or AR. *De novo* germline mutations are also relatively frequent in DC and to date, about 70% DC patients have an identifiable germline mutation (36, 45). These mutations occur in genes responsible for the functioning and maintenance of telomeres (Figure 2A). Currently, there are nine known DC-associated genes (DKC1, TERT, TERC, TINF2, WRAP53, NOP10, NHP2, CTC1, and *RTEL1)* (36, 45).

The first DC-associated gene, X-linked *DKC1,* was discovered by linkage analysis in 1998 (46) (Figure 2B). AD mutations in *TERC,* the RNA component of telomerase, were identified through linkage analysis of a large family (47). Subsequently, a combination of linkage (48) and candidate gene sequencing found mutations in *TERT,* as a cause of DC as well as in cases of isolated aplastic anemia and pulmonary fibrosis (49-52). These findings united the seemingly disparate diseases under the umbrella of DC-associated Telomere Biology Disorders (TBDs) (36, 39).

Linkage mapping was also used to identify AD mutations in *TINF2,* a key component of the shelterin telomere protection complex (53). Homozygosity mapping led to the discovery of AR *NOP10* mutation in a consanguineous family with DC (54). That finding led investigators to follow-up with candidate gene sequencing of related genes and the detection of *NHP2* mutations in two families (55). Candidate gene sequencing also led to the discovery of AR mutations in *WRAP53* (TCAB1) as a cause of DC (56).

Lastly, mutations in two additional genes have been reported in DC, although, their connection with telomere biology is less straightforward. These include an intronic splice variant in Apollo (encoded by *DCLRE1B*) in a patient with HH and normal telomeres (57). On account of this finding, the role of Apollo in telomere biology is currently under investigation. Linkage analysis led to the identification of mutations in C16orf57, a gene with unknown function now called *USB1* (58). C16orf57 mutations were reported in patients with DC and normal telomeres, but also in individuals with Rothmund Thomson syndrome and Poikiloderma with Neutropenia, suggesting an overlapping clinical spectrum (58).

### NGS in DC

Prior to the use of NGS, the genetic cause of DC was known in less than one-half of patients (45). It is notable that mutational analyses have connected Coats plus (CRMCC) and DC based on WES discoveries that compound heterozygous mutations in the telomere capping protein encoded by *CTC1* can cause either disorder (Table 1). Patients with these mutations have short telomeres and features that phenotypically overlapped with DC (9, 59, 60). Subsequently, candidate gene sequence analysis has led to the discovery of AR *CTC1* mutations in a patient with DC (61).

Several groups have independently identified *RTEL1* mutations using WES in families with DC (10, 11, 62). The RTEL1 protein regulates telomere length, may interact with PCNA (proliferating cell nuclear antigen), and also plays a role in DNA repair (10, 11). Most of the *RTEL1* mutations appear to be AR, but AD mutations have been reported (10). It is estimated that the germline genetic cause of DC is now known in about 70% of families with DC due addition of *CTC1* and *RTEL1* to the list of DC-associated genes.

## DIAMOND BLACKFAN ANEMIA (DBA)

### Clinical features and diagnosis

DBA is a rare disorder of erythroid hypoplasia, characterized by macrocytic anemia, usually with normal WBC and platelet counts (Table 1). Patients with DBA are typically diagnosed at birth or within the first year of life (1); it is also associated with congenital anomalies, such as triphalangeal, bifid, or subluxed thumbs, or subtle flattening of the thenar eminence, with a normal radius. Genitoitourinary and heart defects, webbed neck, Klippel-Feil anomaly (fusion of cervical vertebrae), and Sprengel deformity (congenital asymmetric high scapula) have also been reported in DBA (3, 63). Like the other IBMFS, DBA is associated with an increased risk of certain malignancies, including MDS, AML, colon carcinoma, female genital cancers and osteosarcoma (64). The phenotypic spectrum of DBA is very broad, even within families. Some individuals with germline mutations may be silent carriers, or have only mild anemia whereas others are very severely affected. This observation suggests the importance of genetic modifiers, which have yet to be defined.

The diagnosis of DBA is primarily based upon the early onset, in infancy or childhood of persistent severe anemia with reticulocytopenia in the absence of other bone marrow abnormalities (1, 65). The bone marrow of patients with DBA shows erythroblastopenia with normal myeloid and megakaryocytic lineages. Overall, the bone marrow cellularity is usually normal or slightly reduced. An elevated erythrocyte adenosine deaminase (eADA) level in pretransfusion samples supports the diagnosis of DBA, whereas normal eADA levels do not exclude the diagnosis (66, 67).

Red blood cell transfusions are the primary treatment modality for severe anemia until the diagnosis is firmly established (1, 68). Oral corticosteroids may mitigate the need for regular transfusions in some DBA patients; however side effects must be carefully balanced with response (65). In those who do not respond to steroid therapy and require RBC transfusions, iron overload is a major clinical concern and should be treated early.

### Genetics and Pathophysiology of DBA

DBA is AD inherited disease and is usually caused by heterozygous germline mutations in genes encoding key components of small 40S or large 60S ribosomal subunits (Figure 3A) (65, 69). AD germline mutations in *RPS19* were the first known genetic cause of DBA, providing a critical link between DBA and ribosomal biogenesis (70). *RPS19* germline mutations account for approximately 25% of DBA cases. Although there are now nine

DBA-associated genes (*RPS19, RPS17, RPS24, RPS26, RPS10, RPS7*, *RPL35A, RPL5,* and *RPL11*), at least three genes are suspected to be associate with DBA, *RPL36*, *RPS15*, and *RPS27A* (63), but approximately 50% of DBA patients have no known genetic mutation (3). *De novo* mutations and/or variable disease penetrance can also account for DBA in a subset of patients.

Most DBA-associated germline mutations result in abnormal assembly of ribosomal proteins (Figure 3). While ribosome assembly is a highly regulated process, the reason these defects cause a specific defect in erythropoiesis is still not known. Aberrant ribosomal biogenesis due to germline ribosomal protein gene mutations activates cellular stress signaling pathways, such as p53 (71). This change in the balance of the p53 tumor suppressor pathway could disrupt cellular homeostasis and result in increased cancer risk, but further work is required to explain the underlying pathophysiology of the genetic mutations.

### NGS in DBA

An NGS panel of 79 genes encoding ribosomal proteins, including the known DBA-associated genes, has been used to define the genetic cause in some patients and to evaluate potentially novel DBA-associated genes (63, 72-77).

WES has expanded the biological mechanism of DBA and expanded the clinical phenotype. Two families with DBA were found to have germline X-linked *GATA1* mutations by WES. Previously, *GATA1* mutations were only associated with X-linked congenital dyserythropoietic anemia and thrombocytopenia (12, 78). *GATA1* encodes a key component of the GATA family of transcription factors that is important in erythroid development. Notably, the red cell ADA levels were normal in the DBA patients with *GATA1* mutations (13), suggestive of an interaction or a different biological mechanism that is yet to be determined.

As expected based on the other DBA-associated genes, WES has also uncovered mutations in a gene encoding a ribosomal protein, *RPS29* (79). RPS29 is a component of the 40S ribosomal subunit and critical for normal hematopoiesis in a zebrafish model (80). More recently, aCGH also identified deletions in *RPL15* as a novel cause for DBA (14). Similar to RPS26, RPL15 is important in early synthesis of the 60S ribosomal subunit and in the cleavage of the internal transcribed spacer 1 (14).

## THROMBOCYTOPENIA ABSENT RADII (TAR)

### Clinical features and diagnosis

TAR is typically diagnosed in infancy due to the constellation of thrombocytopenia with bilateral absence of radii with the presence of thumbs, albeit abnormal (1, 81, 82). Patients can also have additional bony abnormalities of the ulna or humerus. Occasionally, hip and/or patellar dislocation and other non-specific bony abnormalities are present. The thumbs are always present in TAR, in contrast to FA where the radial ray abnormality results in missing thumbs if radii are absent. Cardiac, gastrointestinal and genitourinary system abnormalities have also been described in TAR (81).

Thrombocytopenia in TAR may be congenital or occur within the first few weeks to months of life (81). The majority of patients present with thrombocytopenia with platelet counts less than 50,000/ul. This is usually transient and significantly improves with time, but usually does not reach normal levels. Cow’s milk allergy is frequent in patients with TAR and can exacerbate thrombocytopenia, however the underlying pathophysiology of this relationship is not completely understood (81). Transient leukemoid reactions have also been reported in

TAR, with white blood cell counts exceeding 35,000 cells/$mm^3$ (15). There have been four reports of leukemia in patients with TAR, which include either AML or ALL (83, 84).

The management of TAR is mainly supportive care of thrombocytopenia with platelet transfusions as needed, and orthopedic treatment of bony abnormalities to improve function of the upper limbs (81, 85).

### TAR Genetics and NGS in TAR

The inheritance of TAR typically fits into either an AR or *de novo* pattern (86). The molecular etiology of TAR was unknown until 2007 when a combination of chromosome GTG-banding and submegabase-resolution whole-genome tiling array CGH was used to identify a microdeletion at chromosome 1q21.1in TAR patients (15). This was further confirmed and fine-mapped to a common deleted region in all 30 individuals with TAR studied. Notably, the deletion was *de novo* in only 25% of affected individuals; an unaffected parent was a carrier of the same deletion in the other families. A subsequent study of 14 patients confirmed the presence of the 1q21.1 deletion in affected individuals and confirmed that unaffected parents also carried the deletion (87). However, the etiology of TAR was not yet been fully defined because unaffected parents can also be carriers of the 1q21.1 deletion. The deleted region of 1q21.1 contains 10 protein-coding genes but the initial candidate gene sequencing across this region failed to identify TAR-associated mutations. However, TAR cases were found to have low-frequency SNPs in regulatory regions of the *RBM8A* gene in the 1q21.1 deleted region (16, 82). The parent without the 1q21.1 deletion was the carrier of the *RBM8A* SNP. Thus, TAR can be caused by the biallelic inheritance of the 1q21.1 deletion from one parent and a rare SNP in *RBM8A* from the other. Notably, the inheritance of two hypomorphic variants in *RBM8A* also appears to cause TAR (16, 82). *RBM8A* encodes the conserved Y14 subunit of the exon-junction complex (EJC) that is essential for RNA processing and expressed in all hematopoietic lineages. These findings suggest that TAR is caused by the loss or significant reduction of *RBMBA* expression (82). This comprehensive NGS approach led to the first report of EJC defects associated with human disease.

## UNCOVERING THE GENETIC ETIOLOGY OF UNCLASSIFIED IBMFS

In some instances patients may present with BMF and select features, such as congenital anomalies or family history, suggestive of an inherited disorder but not consistent with a well defined constellation of features consistent with a known IBMFS. Additionally, atypical presentations of known disorders may also complicate the diagnosis. NGS methods make it possible to uncover the genetic etiology of many previously unclassified patients (88-93) (Table 2). For example, a linkage study did not reveal the genetic cause of BMF and congenital nerve deafness in a family of three siblings and their mother but WES of the four affected individuals led to the discovery of germline mutations in *SRP72* in this family (89). Targeted *SRP72* sequencing identified an additional mother-child pair with BMF and a missense mutation. *SRP72* encodes a component of a signal recognition particle responsible for protein translocation and processing that had not previously been implicated in BMF.

WES of one affected child and the father was used to uncover homozygous mutations in *SBF2* that are likely associated with disease in a family with two children with early onset thrombocytopenia and fair skin (93).The *SBF2* gene encodes a pseudophosphatase, which is a member of the myotubularin-related protein family. Already germline mutations of *SBF2* have been associated with Charcot-Marie-Tooth Diease, type 4B2. Its role in megakaryopoiesis is not understood and the connection between these findings is not known.

Similarly, germline mutations in *VPS45*, a component of congnate syntaxin Tlg2 which is required for membrane traffic through the endosomal system, have been linked to congenital neutropenia and myelofibrosis by WES and homozygosity mapping (92). Cells with the *VPS45* mutation appeared to have defects in the endosomal-lysosomal pathways, a novel finding in IBMFS.

In addition to discovering novel causes of disease, WES and other NGS platforms can uncover mutations in genes associated with other phenotypes. Recently, AR germline mutations in *MPL,* which encodes the thrombopoietin receptor and are primarily responsible for congenital amegakaryocytic thrombocytopenia (CAMT), were linked to childhood onset BMF through exome sequencing of a consanguineous family (90). Evaluation of an additional 33 patients with childhood, but not neonatal BMF, identified another sibling pair with *MPL* mutations. CAMT typically presents during infancy with thrombocytopenia in the absence of physical anomalies but it can also present as BMF without a specific history of thrombocytopenia (94). These findings suggest screening for *MPL* mutations is warranted in individuals with BMF.

Occasionally, an individual with a previously undiagnosed disorder may be found to have two rare syndromes as illustrated in the case of a woman with oculocutaneous albinism, bleeding diathesis, and neutropenia without findings consistent with Hermansky-Pudlak syndrome. Her parents were consanguineous and the combination of WES with homozygosity mapping revealed AR mutations in both *G6PC3*, a congenital neutropenia gene, and *SLC45A2*, a gene associated with oculocutaneous albinism type 4 (88).

## TRANSLATIONAL UTILITY OF GENOMICS IN THE IBMFS

### Mutation identification in individuals with a clinically diagnosed IBMFS and their family members

It is important to identify the germline mutation in individuals with an IBMFS to confirm the diagnosis and tailor medical management accordingly. Genetic testing of a single IBMFS can be expensive and time consuming since there are often many genes that can underlie the main types of IBMFS (Table 1). Logical, sequential testing of specific targeted genes is complicated because data on genotype-phenotype relationships in the IBMFS are limited by sample size and clinical heterogeneity. In many instances, BMF may be the presenting sign for any of the IBMFS described above. For example, patients with FA may not have congenital anomalies, and patients with DC may not have the diagnostic mucocutaneous triad. Importantly, an IBMFS-related cancer, such as head and neck squamous cell cancer, may be the initial manifestation of disorders such as FA and DC. Since many patients with an IBMFS will need an HSCT, knowledge of the causative mutation allows for testing of potential related hematopoietic stem cell donors. This is critical because across the IBMFS spectrum, there is variable penetrance and expressivity of the clinical features. For example, use of a clinically silent carrier of a *TERC* mutation as a HSCT donor for a DC relative led to delayed engraftment and death from infection (95). In another example, HSCT of a DC patient using a sibling, who was a silent carrier of a *TERC* mutation, resulted in non-mobilization of the hematopoietic stem cells and the subsequent need for androgen therapy to maintain blood counts (95). Molecular testing may also help in distinguishing post-HSCT complications, such as chronic graft versus host disease, from late manifestations of DC (96).

NGS multi-gene platforms, specific for certain IBMFS, have been developed and currently are used on a research basis only (18, 19, 21, 63). These panels have the potential to supplant other modalities for establishing the diagnosis while identifying the genetic etiology of IBMFS and other disorders. However, they will not likely replace molecular

diagnostics in IBMFS, but will be used to aid in the diagnostic work-up. For example, increased clastogen-induced chromosome breakage is diagnostic of FA and should continue to be used as a screening test. In FA it is possible that the complementation analysis performed to identify the defective protein could be replaced by NGS gene panels. In this instance, NGS gene panels may prove more cost-effective in identifying the genetic etiology of FA, and possibly other IBMFS.

## Characterization of individuals with a mutation-negative or unclassified IBMFS

The causative mutation is not known in a large number of patients that meet clinical criteria for specific IBMFS. Although aberrations in the ribosomal biogenesis pathway are well known to cause DBA, a mutation has been identified in only 50% of patients with clinical DBA (3). Similarly, in DC the list of associated telomere biology genes is now up to nine, but only about 70% of patients have a mutation in one of those genes (36). Ongoing NGS efforts, including WES, whole genome sequencing, and comprehensive searches for insertions/deletions, will be essential in uncovering the genetic etiology of these disorders.

In some instances, patients may not meet diagnostic criteria for a specific IBMFS but the clinical suspicion of an inherited syndrome remains high. This may include patients with BMF who failed to respond to immunosuppressive therapy, those with a family history of features seen in other IBMFS (*e.g.,* early onset cancers, BMF, pulmonary fibrosis), or a personal history of BMF and other clinical features consistent with, but non-diagnostic of a classic IBMFS (*e.g.,* BMF and nail dysplasia with non-diagnostic telomere lengths). WES, comprehensive gene panels, or the judicious use of other NGS technologies can be extremely valuable in understanding the genetic cause of disease in these patients. It is extremely important to comprehensively evaluate the clinical features of family members of such patients in order to determine whether subtle signs of a familial disorder exist. Sequence analysis should include the affected and unaffected relatives whenever possible. This leads to improved efficiency in filtering of both common and rare genetic variants.

## Genetic Variants in IBMFS Genes May Be Associated with Other Disorders

For the most part, the IBMFS-associated germline mutations are highly penetrant, resulting in clinically significant disease. In AD disorders, variable clinical penetrance of mutations in key telomere biology genes can complicate the diagnosis and genetic counseling. As noted above, AD germline mutations in *TERT* or *TERC* can result in classic DC, but may also result in a TBD lacking the typical DC-associated features (*e.g.,* aplastic anemia, pulmonary fibrosis, or liver disease) (36, 97). Clinically silent carriers of mutations other DC genes and in DBA genes have also been reported (1, 39, 53).

Hereditary breast cancer is associated with monoallelic germline mutations in some of the same DNA repair genes that cause FA in the presence of biallelic mutations. For example, AR inheritance of mutation in *BRCA2* (FANCD1) causes FA whereas AD inheritance of a single mutation can result in a significant predisposition to breast and ovarian cancer (98). The FA-associated *BRCA2* mutations are located in distinct regions from the breast and ovarian cancer susceptibility mutations. Mono-allelic germline mutations in other FA-associated genes, *BRIP1/FANCJ, PALB2/FANCN*, *RAD51C/FANCO,* and *SLX4/FANCP*, were identified as breast cancer susceptibility genes through candidate gene sequencing (99, 100). WES in breast cancer families found rare, deleterious mutations in *FANCC*, *BLM*, and *XRCC2* were associated with breast cancer (101, 102).

As NGS technologies are applied to a broader spectrum of clinical disorders, it is likely that expanded clinical phenotypes will be defined, based, in part, on the underlying molecular pathogenesis. Throughout all of these studies, it will be important to differentiate the highly-

penetrant, clinically-significant germline mutations from those that result in a modest or even relatively minor increased risk of cancer or other disorder.

## GENETIC COUNSELING CONSIDERATIONS

### Genetic Education and Counseling Are Crucial

With the discovery of a compendium of disease mutations comes the responsibility of appropriate genetic counseling of the patients and families. All patients and their family members should receive education and counseling related to the genetics of the disorder for which they are being tested. This includes ensuring their understanding of the concepts of the inheritance of traits and disease, as well as of DNA and how DNA is transcribed to RNA then translated into protein. The molecular consequences of common and very rare, disease-associated DNA nucleotide changes should be described and understood by all individuals undergoing genetic testing. For young children, the parents should be educated and counseled. If the child is of the age of assent (usually around 11 or 12 years of age, depending on institutional policies), age-specific education and counseling should be performed with appropriate assent of the minor child. All individuals undergoing genetic testing should also understand that genetic testing has implications for the entire family. Healthy individuals may be found to be "silent carriers" of a mutation that causes clinically significant disease in their relative.

Couples from IBMFS families are now using pre-implantation genetic diagnosis (PGD) to attempt to have a healthy, unaffected child (103). PGD can only be successful if the causative mutation is known in the family. For example, sequence analysis of *FANCA* in an FA patient with positive FANCA complementation testing will uncover the specific mutations in the patient; the parents are then tested for the specific *FANCA* mutations. However, in mutation-negative families, a comprehensive research-based approach may be required prior to clinical testing. This approach could include WES that leads to the discovery of a new disease-associated gene (*e.g., RTEL1* in DC).

### Return of NGS Results

NGS technologies have been introduced into the clinical diagnosis and management of IBMFS, providing new insights. It is likely that, in the near future, we will know the genetic cause of most of the classic Mendelian disorders. NGS platforms, by the sheer volume of sequence data they generate, identify genetic variants that may or may not be related to the disease being studied. The clinical genetics community agrees that there is some duty to disclose incidental findings in clinical exome and genome sequencing (104). The American College of Medical Genetics and Genomics (ACMG) policy statement recommended a list of conditions, genes, and variants for return of incidental findings to patients. This list includes genes associated with cancer-predisposition disorders, connective tissue diseases, cardiac-related phenotypes, and susceptibility to malignant hyperthermia.

Currently, there appears to be a lack of consensus concerning the return of findings in the research setting. This is especially challenging because many times the investigators seeking to identify the cause of an inherited disorder with a specific phenotype do not have the expertise to identify and prioritize variants in genes important for different phenotypes. This problem is further compounded in studies of pediatric patients (105). Current recommendations include consideration of whether the findings have a known, urgent clinical significance. This includes careful consideration of the balance between knowing about a potential genetic disorder versus the potential risks of anxiety or other psychosocial harm that could result from the knowledge (105). The ACMG statement acknowledged a lack of data on the consequences of return of incidental findings to participants in NGS studies and recommended future longitudinal studies (104).

## SUMMARY

NGS technologies have already had a substantial impact on advancing our knowledge of the etiology of the IBMFS. Ongoing NGS efforts will likely uncover the genetic etiology of the majority of the clinically diagnosed IBMFS. We can also expect to learn more about the pathophysiology of BMF through the study of patients and families with currently unclassified BMF disorders. These findings will advance our understanding of the underlying genetic defect. Follow-up with detailed functional studies will lead to important advances in understanding the underlying pathways and perhaps consider novel approaches towards therapy in the distant future. Patients and their family members need to be involved in the mutation discovery process through genetic education and counseling.

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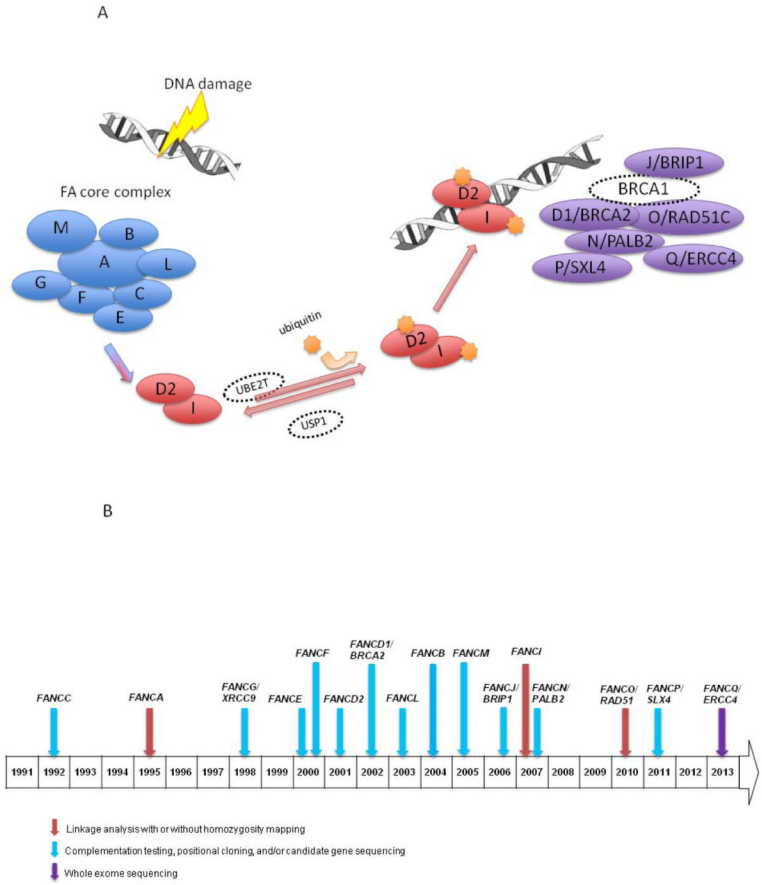


**Figure 1. Fanconi anemia (FA) genetics and biologic pathway**

**A)**FA/BRCA DNA damage response pathway. Following DNA damage, the proteins represented by A, B, C, E, F, G, L, and M form the core complex which is required for ubiquitination of the I and D2 proteins, which are in turn, required for the downstream complex of D2-ubi, I-ubi, and D1/BRCA1, N/PALB2, and J/BACH1/BRIP1 to form foci for DNA repair. Only BRCA1 is not yet known to be a FA gene. Adapted from Shimamura and Alter Blood Reviews 2010;24(3):101-22

**B)** Approximate timeline and methods of gene discovery in FA

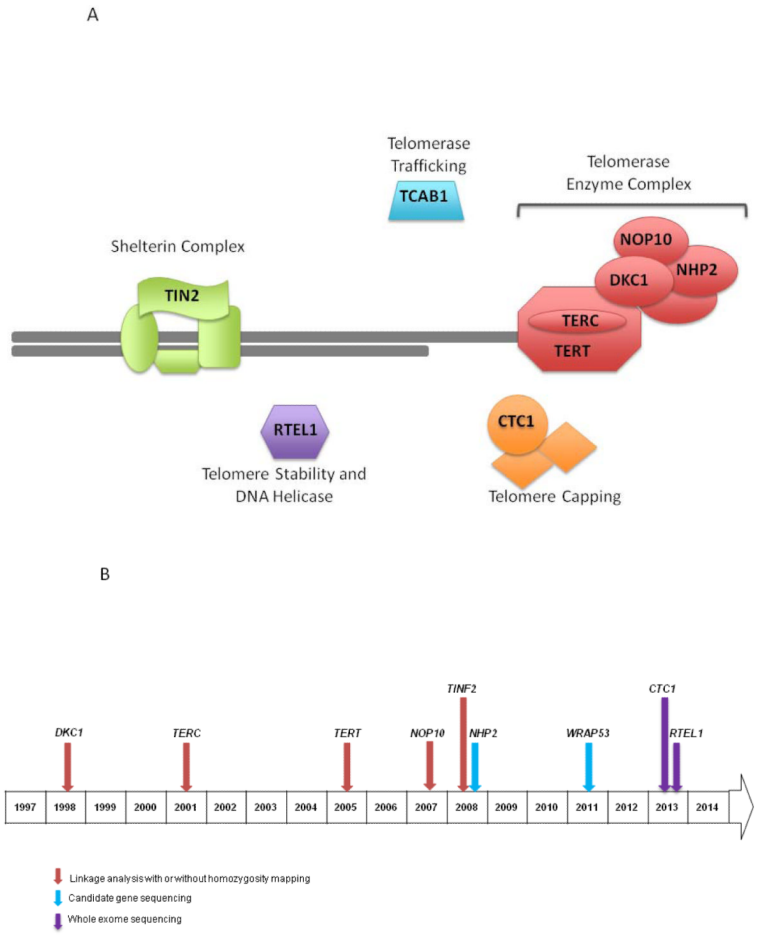


**Figure 2. Dyskeratosis Congenita genetics and biologic pathway**

**A)** Schematic of the telomere and functions of the proteins affected in dyskeratosis congenita and the related telomere biology disorders. Protein names are shown. Abbreviations: TCAB1, telomere Cajal body associated protein 1 (gene name: *WRAP53*); TIN2, TRF1-interacting nuclear factor 2 (*TINF2*); NOP10, NOP10 ribonucleoprotein (*NOP10*); NHP2, NHP2 ribonucleoprotein (*NHP2*); DKC1, dyskerin (*DKC1*); TERC, telomerase RNA component (*TERC*); TERT, telomerase (*TERT*); RTEL1, regulator of telomere elongation helicase 1 (*RTEL1*); CTC1, CTS telomere maintenance complex component 1 (*CTC1*)

**B)** Approximate timeline and methods of gene discovery in DC

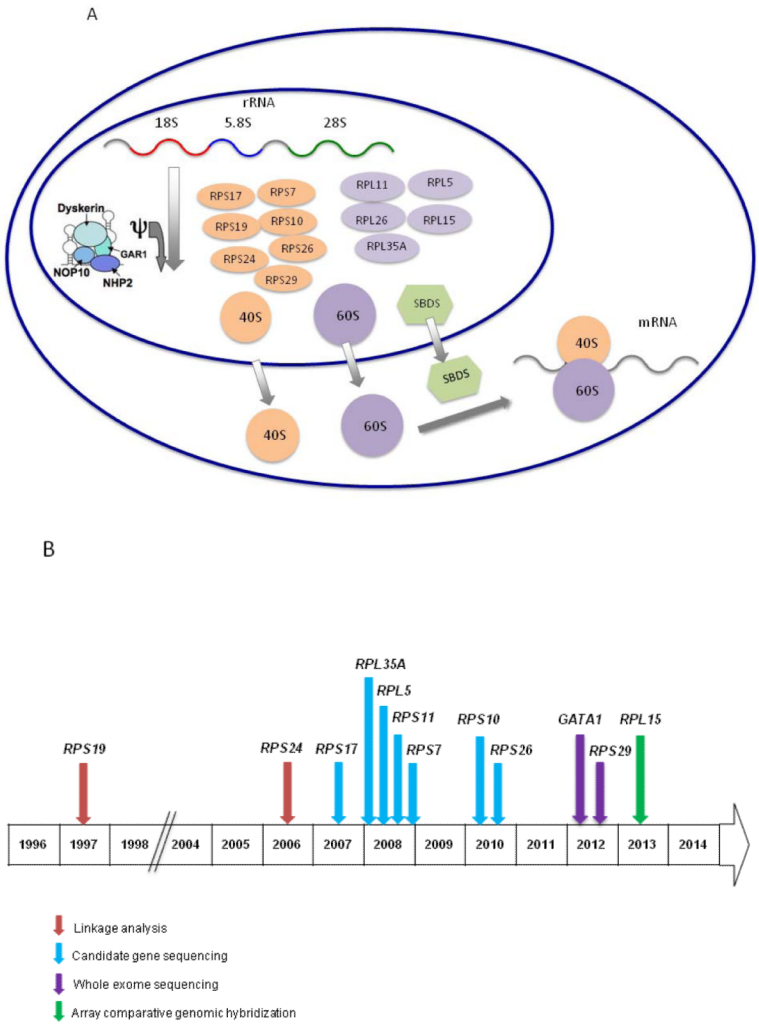


**Figure 3. Schematic of the ribosomal biogenesis pathway associated with DBA**

**A)** Ribosomes consist of a small 40S subunit and a large 60S subunit and catalyze protein synthesis. Small and large subunits are composed of four RNA species and approximately 80 structurally distinct proteins. The DBA-associated proteins are in both the small 40S and large 60S ribosomal subunit. They are encoded by *RPS19, RPS24, RPS1, RPS15, RPS27A, RPS10*, *RPS29* and *RPS26* which belong to the small ribosomal subunit, and by *RPL5, RPL11, RPL35A*, *RPL15* and *RPL36* which are components of the large ribosomal subunit. The *DKC1* gene encodes the dyskerin protein, which has been implicated in ribosomal RNA pseudouridylation (Φ). The SBDS protein appears to be involved in the joining of the 40S and 60S ribosomal subunits to form the mature 80S ribosome. AR mutations in *SBDS*, a key component of ribosomal assembly cause Shwachman-Diamond syndrome (SDS), a disorder of neutropenia and exocrine pancreatic insufficiency. SDS is not discussed herein because NGS studies have not been published in this disorder. Adapted from Shimamura and Alter Blood Reviews 2010;24(3):101-22

**B)** Approximate timeline and methods of gene discovery in DBA

## TABLE 1
### Clinical features of selected inherited bone marrow failure syndromes

All disorders may present with or without family history or as a result of *de novo* mutations in the proband. Patients with these disorders may present with some, but not necessarily all of the features listed. Clinically silent carriers are possible.

| Disorder | Clinical features | Laboratory findings | Biological pathway | Inheritance: known genes | Associated cancers |
|---|---|---|---|---|---|
| Fanconi Anemia | Radial ray anomalies, short stature, microcephaly, café au lait spots, structural renal anomalies, BMF | Increased chromosome breakage in clastogenic assay, macrocytosis, elevated HbF | DNA repair | XLR: *FANCB*<br>AR: *FANCA, FANCC, FANCD1/ BRCA2, FANCD2, FANCE, FANCF, FANCG/ XRCC9, FANCI, FANCJ/BACH1/ BRIP1, FANCL, FANCM, FANCN/PALB2, FANCO/RAD51C FANCP/SLX4, FANCQ (ERCC4)* | Squamous cell cancers of the head, neck, and anogenital region; MDS, AML |
| Dyskeratosis Congenita | Triad of nail dysplasia, lacy skin pigmentation abnormalities, and oral leukoplakia; BMF, pulmonary fibrosis, stenosis of esophagus, lacrimal ducts, or urethra, liver cirrhosis or fibrosis | Very short telomeres, macrocytosis, elevated HbF | Telomere biology | XLR: *DKC1*<br>AD: *TERT, TERC, TINF2, RTEL1*<br>AR: *NOP10, NHP2, WRAP53, RTEL1, TERT, CTC1* | MDS, AML, Head and neck squamous cell cancers |
| Hoyeraal Hreidarsson Syndrome (DC variant) | Features of DC and developmental delay,intra-uterine growth retardation, immunodeficiency, enteropathy | Very short telomeres, macrocytosis, elevated HbF | Telomere biology | XLR:*DKC1,*<br>AD: *TINF2,RTEL1*<br>AR: *TERT,RTEL1* | |
| Revesz Syndrome (DC variant) | Features of DC and bilateral exudative retinopathy, IUGR, intracranial calcifications, developmental delay, fine sparse hair | Very short telomeres, macrocytosis, elevated HbF | Telomere biology | AD: *TINF2* | |
| Coat's plus *or* Cerebroretinal microangiopathy with calcifications and cysts (CRMCC) | Bilateral exudative retinopathy, retinal telangiectasias, growth retardation, intracranial calcifications, neurological symptoms, bone abnormalities, gastrointestinal vascular ectasias, dystrophic nails, sparse or graying hair, and anemia | Very short telomeres, macrocytosis, elevated HbF | Telomere biology | AR: *CTC1* | |
| Diamond-Blackfan Anemia | Severe anemia, typically in infancy, ~25% with birth defects | Elevated RBC ADA, macrocytosis, elevated HbF | Ribosome biogenesis | AD: *RPS19, RPS17, RPS24, RPL35A, RPL5, RPL11,RPS7, RPS26, RPS10*<br>XLR: *GATA1* | MDS, AML, osteosarcoma, colon cancer |
| Thrombocytopenia absent radii | Absent radii with thumbs present, petechiae or hemorrhage in infancy | Thrombocytopenia in infancy/childhood, usually improves with time | Not yet elucidated | AR: 1q21.1 deletion and *RBM8A* variant | AML, ALL |

Abbreviations: DC, dyskeratosis congenita; MDS, myelodysplastic syndrome; AML, acute myeloid leukemia; ALL, acute lymphocytic leukemia; AD, autosomal dominant; AR, autosomal recessive; XLR, X-linked recessive; RBC, red blood cell; ADA, adenosine deaminase; HbF, fetal hemoglobin; BMF, bone marrow failure

## TABLE 2

Examples of studies using whole exome sequencing to uncover the genetic etiology of unclassified IBMFS.

| Disorder/Clinical Features | Inheritance: Gene | Biological pathway | Reference |
|---|---|---|---|
| Primary neutropenia and thrombocytopenia, oculocutaneous albinism, inflammatory bowel disease | AR: *SLC45A2* AR: *G6PC3* | *SLC45A2*: Transcription factor involved in trafficking of melanocyte-specific proteins to melanosomes *G6PC3*: Encodes enzyme involved in glucose-6-phosphate hydrolysis, maintenance of neutrophil viability and regulation of spontaneous neutrophil apoptosis | Culliane *et al.* (2011)(88) |
| MonoMAC syndrome: Monocytopenia, severe infections with nontuberculous Mycobacteria, pulmonary alveolar proteinosis; Emberger syndrome; familial MDS/AML | AD: *GATA2* | Transcription factor involved in development and proliferation of hematopoietic and endocrine cell lineages | Hsu *et al.* (2011)(91) |
| Bone marrow failure, congenital nerve deafness, MDS | AD: *SRP72* | Transcription factor involved in intracellular translocation of proteins by endoplasmic reticulum | Kirwan *et al.* (2012)(89) |
| Familial aplastic anemia presenting as undiagnosed congenital amegakaryocytic thrombocytopenia | AR: *MPL* | Encodes TPO receptor involved in megakaryopoiesis and HSC maintenance | Walne *et al.* (2012)(90) |
| Congenital neutropenia, primary myelofibrosis and bone marrow failure in infancy, bony abnormalities, nephromegaly | AR: *VPS45* | Transcription factor involved in regulation of endosomal system | Stephensky *et al.* (2013)(92) |
| Thrombocytopenia, fair hair and skin | AR: *SBF2* | Biological pathway unknown. | Abuzenadah *et al.* (2013)(93) |

Abbreviations: MDS, myelodysplastic syndrome; AD, autosomal dominant; AR, autosomal recessive; HSC, hematopoietic stem cell; WES, whole exome sequencing