# Chapter 4
# Investigating the Genetic Etiology of Disease in a Patient with Aplastic Anemia


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**Abstract** This study investigates the molecular basis of a male patient presenting with aplastic anemia (AA) and related symptoms of macrocytosis and mild thrombocytopenia. In addition, this patient also presents symptoms not usually found in AA patients, such as fatty liver, liver cirrhosis with portal hypertension, diffuse cerebral and cerebellar atrophy, and congenital left sensorineural hearing loss. As such, it is hypothesized that the patient has AA that is secondary to inherited bone marrow failure syndromes related to telomere biology disorders. Thus, to identify the underlying genetic cause of the disease, relative telomere length (RTL) of proband and family members was determined by qPCR, followed by identification of disease-causing variants through next-generation sequencing of the proband. The proband has a RTL of 0.11 (− 5.96 SD) which is shorter than the 1st percentile (− 2.33 SD). As RTL analysis indicates significant telomere shortening in the patient, it is likely that the patient has a telomere biology disorder. 8 variants in genes (*DKC1, ATXN3, PTPRQ, ABCB4, DIAPH3, TBP, PARP1*) associated with our patient's phenotype, 2 of which were previously reported and were also shortlisted as potential candidates. However, upon curation, these variants were found to be of uncertain significance, and the genetic cause of our patient's condition remains elusive. Nonetheless, we were able to confirm that the patient had significant shortening of telomeres.

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## 4.1 Background and purpose

### *4.1.1 Case Presentation*

A male patient in his 30s presented main symptoms of aplastic anemia (AA), macrocytosis, and mild thrombocytopenia. The patient also presented symptoms uncharacteristic of AA, namely fatty liver, liver cirrhosis with portal hypertension, diffuse cerebral and cerebellar atrophy, and congenital left sensorineural hearing loss.

### *4.1.2 Aplastic Anemia (AA)*

AA is a life-threatening bone marrow failure disorder [1] that can present at any age [2]. The incidence of AA ranges from 1.5 to 7 cases per million people, and the median age at diagnosis ranges from 25 to 60 years globally [3]. In most cases, AA is caused by immune-mediated destruction of hematopoietic stem cells (HSCs), resulting in pancytopenia [4]. However, the patient in this study not only presents symptoms associated with acquired AA such as macrocytosis and thrombocytopenia, but also liver cirrhosis with portal hypertension and fatty liver, which have been associated with certain inherited bone marrow failure syndromes (IBMFSs) that are telomere biology disorders (TBDs) [5, 6]. TBDs are characterized by short telomeres and may cause AA [7]. Telomere loss in HSCs can result in early apoptosis of these cells [8] and decrease their proliferative potential in vitro [9], causing them to be unable to mature and differentiate into blood cells [10]. This patient also presents with diffuse cerebral and cerebellar atrophy and congenital left sensorineural hearing loss (SHL) that are uncharacteristic of AA. Thus, it is possible that the patient's phenotype might be attributed to AA that is secondary to IBMFS [11] including dyskeratosis congenita (DC), Diamond Blackfan anemia (DBA), and Shwachman-Diamond syndrome (SDS), occurring from germline mutations inherited from parents or arising *de novo* [12].

### *4.1.3 Importance and Challenges of an Accurate Diagnosis*

It is crucial to provide an accurate diagnosis and distinguish between acquired AA and AA resulting from IBMFS to effectively manage symptoms and improve patient outcomes [13] as clinical treatments, and approaches are different for the two diagnoses [7, 11]. Patients with congenital disorders may also present additional symptoms that must be treated. Thus, an accurate diagnosis is required to rule out other underlying disorders, primarily IBMFS, and assess for specific etiologies and associations [11]. However, it is difficult to distinguish between inherited AA and IBMFS,

especially if the patient has a *de novo* mutation, or a mutation with low-disease penetration, or is not presenting any other classical congenital anomalies [11]. Besides, there are gaps in literature, where the list of genetic mutations that causes specific genetic disorders is incomplete, in addition to a large number of genes reported to be involved in a specific disease. Due to genotype heterogeneity, a specific genetic mutation can also give rise to multiple diseases. Additionally, symptoms that are not unique to a particular disease can overlap and vary in severity, making it challenging to provide an accurate diagnosis.

### *4.1.4 Aim*

The aim of this project is to investigate the underlying cause and genetic etiology of AA in a patient presenting with multiple associated symptoms. This will be done by (1) determining the relative telomere length measurements and (2) identifying presence of any pathogenic genetic variants.

## 4.2 Hypothesis

Based on this patient's primary presentation of AA and taking into consideration that he presented other symptoms that are not characteristic to acquired AA, as detailed above, it is hypothesized that a telomere biology disorder may be involved. From literature review, AA can be a symptom of several IBMFSs that are TBD, including DC [14] and SDS [15].

## 4.3 Materials and Method

### *4.3.1 Literature Review*

Literature review was initially conducted to examine the spectrum of clinical symptoms of IBMFS such as DC and AA. The respective causal genes, methods to identify the causes of the diseases, treatment options, and all overlapping symptoms were identified. The testing methods for IBMFS and AA such as next-generation sequencing for genetic testing, flow-FISH, and quantitative PCR for telomere length testing were also reviewed to determine the most suitable methodologies to be used as laboratory investigations to support a diagnosis.

### 4.3.2 Relative Telomere Length (RTL) Assay

The RTL was obtained from quantitative PCR (qPCR) data, using extracted genomic DNA from peripheral blood of patient, brother, mother, and father. Two references (NM914) and (NM917) were used as the positive controls, and a no-template control was used as the negative control. Telomere qPCRs and single-copy gene (*HBG* gene) qPCRs were performed in separate wells using primers [16, 17] as follows. Telomere (A) (5′-CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT-3′) and Telomere (B) (5′-GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT-3′); *HBG1* (5′-GCT TCT GAC ACA ACT GTG TTC ACT AGC-3′) and *HBG2* (5′-CAC CAA CTT CAT CCA CGT TCA CC-3′). A final volume of 10μl amplification reaction for both qPCRs using the QuantiNova SYBR Green PCR Kit - Qiagen 208056 contained 5 μl of 2x SYBR PCR Master Mix, 0.2 μl forward primer (10μM), 0.2 μl reverse primer (10 μM), 0.1 μl template DNA (10 ng/μl), and 4.5 μl RNase-Free water. Each sample was run in triplicates. PCR cycling conditions for telomere amplification started with 1 cycle of 95 °C denaturation for 2 min, followed by 35 cycles of 95 °C for 5 and 10 s at 58 °C for telomere amplification, and 56 °C for *HBG* amplification [16]. The PCR efficiency was > 90%, and the linear correlation coefficient values for both reactions were > 0.99.

### 4.3.3 RTL Determination

Telomere (T) signals and single-copy gene (S) signals were obtained from qPCR. The T/S ratio of each individual run was calculated by dividing the telomere starting quantity by the single-copy gene *HBG* starting quantity [16]. The mean of the triplicates was then calculated to give the mean T/S ratio, which represents the RTL value. Next, the standard deviation of the triplicates and the coefficient of variation (CV) were calculated to ensure the quality of data collected [16].

### 4.3.4 Statistical Analysis of RTL Methodology

*Z*-scores of patient and brother who were in their 30 s as well as father and mother who were in their 60 s were calculated using the formula $Z\text{-score} = (X - \mu)/\sigma$, where $X$ = RTL of the patient; $\mu$ = the mean RTL of age-matched controls; and $\sigma$ = the standard deviation (SD) of age-matched controls [18].

### 4.3.5 *Next-Generation Sequencing (NGS)*

As no suitable candidates were identified, the variants were re-filtered using subsequent sets of less stringent criteria. 2nd pass candidates were filtered for MAF < 0.05 instead of MAF < 0.01 before continuing with the previously described pipeline. 3rd pass candidates were filtered for MAF < 0.01 with non-coding and synonymous variants removed, however, these variants were prioritized for curation and pathogenicity prediction based on the genes' association with the patient's phenotype, as well as the results of computational predictive programs. The computational predictive programs used were SIFT, PolyPhen2-HDIV, PolyPhen2-HVAR, MutationTaster, mutation assessor, and FATHMM for exonic missense variants and NetGene2, BDGP, MutationTaster, varSEAK for intronic variants near a splice site. 4th pass candidates were filtered for non-synonymous variants occurring in AA genes with a MAF of < 0.01, but inclusive of non-coding variants, before curation and pathogenicity prediction (Fig. 4.1; Table 4.1).

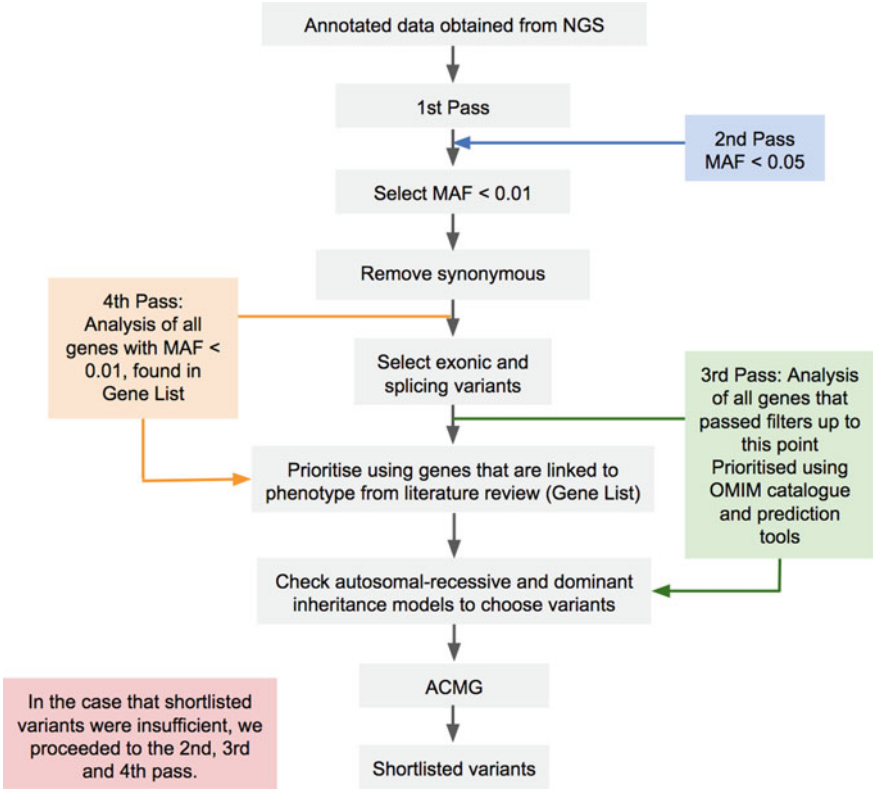


**Fig. 4.1** NGS pipeline: Pathogenic variants were identified by passing annotated data obtained from NGS through the pipeline containing 4 passes

**Table 4.1** RTL results showing raw RTL and *Z*-score calculated relative to age-matched normal controls

| Sample | RTL | *Z*-score/SD |
|---|---|---|
| Patient | 0.11 | − 5.96 |
| Patient's brother | 0.13 | − 5.56 |
| Patient's mother | 0.14 | − 7.50 |
| Patient's father | 0.16 | − 6.66 |

## 4.4 Results and Discussion

### *4.4.1 RTL Z-Score Analysis*

The *Z*-score compared the telomere measurement in each individual with the age-matched mean and SD of the values in the normal controls, accounting for the known wide inter-individual telomere length variability. Telomere shortening and TBD were considered when the *Z*-score was below the 10th percentile of a normal distribution (− 1.28 SD) [19]. The patient was observed to have a RTL of 0.11 (− 5.96 SD), which is shorter than the 1st percentile (− 2.33 SD) and has been cited by literature to be 91% specific for DC [20]. However, the patient also presents with left SHL, diffuse cerebral, and cerebellar atrophy which have not been linked to DC, suggesting alternative underlying genetic mutations. Both the patient's mother and father also have RTLs shorter than the 1st percentile when compared to age-matched normal controls, suggesting the possibility of a TBD. However, only the patient and his brother presented symptoms while their parents are phenotypically normal. Telomere length decreases progressively with advancing age [21], but the patient and his brother who are in their 30 s have shorter RTL than their parents who are in their 60 s. Hence, both patient and brother might be suffering from a TBD that causes shorter RTL. The brother has milder symptoms and a longer RTL compared to the patient, indicating that the brother might have a milder form of TBD. The mother has a shorter RTL of 0.14 as compared to the father who has a RTL of 0.16. Thus, it is possible that the mother is an asymptomatic mutation carrier [7] for an X-linked TBD such as DC, while telomere shortening in the father may be due to other factors such as chronic stress, poor lifestyle choices [22], and paternal age at birth [23]. To identify potential genetic causes of the patient's phenotype, exome sequencing was conducted on the patient's genome.

### *4.4.2 NGS Analysis*

Table 4.2 describes the 8 variants shortlisted from patient's WES data of which the *DKC1, ATXN3, ABCB4,* and *PTPRQ* variants will be discussed in detail. A homozygous *DKC1* variant NM_001363.3:c.771+3A>G was identified in the patient. This mutation has not been reported in any variant database and is absent in any

**Table 4.2** 8 shortlisted variants with ACMG curation

| Gene | Disease and inheritance | Variant | Variant zygosity | ACMG | Prior reports/ClinVar | Deleterious predictions |
|---|---|---|---|---|---|---|
| DKC1 | Dyskeratosis congenita, X-linked [25] | NM_001363.3:c.771+3A>G | Homo | VUS: PM2 | Variant not reported | 2/4 |
| ATXN3 | Machado-Joseph disease (AD) [29] | NM_004993:exon10:c.915_916insCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGC: p.G306fs | Compound het | VUS: PM2 | Variant not reported | NA |
| ATXN3 | Machado-Joseph disease (AD) [29] | NM_004993:exon10:c.915_916insCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAG: p.G306delinsQQQQQQQQQQQQG | Compound het | VUS: PM2 | Variant not reported | NA |
| ABCB4 | Cholestasis, progressive familial intrahepatic 3 (AR); gallbladder disease 1 (AD) [30] | NM_000443:exon13:c.A1529G:p.N510S | Het | VUS: PM2, PP3, PP5 | Reported likely pathogenic; pathogenic; uncertain significance | 6/7 |
| PTPRQ | Deafness, autosomal recessive 84A (AR); deafness, autosomal dominant 73 (AD) [31] | NM_001145026:exon3:c.C206G:p.S69C | Het | VUS: PM2, PP3 | Variant not reported | 4/7 |

(continued)

**Table 4.2** (continued)

| Gene | Disease and inheritance | Variant | Variant zygosity | ACMG | Prior reports/ClinVar | Deleterious predictions |
|---|---|---|---|---|---|---|
| *DIAPH3* | Auditory neuropathy, autosomal dominant, 1 (AD) [32] | NM_001042517:exon16:c.C1787T:p.P596L | Het | VUS: PM2, PP3, BP6 | Reported benign | 4/7 |
| TBP | {Parkinson disease, susceptibility to}; spinocerebellar ataxia 17 (AD) [33] | NM_003194:exon3:c.222_223insCAGCAGCAG:p.Q74delinsQQQQ | Het | VUS: PM2 | Variant not reported | NA |
| PARP1 | Fanconi anemia [34] | NM_001618:exon21:c.A2819G:p.K940R | Het | VUS: PM2, PP3 | Variant not reported | 5/7 |

population databases used. Since this mutation occurs 3 bases away from an intron-exon boundary, it is predicted to affect splicing by disrupting the highly conserved sequences that define the intron-exon boundary [24]. MutationTaster predicts that this variant causes a gain in splice donor site, with a score of 0.76. varSEAK predicts that this variant is deleterious with likely loss of splice site. BDGP and NetGene2 predict that this variant is not likely to cause a loss or gain of splice sites, with NetGene2 predicting that confidence of the donor splice site (direct strand) decreases from 0.83 to 0.71. However, BDGP and NetGene2 both show that this variant occurs close to the predicted splice site and might affect splicing through activating cryptic splice sites or affecting the binding of splicing enhancers and silencers [24]. Thus, this variant is likely to affect splicing and compromise the function of the *DKC1* gene product dyskerin, which is a component of the telomerase complex, possibly causing a defect in the maintenance of telomeres [25].

*DKC1* is associated with X-linked dyskeratosis congenita (DC) [25], with 20–25% of DC cases being caused by a mutation in the *DKC1* gene [26]. DC is a TBD and has also been associated with liver cirrhosis and cerebellar ataxia [27]. Approximately 80–90% of patients with DC develop AA before age 30, with the severity of bone marrow failure varying [28]. While the patient does not display all the symptoms of DC such as the classical triad of nail dystrophy, skin hyperpigmentation, and oral leukoplakia [20], DC has a broad phenotypic spectrum [20], so patients may not display the full or classical phenotype [20]. Based on our ACMG curation, this variant is classified as variant of uncertain significance (VUS): PM2. However, considering that the variant occurs close to the predicted splice site, likely affecting splicing and protein function, and since the patient's phenotype corresponds with the symptoms of DC, including AA, thrombocytopenia, liver cirrhosis, and cerebellar ataxia, in addition to the short telomeres below the 1st percentile that is characteristic of DC patients [18], we consider this variant to be a strong causative candidate for our patient's condition.

Two novel compound heterozygous *ATXN3* variants NM_004993:exon10:c.915_916insCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCA:p.G306fs and NM_004993:exon10:c.915_916insCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAG:p.G306delinsQQQQQQQQQQQQQG were also observed in the patient. Mutations in *ATXN3* have been associated with Machado-Joseph disease (MJD) [29, 35], also known as spinocerebellar ataxia, which includes symptoms of cerebellar atrophy. While the patient does not present the full range of symptoms of MJD including eye abnormalities and limb ataxia, MJD has wide clinical variability [29]. There is variability in age of onset and severity of disease, with age of onset varying from early teens to late-adulthood [36]. In this variant, there is an expansion of 26 CAG repeats and a CA insertion, resulting in a frameshift mutation. MJD is known to result from an expansion of a (CAG)*n* repeat in the *ATXN3* gene [37]. Additionally, a-1 frameshift with an expanded CAG repeat has been reported to have a deleterious effect on *Drosophila* and mammalian neurons since a GCA frame would be translated, producing polyalanine instead of polyglutamine [38].

The patient harbored a heterozygous *ABCB4* variant, NM_000443:exon13:c.A1529G:p.N510S. While homozygous mutations in *ABCB4*, also known as *MDR3*, have been associated with cholestasis, progressive familial intrahepatic 3 autosomal recessive [30], heterozygous mutations in *ABCB4* variants have been reported to result in less severe clinical patterns [39]. Mutations in the *ABCB4* gene present clinical symptoms including adult idiopathic cirrhosis [39] and drug-induced liver injury [40]. As such, this mutation in the *ABCB4* gene might be related to the fatty liver and liver cirrhosis with portal hypertension that ML1 presents with.

Our analysis also identified a heterozygous *PTPRQ* variant, NM_001145026:exon3:c.C206G:p.S69C, in the patient. PTPRQ has been associated with deafness, autosomal dominant 73 [31]. This variant occurs in the Fibronectin Type III (FN3) domain. PTPRQ has 17 FN3 domains which mediate binding to extracellular proteins [41]. Mutations in the FN3 domain can lead to malformations of shaft connectors and incomplete maturation of cochlear hair bundles, resulting in SHL [42]. Hence, mutations in this domain are likely to deleteriously impact function. Since mutations in the *PTPRQ* gene have been associated with the patient's phenotype of congenital left SHL, and the affected FN3 domain may result in SHL, this variant may be another potential contributing candidate for our patient's phenotype.

Although 8 variants were shortlisted for the patient, we were unable to classify any of them as clinically pathogenic under the ACMG guidelines. There are limitations of WES, where there may be damaging deep intronic pathogenic variants, copy number variations, or chromosomal relocations that occurred but were undetected. To address these limitations, whole-genome sequencing can be performed to identify variants in the entire genome. If no pathogenic variants are identified, further studies on potential underlying cause of the patient's disease can be made through investigating the phenotype and genotype of the patient's relatives using WES. This would enable us to analyze the cosegregation of variants with the disease in the family, aiding in the classification of pathogenicity of variants with uncertain significance. Furthermore, in vitro or in vivo functional studies can be carried out on shortlisted variants to help determine their pathogenicity.

Although the causal gene underlying a TBD could not be conclusively identified, the telomere measurements study points to the latter as being associated with the condition in the patient. It is noted that absolute telomere measurements were not performed due to the complexity of the experiments, nonetheless RTL assays are commonly accepted as reliable and have been used in studies to detect telomere disorders [43], with short RTL measurements being used as a criteria for diagnosis in DC patients [44]. As to whether the telomere shortening could be a consequence and not cause of the disease, the symptoms of the patient manifesting phenotypes suggestive of telomere disorder lends support that the latter is probably the cause of disease.

## 4.5 Conclusion

In conclusion, the patient is likely to be suffering from a TBD since telomere shortening is evident, with the patient having a relatively shorter RTL of 0.11 (− 5.96 SD), which is below the 1st percentile (− 2.33 SD) with respect to age-matched normal subjects. Through comprehensive analysis of the patient's genome, we have shortlisted 8 variants in genes (*DKC1, ATXN3, PTPRQ, ABCB4, DIAPH3, TBP, PARP1*) associated with our patient's phenotype as potential candidates. Particularly, we identified a mutation in the *DKC1* gene (NM_001363.3:c.771+3A>G) near the intron-exon boundary, which could likely affect splicing and dyskerin function, resulting in reduced telomerase function [25]. However, upon curation of our shortlisted variants, all the variants were found to be of uncertain significance due to lack of supporting evidence in literature. Hence, the genetic cause of disease in our patient could not be confirmed. Nevertheless, these shortlisted variants are promising potential candidates, and their pathogenicity can be confirmed through further studies such as in vitro or in vivo functional studies if they are also found in other similar patients elsewhere.

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