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Dyskeratosis congenita as a multifaceted bone marrow disorder

Maciej Mazurek, Wojciech Młynarski, Joanna Madzio*

Department of Pediatrics, Oncology & Hematology, Medical University of Lodz, Łódź, Poland

Abstract

Dyskeratosis congenita (DC) is a rare multisystem clinical entity caused by genetic mutations associated with telomere biology disorder. The symptoms include bone marrow dysfunction as well as skin and mucosal abnormalities. In severe cases, DC is characterized by high mortality rates among children. In milder subtypes, it is less detectable in adults, due to the occurrence of cryptic forms of the disease. To date, more than 15 mutated genes have been shown as causative for DC.

The aim of this study was to provide a brief description of the currently known clinical and genetic characteristics of DC, and to elucidate the molecular pathogenesis.

Key words: bone marrow failure, dyskeratosis congenita, mutation

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Molecular background

The integrity of the genome is a crucial aspect of maintaining all processes in a living cell. Any disruptions related to this have far-reaching consequences, leading to cell death through apoptosis [1]. If this mechanism fails, disruptions in the cell cycle and division can lead to further rearrangements, ultimately resulting in transformation and malignancy [2, 3].

One of the important mechanisms involved in genome maintenance are telomeres [4]. Telomeres are nucleoprotein complexes located at the ends of eukaryotic chromosomes, consisting of protective proteins and a double, non-coding DNA strand containing G-rich repeats (TTAGGG)n, ending with a single strand at the 3' end. Telomere length depends on many aspects, including interspecies differences, interindividual differences, and differences between cells of the same organism [5–7]. Variable telomere lengths have been observed depending on the cell type, its metabolic rate, age, or inheritance. In the case of mammalian cells, their length ranges from 2 kb to 14 kb, for example, in sperm, the length is estimated to be between 10 kb and 14 kb, while in somatic cells, it is a few thousand base pairs [7]. In the umbilical cord blood of a newborn, the average telomere length is around 10 kb [8].

The main function of telomeres is to maintain the integrity of the genome during replication. Being located at the ends of chromosomes, they prevent their fusion and thus limit the possibility of random recombination with other chromosomes. Along with proteins, they form a specific secondary structure at the ends of telomeres, a protruding single 3' strand (overhang), called the T-loop. Loops protect the ends of chromosomes from the accidental response of repair systems during replication [9, 10].

Telomeres are also a kind of response to the so-called 'end replication' problem, which was first described over 50 years ago [11]. This is a result of the imperfection of the replication process, or rather the activity of DNA polymerase, which synthesizes a new strand only in one direction from the 5' to the 3' end. For replication to proceed in

*Address for correspondence: Joanna Madzio, Department of Pediatrics, Oncology and Hematology, Medical University of Lodz, Sporna 36/50, 91–738 Łódź, Poland, e-mail: joanna.madzio@umed.lodz.pl

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both directions, DNA on the lagging strand must be synthesized in short Okazaki fragments from RNA primers. When the replicase reaches the end of the chromosome, the nearest RNA primer on the lagging strand cannot be replaced with DNA or it would fall off the chromosomal DNA. Therefore, the 3' end of the strand lacks a synthesized DNA fragment. In this case, the telomeric DNA on the sister strand serves as a template, according to which the missing fragment of chromosomal DNA is synthesized, and the loss of the fragment of the 3' end strand is on the side of the telomeric DNA [12–16]. This mechanism is considered a molecular biological clock that determines the limit of cell division, and this is called the Hayflick limit [17, 18].

Several proteins are responsible for maintaining the integrity and functionality of telomeres, including three key protein complexes: 1) telomerase, responsible for elongating telomeres; 2) shelterin complex, binding to the single-stranded ends of telomeres; and 3) CST complex, which also modulates the activity of telomerase by limiting its access to telomere ends.

Telomerase is a ribonucleoprotein DNA polymerase dependent on RNA with properties of reverse transcriptase, responsible for synthesizing telomeres during the S and M phases of the cell cycle [19]. Telomerase is a holoenzyme composed of several key subunits, with TERT (hTERT in humans) responsible for the main elongation activity of the complex, which is a subunit with reverse transcriptase activity that catalyzes the elongation of telomeric DNA. The TERC/TR subunit (hTR in humans) is responsible for the RNA matrix, on which the new DNA strand is synthesized [20, 21]. The remaining subunits are associated with the biogenesis and stability of the entire complex: dyskerin (pseudouridine synthase) binds to a specific TERC RNA motif (HA/ACA box), stabilizing this structure and thus contributing to the enzymatic stability of the entire complex.

In addition, the NOP10, NHP2, and GAR1 subunits stabilize the process of TERC maturation, performing isomerization of uridine to pseudouridine, stabilizing the spatial conformation of rRNA [22]. During maturation, NAF1 is a chaperone protein that supports TARC maturation, and during this process it is replaced by GAR1 which stabilizes the mature form [23]. Other important elements associated with telomerase complex maturation include the PARN ribonuclease and the TCAB1 protein. The former is responsible for the maturation of the TERC transcript, as well as DKC1 and TRF1 by removing the poly(A) tail [24, 25]. The latter is a protein responsible for recruiting the complex to the Cajal bodies at the ends of telomeres [26].

The attachment of telomerase to telomeres is tightly controlled, and one of the mechanisms of control is the six-protein complex shelterin, which controls the recruitment of telomerase to telomeric DNA [27]. The main proteins that stabilize the binding to DNA are the TRF1 and TRF2 subunits. Both of these bind to the double-stranded structure and inhibit the attachment of telomerase by creating a spatial structure (t-loop) [28]. These subunits are structurally stabilized by TIN2 [29], which also provides a scaffold for the next protein — TPP1, responsible for the recruitment and attachment of telomerase [30]. An extremely important component of the protective complex is POT1, which binds to the single strand at the 3' end of the telomere, creating a specific secondary structure (D-loop) that protects the end from recognition by RPA1, one of the activators of the homologous repair system [31–34]. The RAP1 subunit, along with TRF2, is responsible for inhibiting non-homologous end joining (NHEJ) [35, 36].

An additional complex that regulates the activity of telomerase and interacts with the protective complex is the CST complex, consisting of three subunits [37]. The main component is CTC1, which along with the STN1 subunit in mammalian cells is responsible for inhibiting the attachment of telomerase to telomeres by physical interaction with POT1 and TPP1 [38]. Both subunits have the ability to directly bind to the single strand of telomeric DNA. The third, smallest subunit (TEN1) does not have DNA binding properties, but is responsible for stabilizing the interaction between the other components. In addition, the CTC1 subunit is responsible for recruiting DNA polymerase α, which is responsible for replicating chromosomes during cell division [39]. The relationship among the various complexes involved in telomere length maintenance is shown in Figure 1.

In humans, telomerase expression is high during early embryogenesis, but in most somatic cells it is silenced, with the exception of hematopoietic cells, stem cells, activated lymphocytes, and male germ cells [40, 41]. Furthermore, it is estimated that increased overexpression and overactivity of telomerase occurs in c.80% of human somatic cell tumors, clearly linked to escaping cell cycle checkpoints and abolishing the Hayflick limit [42–44]. Interestingly, the expression of the TERT subunit can be stimulated in infected cells by certain viruses, such as Epstein-Bárr virus (EBV), cytomegalovirus (CMV), human papillomavirus (HPV), hepatitis B virus (HBV), hepatitis B virus (HCV), and Kaposi's sarcoma-associated herpesvirus (KSHV) [45].

As described, each of the individual components of the complexes has a significant importance in the biogenesis, maintenance, and stability of telomeres. Disruptions in any of them can lead to diseases associated with telomere biology disorders (as presented in Table I). This review will discuss the most important diseases associated with telomere biology disorders.

Dyskeratosis congenita: clinical manifestations

The first reports of a lethal, genetic disease characterized by mucocutaneous and hematological disorders were published in 1906 by Zinsser, and later in 1926 and 1930

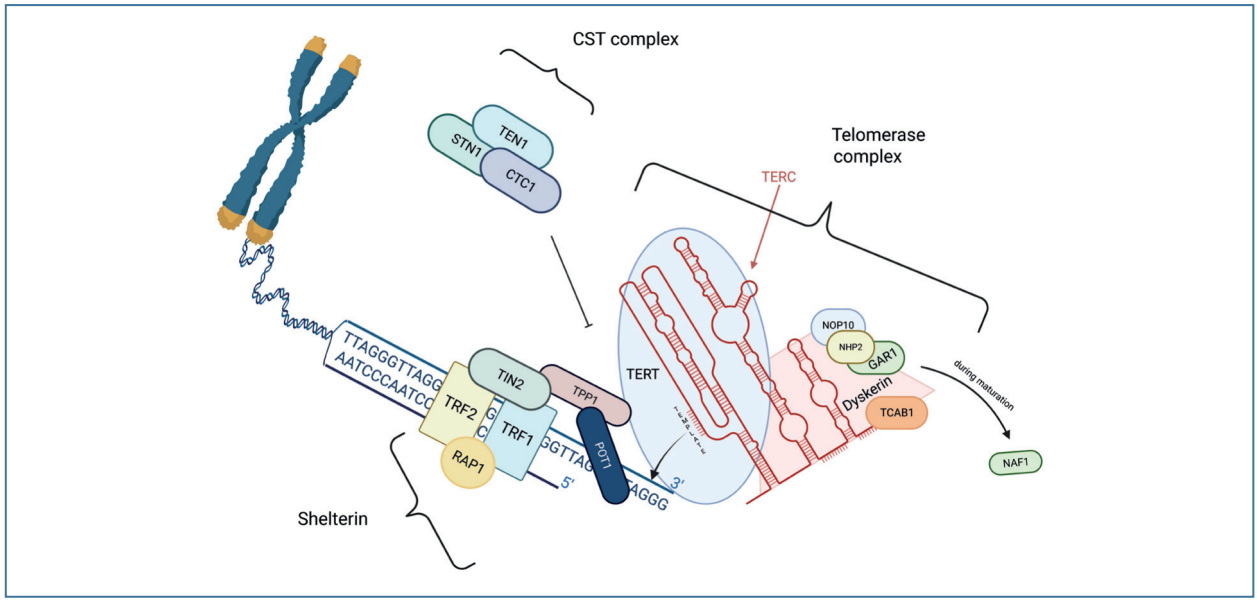

Figure 1. Three crucial complexes and their particular subunits involving in maintenance telomers length, each is of which is essential for proper telomer maintenance machinery

Table I. Germinal mutations associated with telomere biology disorder and other diseases (in brackets phenotype OMIM database number)

Localization	Gene (protein/ /product)	Cytogenetics	Function	Loss of function	Inheritance model	Clinical presentation	Non-TBD syndrome	Mutation frequency of TBD
Telomerase complex	*TERT* (hTERT)	5p15.33	Recruitment of the complex, telomere elongation	Inhibition of recruitment, elongation and telomerase activity	AD	DC (613989), AA, PF, LD, MDS, AML (601626)	CMM9 (615134)	10–20%
					AR	HH		<1%
	TERC telomerase RNA component (hTR)	3q26.2	RNA template, telomere elongation	Inhibition of telomerase activity	AD	DC (127550), AA, PF (614743), LD, HH, MDS, AML		10–20%
	DKC1 (dyskerin)	Xq28	Complex assembly, hTR stabilization	Decreased hTR stability and telomerase activity	XLR (X-linked)	DC (305000), HH, PF		10–20% of males
	NOP10 (NOP10, NOLA3)	15q14	Biogenesis and stabilization of hTR	Decreased hTR stability and telomerase activity	AR	DC (224230)		<1%
	NHP2 (NHP2, NOLA2)	5q35.3	Biogenesis and stabilization of hTR	Decreased hTR stability and telomerase activity	AR	DC (613987)		<1%
	NAF1	4q32.2	Biogenesis and stabilization of hTR	Decreased hTR stability and telomerase activity	AD	LD, PF (620365), MDS		<1%
	WRAP53 (TCAB1)	17p13.1	Trafficking through Cajal bodies, telomerase recruitment	Inhibition of telomerase recruitment to telomeres	AR	DC (613988), HH		<1%

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Table I. cont. Germinal mutations associated with telomere biology disorder and other diseases (in brackets phenotype OMIM database number)

Localization	Gene (protein/ /product)	Cytogenetics	Function	Loss of function	Inheritance model	Clinical presentation	Non-TBD syndrome	Mutation frequency of TBD
Shelterin complex	*TINF2* TIN2	14q12	Regulation of telomerase activity, recruitment of Shelterin to telomeres, protection of telomeres from DDR	Disruption of telomere maintenance	AD, S (*de novo*)	DC (613990), HH, PF, RS (268130)		10–20%
	ACD (TPP1)	16q22.1	Recruitment of telomerase and telomerase activity	Disruption of telomerase recruitment	AD	DC (616553), AA		<1%
					AR	DC, HH (616553)		<1%
	POT1	7q31.33	Interaction with CST complex, telomere protection from DDR, telomerase inhibition	Disruption of telomere replication and telomerase negative	AD	PF (620367)	GLM9 (606478), CMM10 (615848)	–
					AR	CP (620368)		<1%
CST complex	*CTC1*	17p13.1	Telomere replication, telomerase inhibition	Disruption of telomere replication and telomerase inhibition	AR	DC, CP (612199)		<1%
	STN1	10q24.33	Telomere replication, telomerase inhibition	Disruption of telomere replication and telomerase inhibition	AR	CP (617341)		<1%
	RTEL1 (RTEL1)	20q13.33	Telomere replication, T-loop stability	Disruption of telomere replication	AD	DC (615190), AA, PF, LD,		10–20%
					AR	HH, DC		<10%
	PARN	16p13.12	Maturation and stability of hTR	Decrease in hTR stability, inhibition of telomerase activity	AD	PF (616371)		10–20%
					AR	DC (616353), HH		<1%
	ZCCHC8 (ZCHC8)	12q24.13	Maturation of hTR	Impaired of telomerase function	AD	PF (618674)		<1%
	DCLRE1B (Apollo)	1p.13.2	Interacts with TRF2 at telomers, involving on cross-link DNA repair	Chromosome instability	AR	DC (620133), HH		<1%

AA — aplastic anemia; AD — autosomal dominant; AR — autosomal recessive; CMM — cutaneous malignant melanoma; CP — Coats plus; DC — dyskeratosis congenita; DDR — DNA damage response; GLM — glioma susceptibility; HH — Hoyeraal-Hreidarsson syndrome; LD — liver disease; PF — pulmonary fibrosis; RS — Revesz syndrome; S — somatic; XLR — X-linked

by Engman and Cole. The disease was then called Zinsser-Engman-Cole syndrome [46].

Today, it is known as congenital dyskeratosis (DC), a rare multi-systemic disease caused by disturbances in telomere biology, primarily their shortening, which is documented in 90% of patients [47]. DC strongly predominantly affects males. Clinical features vary widely. DC can manifest as the classical triad associated with dermatological disorders: reticular hyperpigmentation, nail dystrophy, and leukoplakia. Other characteristics include premature

graying of the hair, osteoporosis, lacrimation, dental abnormalities, and gastrointestinal diseases. Additionally, testicular atrophy may occur. Furthermore, the disorders can involve the bone marrow, leading to bone marrow failure (BMF) and predisposing to malignancies, as well as contributing to pulmonary and hepatic fibrosis. The estimated prevalence of congenital dyskeratosis is 1–9 per 1,000,000 individuals (ORPHA code: 1775, OMIM phenotype number for DKC1 variant: 305000).

Mucocutaneous symptoms are very common in DC. Reticulated pigmentation changes are observed in 90% of cases, often appearing in the first decade of life they can also be present as congenital pigmentation changes [48]. Nail dystrophy can manifest in the early months of life or in late adolescence, and occurs in 88% of patients diagnosed with DC. Leukoplakia occurs in 80% of cases and exhibits variable progression. It can be observed at birth or in patients several months old. It may be visible on the tongue, the cheek, or on both simultaneously. Additionally, 16% of patients experience hair and eyebrow changes, including premature graying or loss [49, 50].

In addition, idiopathic pulmonary fibrosis can develop in patients with DC and, for many of them, this may be one of the first symptoms of telomere-related disorders. They can be revealed by dry rales, dry cough, or dyspnea, and hypoxemia with exertion is usually one of the earliest signs. With disease progression, hypoxemia occurs at rest [51].

Therefore, in the case of such a symptom occurring in a young individual, DC should be considered in the diagnostic process. In these cases, mutations are most commonly autosomal dominant in nature, and are associated with particular genes: *TERT*, *RTEL1*, *TERC*, *TINF2*, *DKC1*, *PARN* [52]. Furthermore, pulmonary fibrosis is one of the more severe complications/consequences associated with hematopoietic stem cell transplantation (HCT), which is the only treatment option for patients with BMF. In the long term, this can be one of the leading causes of death due to infections and impairment of lung function, such as reduced lung capacity and impaired gas exchange [53, 54]. Additionally, it has been reported that pulmonary arteriovenous malformations, without fibrosis features, can occur in patients with DC [55, 56].

Progressive bone marrow failure is common and occurs in 80% of cases [57]. Due to pancytopenia and dysfunctional immune cells, the majority of deaths are caused by viral infections such as CMV, HBV, herpes simplex virus (HSV), and fungal infections including *Candida spp.*, *Pneumocystis jiroveci,* and *Aspergillus spp.*, as well as bacterial infections including *Pseudomonas aeruginosa* infection leading to sepsis [49, 58–60]. Patients with DC are highly susceptible to developing leukemia and squamous cell tumors in the head and neck region. The symptoms and course of the disease depend on the specific gene mutations involved (set out in Table I).

Genetics of DC

To date, several genes directly associated with telomere activity, maturation, and biogenesis have been described including *ACD*, *DCK1*, *TER*, *TERC*, *NOP10*, *NHP2*, *NAF1*, *TINF2*, *POT1*, *WRAP53*, *CTC1*, *STN1*, *TEN1*, *RTEL1*, *PARN*, *DCLRE1B* and *ZCCHC8*. The inheritance pattern is diverse and mainly involves germline mutations, including autosomal dominant and recessive inheritance, as well as X-linked inheritance [56]. The most common type is X-linked inheritance, as evidenced by a male-to-female disease incidence ratio of 13:1 [61]. The type of inheritance depends on the specific mutations and is associated with the types of symptoms and the course of the disease. The severity of the disease correlates with the age at symptom onset: the earlier the onset, the more severe the course. In very severe cases, symptoms can appear in newborns or even during fetal life, manifesting as multi-systemic dysfunction. In adolescence or adulthood, the classical clinical trial PF is not always observed, and DC may present in cryptic variants, only manifesting as respiratory, liver function, or hematological disorders [62, 63]. Based on the above description, DC can be divided into two main types, cryptic and severe, taking into account the severity of symptoms.

Cryptic variants of DC

Cryptic variants often manifest as single or oligosymptomatic disorders that occur in early adulthood or middle age. In these cases, DC manifests as diseases that primarily cause other disorders and initially show no apparent links to telomere biology (TBD). Due to the absence of the classical triad and the late clinical manifestation, patients are often misdiagnosed. The most common conditions associated with cryptic variants are aplastic anemia (AA) and idiopathic pulmonary fibrosis (PF) [62, 63]. Similarly as in the 'classical' form, patients are at risk of developing myelodysplastic syndrome (MDS) and acute myeloblastic leukemia (AML) [64].

Heterozygous variants in *TERT*, *TERC*, and *RTEL1* are typically detected in cryptic TBD, presenting as PF or BMF [65, 66]. These mutations lead to the inhibition of telomerase activity (preventing telomere elongation) and disruptions in telomere replication during cell division.

Severe DC variants

Hoyeraal-Hreidarsson

Hoyeraal-Hreidarsson syndrome (HHS or HH) is a severe variant of DC that manifests with clinical severity from a very young age. In addition to the classical symptoms associated with DC, patients with HHS suffer from immune deficiencies, intrauterine growth retardation, and neurological disorders such as developmental delay and

cerebellar aplasia, which are characteristic features of this syndrome. Molecular studies have shown exceptionally short telomeres in leukocytes compared to patients with other subtypes of DC [67]. The cause of the severe disease course and additional symptoms are mostly recessive or X-linked mutations characterized by the absence of a second 'healthy' allele. This is evident in specific genes such as *TERT*, *WRAP53*, *TPP1*, and *PARN*, where autosomal recessive (AR) mutations lead to the development of HHS, similar to mutations in the *DKC1* gene [67, 68].

Furthermore, an additional biallelic mutation in the *RTEL1* gene has been associated with the occurrence of HHS [69]. However, mutations in the *TINF2* and *TERC* genes have also been reported to be inherited in an autosomal dominant manner, where a single 'healthy' copy of the allele is insufficient to prevent the manifestation of symptoms.

Revesz syndrome

Revesz syndrome (RS) is an extremely rare subtype of DC and, similar to HHS, is characterized by extremely short telomeres, even compared to other subtypes of DC. So far, only a few cases of RS have been described. The variant is characterized by the classical triad of DC symptoms, along with severe bone marrow disorders manifested as aplastic anemia, noticeable in children up to the age of 2, and in milder courses, in patients up to the age of 6 [70, 71]. Additionally, neurological symptoms such as cerebellar hypoplasia and intracranial calcifications are observed. Moreover, a characteristic feature is bilateral exudative retinopathy, which can manifest from 6 months. The disease often exhibits a very severe course, leading to the death of the patient in childhood or adolescence, with patients rarely surviving past the age of 12: median survival is 6.5 years [71].

The occurrence of this syndrome is attributed to a mutation in the *TINF2* gene, inherited in an autosomal dominant manner. However, this mutation does not always lead to RS but can also result in HHS or a milder course, with clinical characteristics closer to classical DC [71, 72].

Coats plus syndrome or cerebroretinal microangiopathy with calcifications and cysts (CRMCC)

Coats plus syndrome (CP), similar to other severe subtypes of DC, is characterized by multisystemic disorders, with predominant neurological symptoms including leukodystrophy, intracranial calcifications, and brain cysts. These pathological changes in the brain lead to cognitive impairment, spasticity, dystonia, and ataxia. Additionally, CP presents with Coats syndrome-like symptoms such as telangiectasia and retinal exudates. There is also a high risk of gastrointestinal bleeding due to vascular abnormalities (ectasia) [73]. Some patients exhibit classical mucocutaneous manifestations, as well as hematological disorders such as thrombocytopenia and anemia [74, 75].

The midbrain changes have a similar characteristic to Labrune syndrome, but these two diseases differ in terms of genetic abnormalities [75]. The syndrome is associated with autosomal recessive mutations in the CST complex involving the genes *STN1* and *CTC1*, which form this complex, as well as the *POT1* gene, whose product directly interacts with CST [76, 77]. These mutations lead to telomere replication disorders.

Diagnosis

Due to the complex clinical picture of congenital dyskeratosis, establishing the correct diagnosis can be challenging, especially in cryptic variants. Therefore, in the diagnostic process, Fanconi anemia (FA) and other inherited BMF disorders such as Shwachman-Diamond syndrome (SDS) or SDS-like Diamond-Blackfan anemia should be ruled out — for example, by determining chromosomal fragility, which allows FA to be distinguished from DC. Additionally, in differential diagnosis, it is worth excluding other genetic syndromes associated with nail dysplasia, such as twenty-nail dystrophy, nail-patella syndrome, and poikiloderma with neutropenia [56].

If the following variants occur, the patient should be considered as having congenital dyskeratosis: 1) if classical mucocutaneous symptoms are present (at least one of them) along with accompanying bone marrow failure; 2) if all three 'classical' mucocutaneous symptoms are present; 3) patients with AA, MDS, PF in whom other etiologies have been excluded and mutations related to telomere biology (presented in Table I) have been confirmed; 4) patients presenting specific symptoms associated with severe progression related to subtypes: HHS, RS, and CP, such as cerebellar hypoplasia, underdevelopment, BMF, or retinopathy and referred for further diagnosis [78, 79].

The gold standard diagnostic methods for suspected DC encompass genetic tests employing next-generation sequencing (NGS) and flow-fluorescence in situ hybridization (flow-FISH) cytometric tests for telomere length determination. Flow-FISH tests are based on determining the total telomere length in the tested material. It is essential to consider age, tissue type, and cell characteristics when selecting appropriate controls for diagnostic purposes. A diagnosis pointing towards DC is deemed affirmative when the results fall below the 1% percentile of telomere length observed in healthy controls (e.g. in bone marrow this is below 8 kb up to the age of 5, whereas for a healthy individual of the same age, 100% corresponds to approximately 12 kb). An alarming result is considered within the range of 10% to 1% percentile (approximately

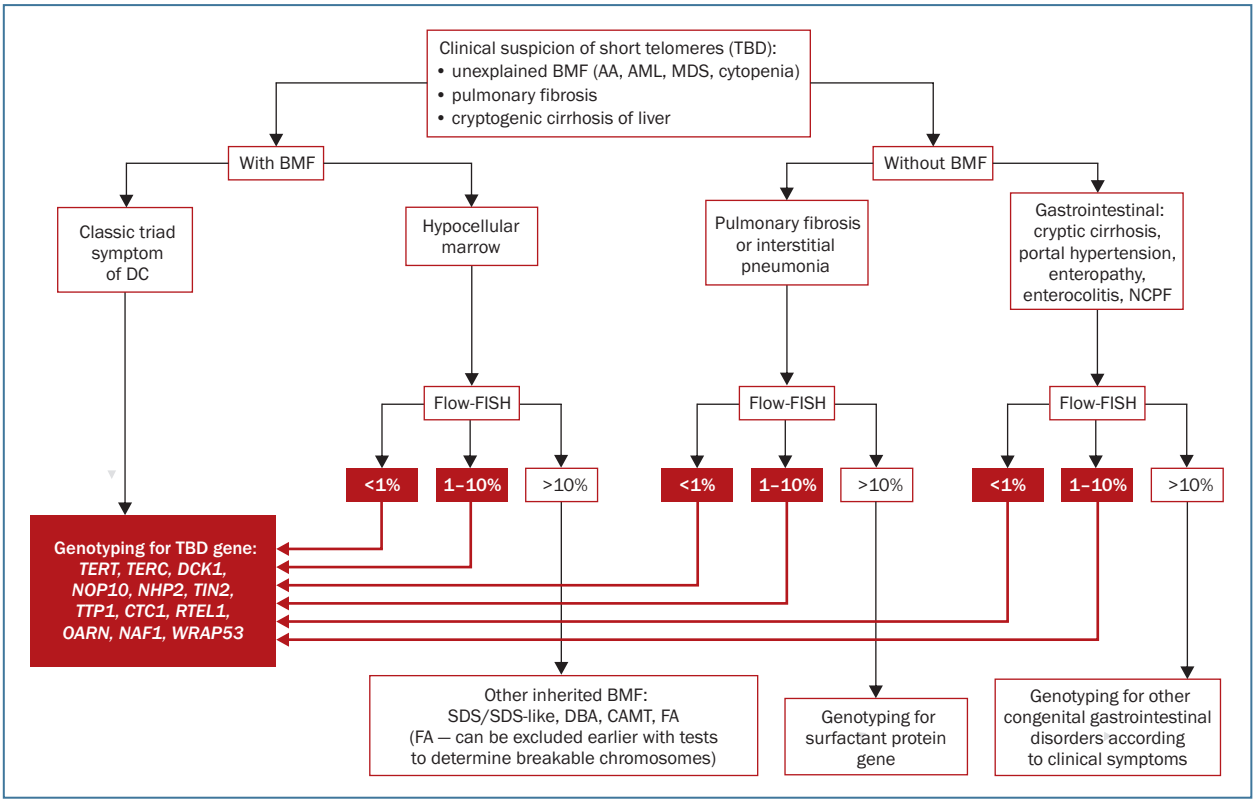

Figure 2. Dyskeratosis congenita diagnostic schema; AA — aplastic anemia; AML — acute myeloblastic leukemia; BMF — bone marrow failure; CAMT — congenital amegakaryocytic thrombocytopenia; DC — dyskeratosis congenita; DBA — Blackfan-Dimond anemia; FA — Fanconi anemia; flow-FISH — flow-fluorescence *in situ* hybridization; MDS — myelodysplastic syndrome; NCPF — non-cirrhotic portal fibrosis; SDS — Shwachman-Diamond syndrome; TBD — telomere biology disorder

9 kb to 8 kb) compared to a healthy control [80]. In both of the aforementioned cases, further diagnostic procedures, such as genetic testing, should be pursued to confirm the result. The first-line tool for qualitative genetic changes is targeted sequencing should encompass genes most frequently associated with telomere biology disorders (TBD). To identify the copy number variations (CNVs), a molecular cytogenetic method such as a comparative genomic hybridization (CGH) array or SNP array should be applied. The last one additionally identifies the single nucleotide variants [81]. If previous methods give negative results and the patient's symptoms indicate DC, it is necessary to extend the analysis using whole exome sequencing (WES). The latest, promising, method of detecting large-scale structural variants is optical genome mapping (OGM) which can be used for DC diagnostics [82]. A flowchart representation of the diagnostic schema is presented in Figure 2.

Conclusions

Today, we have increasing knowledge about telomere biology disorders (TBD). However, there are clinical cases that appear to be unrelated to TBD but still exhibit telomere dysfunction, as demonstrated by Janczar et al. [83]. Furthermore, the number of patients with cryptic variants of the disease is unknown. Currently, treatment is symptomatic and depends on the type and severity of symptoms.

A promising hypothesis for targeted therapy is androgen therapy. This is based on the hypothesis that androgens increase telomerase activity through the TERT promoter, which may be associated with estrogen response, although the detailed molecular mechanism is not yet fully understood. Preclinical *in vivo* studies in mice and *in vitro* studies on cell lines have shown associations between androgen treatment and telomere elongation. However, the results of clinical studies are inconclusive. Two out of four studies have demonstrated telomere lengthening after the administration of androgens (using danazol, oxymetholone, and nandrolone) using flow-FISH and qPCR tests. It is worth noting, however, that these were small studies with small representative groups. Little is known about the long-term effects of using specific androgens in TBD [84, 85].

Therefore, it will be important to further explore the topic of telomere disorders, especially cryptic variants, and investigate the possibilities of their occurrence in conjunction with other medical conditions. Improving and disseminating diagnostic protocols is also crucial.

Author's contributions

MM initiated the review article, reviewed the literature, drew the figures and prepared drafts of the manuscript. WM contributed to the conception and provided input throughout. JM evaluated the literature, edited drafts of the manuscript and finalized the manuscript for submission. All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

Conflict of interest

The authors declare no conflict of interest.

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Ethics

The work described in this article has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; EU Directive 2010/63/EU for animal experiments and uniform requirements for manuscripts submitted to biomedical journals.

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