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REVIEW

Telomere Length and Telomerase Activity as Biomarkers in the Diagnostics and Prognostics of Pathological Conditions

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Abstract—Telomere biology still remains a topic of interest in life sciences. Analysis of several thousand clinical samples from healthy individuals performed in recent years has shown that the telomere length (TL) in peripheral blood leukocytes correlates with the TL in cells of internal organ and reflects their condition. TL decreases under the influence of damaging factors and can serve as an indicator of health status. The telomere shortening leads to the cell proliferation arrest and is considered as a marker of replicative aging of proliferating cells. A decrease in the TL in peripheral blood leukocytes is viewed as an indicator of organism aging. Recent studies have allowed to formulate the concept on the role of the CST–polymerase α/primase in the C-strand fill in after completion of 3′G overhang synthesis by telomerase during telomere replication. The discovery of the telomeric RNA (TERRA) and its role in the regulation of telomerase activity (TA) and alternative lengthening of telomeres, as well as the possibility of TERRA translation, has provided evidence of the complex epigenetic regulation of the TL maintenance. Analysis of the published data indicates that telomeres are dynamic structures, whose length undergoes significant changes under the influence of damaging factors. TL is determined not only by the chronological age, but also by the exposure to the exogenous and endogenous deleterious factors during the lifetime. A decrease in the TL due to inherited mutations in the genes coding for proteins involved in the telomere structure formation and telomere replication (primarily, proteins of the shelterin and CST complexes and telomerase) has been found in a number of hereditary diseases – telomeropathies. The assessment of TL and TA is of great importance for the diagnostics of telomeropathies and can be useful in the diagnostics of cancer. Analysis of TL can be used for monitoring the health status (e.g., in the case of exposure to ionizing radiation and space flight factors), as well as predicting individual's sensitivity to the action of various damaging agents. The application of modern advancement in genetic technologies in the analysis of TL and TA makes it available for the use in clinical and epidemiological studies, diagnostics of telomeropathies, and monitoring of astronauts' health.

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INTRODUCTION

About 50 years ago, the Russian journal *Proceeding of the USSR Academy of Sciences* (*Doklady Akademii Nauk SSSR*) in 1971 and then the *Journal of Theoretical Biology* in 1973 published the visionary articles of the research scientist and theoretician Alexey Matveevich Olovnikov (1936-2022), the author of the terminal underreplication theory that explained the shortening of the chromosomal ends with each cell division [1, 2]. The ideas presented in these articles had been developed long before the discovery of telomere shortening and its molecular mechanisms. The studies of telomeres and mechanisms preserving their length with the involvement of special polymerase have been initiated due to the publication of the famous work by Leonard Hayflick, who stated that normal somatic cells (fibroblasts) cultured *in vitro* are unable to proliferate indefinitely and stop dividing after approximately 50 divisions [3].

To explain this phenomenon, Olovnikov suggested that during preparation for the cell division, the chromosomal DNA cannot not be fully replicated and becomes shorter at the ends after each doubling. DNA shortening to a certain critical length causes the malfunctioning of genes located close to the telomeres, eventually leading to the cell death. According to the theory of telomere aging, the shortening of chromosomal ends is a timer mechanism that determines the number of cell divisions and explains the Hayflick limit.

These ideas have been confirmed by subsequent discoveries. First, Greider and Blackburn found a new enzyme, telomere terminal transferase (later named telomerase), in the extracts of *Tetrahymena* cells [4]. The same authors demonstrated that telomerase contains RNA essential for the synthesis of telomeric repeats [5]. Inspired by the work of Olovnikov, Carol W. Greider conducted similar studies in human fibroblasts [6], resulting in the discovery of telomerase in human cells and cells of other eukaryotic organisms. It was demonstrated that the aging of cultured human fibroblasts was indeed accompanied by telomere shortening [7, 8]. The results obtained by Greider and Blackburn, who were awarded the Nobel Prize in Physiology or Medicine in 2004, have fully proven the Olovnikov's hypothesis on the mechanisms of somatic cell aging. Moreover, these studies have opened a new era in the research of the role and mechanisms of telomere length (TL) maintenance in normal cells and in various pathological processes, including those caused by deleterious factors, such as ionizing radiation (IR), and space flight factors. *Biochemistry (Moscow)* [9] and *Biogerontology* [10] have dedicated special issues to the memory of A. M. Olovnikov and modern developments in the concepts of telomere structure he had predicted.

The structure of chromosomal telomeric regions is characterized by a number of features that make them particularly sensitive to the effects of genotoxic factors. A decrease in the TL can lead to irreversible disturbances in cell functioning and can serve as a marker for the individual's increased sensitivity to the effects of endogenous and exogenous damaging factors. Current achievements in biochemistry and molecular genetics and introduction of methods for the analysis of large amounts of data have significantly expanded the possibilities of medical and biological studies of the state of telomeres.

The aim of this review was to analyze the results of studies on the mechanisms of telomere maintenance in human cells under the influence of damaging factors and in diseases caused by disturbances in the telomere biology. Such analysis can be helpful in selection of prognostic markers of the increased sensitivity of patients to radiotherapy and chemotherapy, which would allow to evaluate the risk of developing unwanted late complications. Here, we addressed current concepts on the telomere structure, mechanisms of TL maintenance, regulation of telomerase activity (TA), telomere sensitivity to irradiation, oxidative stress, and space flight factors, state of the TA-TL system, and technologies for the TA and TL assessments that can be used in clinical and epidemiological studies.

TELOMERE STRUCTURE AND MECHANISMS OF TELOMERE MAINTENANCE

Telomere structure. Telomeres are specialized DNA sequences at the ends of eukaryotic chromosomes. In humans, they consist of the repeating non-coding six-nucleotide sequence TTAGGG. The underreplication of chromosomal DNA due to the properties of DNA polymerase functioning results in the generation of free single-stranded GGG overhangs at the telomere 3′-ends in the course of DNA replication. During DNA replication in the S phase of the cell cycle, telomeres exist in the open linear form, while in other phases of the cell cycle, telomeres form loops that close the ends of the chromosomes (Fig. 1).

Electron microscopy studies of the *in situ* configuration of human and mouse telomeric DNA [11] showed the presence of large loops formed by folding back the telomere ends. The circular segment of the loops consisted of the telomeric DNA duplex called the T-loop. The invasion of the 3′ telomeric overhang into the duplex telomeric repeat array results in the displacement of the TTAGGG repeat strand at the loop-tail junction. Based on the binding with a single-stranded DNA, this displacement loop of TTAGGG repeats (D-loop, Fig. 1) was deduced to be

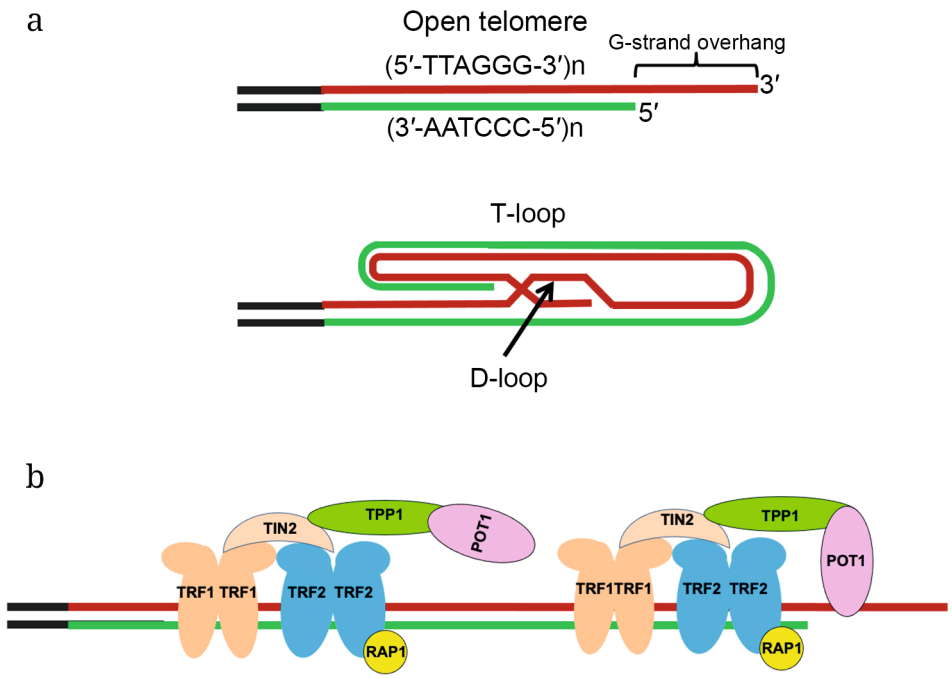

Fig. 1. Open telomere and telomere as the T-loop with D-loop at the site of the single-stranded telomere overhang insertion (a); the binding of shelterins to the double-stranded (TRF1 and TRF2) and single-stranded (POT1) telomeric DNA (b). Shelterin complex proteins cover the entire telomere. According to [11, 15].

approximately several hundred nucleotides long in many T-loops. Although the exact site of the 3′-end invasion has not been identified, in most cases the loop was very large (many kilobases) and sometimes encompassed the entire telomere. A close correlation was found between the length of the telomeric repeats and the T-loop size [11]. Figure 1 shows the structure of the T-loop and provides a general overview of how the loops can form to protect the telomeric ends, although the possibility of a different organization of the loops cannot be ruled out [12, 13].

Human telomeres contain a large number of G-quadruplexes (G4s). G4s are non-canonical four-stranded structures that can form in guanine-rich DNA and RNA sequences. The core structure of G4s consists of stacked guanine tetrads (G-tetrads), square planar platforms of four guanine bases held together by Hoogsteen hydrogen bonds. Formation of G4s requires cations, in particular, K^+ or Na^+, to stabilize the stacked guanine tetrads through coordination with the O6 atoms in guanines. G4s can promote the T-loop formation and provide intermolecular DNA–RNA interactions [14].

An important role in the formation and stabilization of T-loops and, thus, in the protection of telomeric ends, belongs to the proteins of the shelterin complex (Fig. 1b) [15-18]. These proteins bind to the telomeric DNA and determine the shape of the telomeric ends. They protect telomeres from the action of DNA repair enzymes and perform a number of other functions resulting in telomere protection [15, 17, 19]. The shelterin complex consists of six proteins listed in Table 1.

The POT1–TPP1 complex is a structural component of the telomere that increases the activity and processivity of telomerase core enzyme and provides the TL increase during DNA replication [21, 22].

Telomeres are also associated with a number of proteins (Mre11/Rad50/Nbs1, ERCC1/XPF, DNA-PK, PARP2) involved in recombination and DNA repair, including homologous recombination repair. These proteins interact with the shelterin complex proteins and participate in the formation and maintenance of T-loops [15]. Beside the formation of T-loops to prevent the recognition of chromosomal ends as double-strand DNA breaks (DSBs), other cellular mechanisms have been discovered that suppress the development of the DNA damage response (DDR) at the telomeres. These mechanisms involve proteins of the shelterin complex and have been described in detail in [12, 17, 23].

In his discussion of the connection between the telomere shortening, decreased cell survival, and aging, Yegorov [24] pointed out the strained conformation of DNA in the T-loop. Gradual shortening of telomeric DNA may come to a point when the TL becomes insufficient for the telomeric DNA to form a loop. The telomere acquires a free open conformation and is perceived by the cell as a DSB, which triggers the DDR and results in telomere damage [24]. This hypothesis explains the connection between the

Table 1. Structural proteins of the shelterin complex and their functions [15, 20]

Abbreviation	Full name	Protein function
TRF1	telomere repeat factor 1	TRF1 homodimer binds to the double-stranded TTAGGG telomere region and inhibits telomere elongation by telomerase
TRF2	telomere repeat factor 2	TRF2 homodimer binds to the same region as TRF1, but prevents recognition of double-strand DNA breaks (DSBs) as a type of DNA damage that requires repair
POT1	protection of telomere 1	exhibits affinity for single-stranded guanine-rich telomere regions, protects them from nucleases
RAP1	repressor/activator protein 1	stabilizes TRF2
TIN2	TRF1- and TRF2-interacting nuclear protein 2	stabilizes the complex of TPP1-POT1 with TRF1 and TRF2
TPP1 (TINT1, PTOP, PIP1)	telomere-protecting protein 1	binds POT1 to TIN2 and is required for POT1 functioning

decrease in the TL, arrest of cell proliferation, and cell aging and death.

During each cell cycle, telomeres shorten by 50-150 bp at the DNA replication stage [25]. Critical shortening of telomeres impairs their function, leading to the cell aging or apoptotic death. Chromosomes lacking telomeres may undergo further shortening, resulting in the loss of coding genes or fusion with other chromosomes, which causes genetic instability and increases the risk of cell malignant transformation [26].

A decrease in the TL or prolonged DDR lead to the cessation of proliferation and activation of cell senescence. Senescent cells actively secrete proinflammatory cytokines (IL-6, IL-8, TNFα, IL-1, and CCL), growth factors (HGF and IGFBP), metalloproteinases (enzymes that degrade the extracellular matrix), reactive oxygen species (ROS), and nitric oxide, i.e., acquire the so-called senescence-associated secretory phenotype (SASP). The cytokines secreted by senescent cells can induce senescence of neighboring cells and provoke body aging [27-29]. Therefore, telomere shortening can be considered as a marker of replicative aging of proliferating cells, while stable TL is an indicator of a healthy state.

The TL as a biomarker of aging or some pathologies is typically assessed in leukocytes or peripheral blood lymphocytes. Analysis of human tissues from the Genotype-Tissue Expression (GTEx) Project collection [30, 31] allowed to characterize the TL in more than 6300 samples from over 20 tissue types obtained from 952 individuals. It was shown that variations in the TL are determined by the tissue type, donor, donor age, and to a lesser extent by race or ethnicity, smoking, and hereditary variants. Generally, the TL in the whole blood cells correlated with the TL in most tissues. The TL was the shortest in the whole blood cells and the longest in the testis. In most tissues, the TL was inversely associated with age, and this association was the strongest in the tissues with a shorter average TL. The results of this study demonstrated that changes in the TL in the blood cells reflected the TL features characteristic of all other tissues of an examined individual [31].

Similar results were obtained for the comparison of the TL in human brain and peripheral tissues [32]. DNA was isolated from saliva, buccal epithelium, blood, and brain tissue from the patients undergoing neurosurgery for the intractable epilepsy, and average TL was assessed by qPCR. The highest correlation was detected between the TL values in the brain and buccal samples. This study was unique, because the authors were able to directly compare the TL in the brain and peripheral tissues from living subjects. The correlation between the TL values in simultaneously collected buccal samples and leukocytes was sufficient to suggest the use of buccal DNA as a reliable non-invasive source for determining the TL in humans [33].

Currently, the TL is commonly considered as an indicator of human health. The maximal TL is observed in children at the age of 18 months. Then, the TL rapidly decreases, gets stabilized by the age of 5, and remains at ~12 kb until the age of 25. After this, the TL steadily decreases and could reach 5 kb by the age of 80 [34, 35]. The TL depends not only on the age, but also on the individual's genetic features and the action of various damaging factors experienced by a person throughout the lifetime.

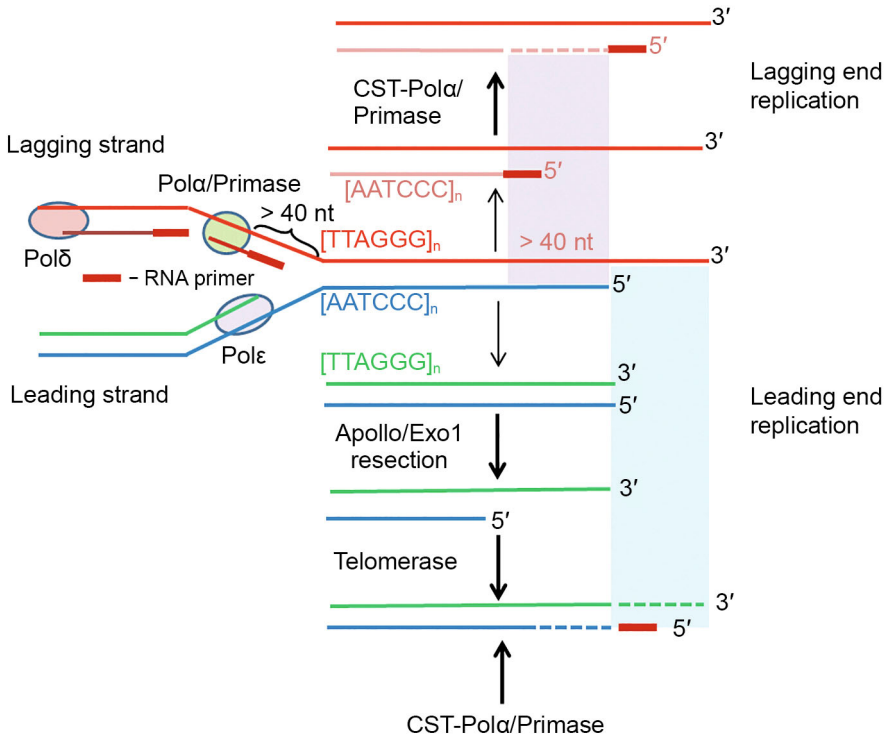

Fig. 2. The role of CST–Polα/Prim in the synthesis of telomere ends. Telomerase elongates the G-overhang of the leading DNA strand, while CST–Polα/Prim fills in the complementary C-strands of the lagging and leading DNA strands at the telomere end. When the replisome reaches the telomere end, the synthesis of the last Okazaki fragment begins >40 nucleotides (nt) from the site of the double-stranded DNA unwinding (according to [46]). Polδ and Polε are DNA polymerases δ and ε, respectively.

Telomere maintenance mechanisms. As mentioned above [4, 7], the TL in proliferating cells is maintained by telomerase and CST–Polymerase α/primase (CST–Polα/Prim) [36, 37]. In some normal and tumor cells, the TL is maintained by the homologous recombination of telomeric DNA. During this process, the 3′ overhang of a short telomere of one chromosome is inserted into the telomere of another "long" chromosome and uses it as a template for DNA synthesis with the participation of enzymes and factors required for homologous recombination. This mechanism is called alternative lengthening of telomeres (ALT) [26, 38, 39]. Under certain conditions (e.g., when DNA is damaged), the elongation of telomeres may switch from the telomerase-catalyzed process to the ALT [40]. In most proliferating cells, the main mechanism of the TL maintenance is the synthesis of telomeric DNA by telomerase and CST–Polα/Prim, but under stress conditions, even these cells can simultaneously use the ALT mechanism.

Telomerase and CST–polymerase α/primase. Telomerase is a ribonucleoprotein complex consisting of the catalytic subunit (telomerase reverse transcriptase, TERT) and the RNA component (TR, or TERC) that acts as a template for the synthesis of telomeric repeats at the ends of eukaryotic chromosomes [4, 7]. The template for the synthesis of telomeric repeats in the hTR is the 5′-CUAACCCU-3′ sequence. The template binds complementarily to the protruding 3′ end of the telomere and is used to synthesize new telomeric 5′-TTAGGG-3′ repeats in vertebrates [41-43]. Telomerase adds G-rich telomeric repeats $(TTAGGG)_n$ to the 3′ ends of telomeres, thereby counteracting telomere shortening caused by the loss of telomeric 3′ overhangs during the synthesis of the DNA leading strand. However, telomerase does not compensate for the loss of DNA sequence at the 5′ ends of chromosomal DNA, which occurs during the synthesis of the lagging DNA strand, as well as by partial degradation of the terminal fragments of telomeric DNA by nucleases.

Until recently, the mechanism of synthesis of the $(CCCTAA)_n$ ends of the telomere complementary C-strand had remained poorly understood [44]. In recent years, several excellent experimental papers and reviews on this problem have been published [36, 37, 45, 46].

The replication of the C-strand end region of telomeres *in vivo* requires the CST protein complex that acts as an auxiliary factor for the activity of CST–Polα/Prim received its name from the first letters of the names of proteins in its content: CTC1 (conserved telomere component 1), STN1 (suppressor of cdc thirteen), and TEN1 (telomeric pathways in association with STN1, number 1) [44, 47]. The elongation of telomeres

begins at the end of the S phase. First, the 3′ G-end is elongated by telomerase, and then the complementary C-strand is synthesized by CST–Polα/Prim (Fig. 2).

Regulation of telomerase activity. The catalytic core of human telomerase exists *in vivo* as a functionally cooperative dimer of two reverse transcriptase subunits (hTERT and hTR) or as a multimer [48]. In addition to the proteins of the shelterin and CST complexes, hTERT can bind p23/p90 (a chaperone responsible for the complex assembly and conformation), nuclear localization protein 14-3-3, and TP1 protein with an unknown function [49]. hTR can bind hGAR1, Dyskerin/NAP57, hNHP2, and C1/C2 (proteins responsible for the RNA stability, maturation, and localization), proteins providing binding to the telomeres, and some other proteins [50].

The gene coding for hTERT consists of 16 exons. Its length is 37 kb, of which ~33 kb are introns and the rest ~4 kb encodes the transcript [51]. The TA can be regulated through different mechanisms, including gene expression, phosphorylation/dephosphorylation, alternative splicing, etc. [52, 53]. Humans express more than 20 telomerase isoforms. The N-terminal fragment of telomerase contains the nuclear localization signal, RNA interaction domains, and a sequence that directs it to the mitochondria. The C-terminal part contains regions that determine the reverse transcriptase activity of hTERT. In most telomerase isoforms, these critical elements of protein structure are disrupted to various degrees. Only the full-length enzyme, whose mRNA contains all 16 exons, displays a high TA. All known hTERT splice variants in human and mouse cells are inactive and, moreover, inhibit the TA. This suggests that the hTERT isoforms formed by alternative splicing, play an important role in the TA regulation and implementation of telomerase non-canonical functions unrelated to its catalytic activity [54].

The recruitment of telomerase to telomeres is mediated by the shelterin complex proteins POT1–TPP1 [42], whereas timely termination of telomere elongation occurs with involvement of the CST heterotrimeric complex composed of the CTC1, STN1, and TEN proteins [55, 56]. The CST complex competes with the telomerase processivity factors POT1–TPP1 for binding to the single-stranded region of telomeric DNA and inhibits the TA by preventing enzyme binding to the single-stranded region of DNA and its physical interaction with POT1–TPP1. The binding of the CST complex to the telomere increases during the late S/G2 stages only when the telomerase is active and results in the inhibition of its activity. Removal of the CST complex leads to the excessive TA and promotes excessive telomere elongation. By binding to the elongated telomere, the CST complex interrupts the action of telomerase. Therefore, the elongation of the telomere 3′ G-end is determined by the sequence of three events (Fig. 2) which first turn on and then stop the telomerase-mediated telomere elongation: (i) telomerase recruitment to telomeres in the region of the single-stranded 3′ G-end with participation of the POT1–TPP1 proteins; (ii) elongation of the telomere 3′ G-end by telomerase; (iii) displacement of the POT1–TPP1 proteins from the complex with the single-stranded 3′ G-end by the CST complex and blockade of telomerase binding to the 3′ G-overhang, resulting in the cessation of telomere elongation [37, 45, 47, 49]. The synthesis of the complementary C-strand by CST–Polα/Prim begins after completion of the G-strand elongation [46, 57].

Hence, the CST complex plays a key role in the regulation of two steps of telomere replication: termination of the G-strand elongation by telomerase and recruitment of CST–Polα/Prim to the newly synthesized G-overhang and its activation for the synthesis of the complementary C-strand.

A long non-coding RNA with the telomeric repeats at the 3′ end, called TERRA (telomeric repeat-containing RNA), may play an important role in the TA regulation at the epigenetic level [58] (see reviews [59, 60] for the TERRA formation and properties). It was shown that telomeres can be actively transcribed by RNA polymerase II from promoters located in the subtelomeric regions in the direction from the centromere toward the end of telomeres with the formation of the TERRA. The length of this RNA can vary from 100 nt to 9 kb [58]. The 5′-UUAGGG-3′ repeats in TERRA interact with the hTR sequence. In addition, TERRA binds to the TERT protein independently of its interaction with hTR. TERRA is not used as a telomerase substrate. Instead, it acts as a potent competitive inhibitor of telomerase interaction with the telomeric DNA [61]. TERRA colocalizes with the telomerase hTR in the nucleoplasm and at telomeres at different phases of cell cycle.

Most TERRA molecules are diffusely located in the nucleoplasm. One of the functions of this soluble pool is telomerase binding and regulation of its activity. TERRA controls the TL homeostasis by regulating the TA at the telomeres. High TERRA expression was found to correlate with the content of short telomeres in various cell lines, which supports the model of TERRA as a negative regulator of TA [62].

TERRA may be regarded as a structural component of the telomere binding to the telomere through the shelterin complex proteins TRF1 and TRF2, heterochromatin protein 1, histone H3trimethylK9, and some other proteins [63]. Telomeric DNA exists in the cell as a component of heterochromatin. TERRA is currently considered as a key regulator of telomere maintenance and heterochromatin formation at telomeres [59, 63].

Xu and Komiyama [64] summarized the data on the structure and conformation of G4s in DNA, RNA, and DNA–RNA hybrids in human telomeres and showed that TERRA plays a crucial role in the telomere capping through the formation of telomeric G4s [64]. Notably, TERRA exhibits a preference for the formation of G4s in the DNA–RNA hybrids at the 3′ end of telomeric DNA. Intramolecular G4s with different folding structures and loop conformations and experimentally determined molecular structures have been shown for the parallel, hybrid, and basket G4s in [14, 64] and for intermolecular hybrid DNA–RNA G4s in [64]. It should be noted that G4s are mobile structures capable of unfolding, in particular, with the participation of POT1 protein [65]. Some small G4 ligands stabilize G4s, resulting in telomerase inhibition [66]. Telomere damage triggers TERRA transcription, so that the upregulation of TERRA can be considered as a molecular marker of telomere destabilization [67].

TERRA can form RNA–DNA hybrids with the cytosine-rich telomeric DNA strands. The telomeric strand containing the TTAGGG repeats is displaced, resulting in the formation of the R-loop structure [68] crucial for the telomere maintenance [69]. Excessive TERRA R-loops can cause replicative stress, chromosome damage, and loss of telomeres [69, 70]. TERRA and TERRA R-loops are highly abundant in human cancer cells, in which telomere elongation occurs via the ALT mechanism [71].

It has been recently shown that translation of TERRA yields two proteins consisting of repeating dipeptides: a highly charged protein formed by valine-arginine (VR) repeats and a hydrophobic protein formed by the glycine–leucine (GL) repeats [72]. The VR protein binds nucleic acids and localizes at the replication forks. Both VR and GL proteins form long 8-nm filaments with the amyloid properties. The authors showed that cells with the elevated levels of TERRA in the nuclei contained three to four times more VR protein than the original cell line. Deletion of TRF2 caused the dysfunction of telomere and increased the content of VR protein, while manipulation of TERRA levels using specific approaches resulted in the appearance of large VR aggregates in the nucleus. These data suggest that telomeres, particularly in cells with dysfunctional telomeres, can express two unusual dipeptide-repeat proteins with potentially important biological properties [72]. These new findings suggest that despite extensive research into the role of TERRA in the regulation of telomere function, many unanswered questions still remain.

Alternative functions of TERT and TR. The extensive studies of telomerase have provided new data on the functions of holoenzyme components that are not associated with the telomere elongation *per se*, but play an important role in ensuring cell survival under the influence of damaging factors. Both TERT and TR may be involved in the regulation of various intracellular processes, including gene expression and stress response.

Alternative functions of TERT have been discussed in [43, 73-76]. An important property of TERT is its ability to protect cells from the damage caused by oxidative stress. Alternative functions of TERT include not only the antiapoptotic and antioxidant effects, but also the protection against specific DNA-damaging agents. TERT can translocate between the nucleus, cytoplasm, and mitochondria due to the nuclear export signal identified at the protein N-terminus. In the case of oxidative stress, TERT is phosphorylated at the tyrosine residue 707 by the Src kinase, which triggers its nuclear export. In addition, TERT contains a specific N-terminal 20-amino acid sequence that serves as a mitochondrial localization signal. The transport of TERT into the mitochondrial matrix involves translocases of the outer and inner mitochondrial membranes.

The role of TERT in the mitochondria was discussed in [75]. hTERT-overexpressing non-proliferating human fibroblasts were characterized by the reduced ROS generation and upregulated expression of antioxidant defense proteins. In these cells, the oxidative stress induced by X-rays or H_2O_2 caused less damage to the mitochondrial membrane potential and mitochondrial morphology than in normal fibroblasts. Both genotoxic factors (X-rays and H_2O_2) significantly increased the number of γH2AX foci (phosphorylated form of H2AX histone, DNA damage marker). The DNA damage induced by X-ray irradiation in the control cells persisted for many days, while in the hTERT-overexpressing fibroblasts, this damage was considerably less and DNA was fully repaired within the following days.

It should be noted that the early studies on the telomerase content in different tissues have shown the presence of hTERT in proliferating cells, while hTR has been found in almost all tissues [41], suggesting that hTR may have the functions unrelated to the TA regulation. It was later discovered that the hTR knockdown reproducibly induced apoptosis in the absence of any detectable telomere shortening or DDR activation. In contrast, the knockdown of *hTERT* did not induce apoptosis [76]. The authors suggested that hTR can function as a non-coding RNA that protects cells from apoptosis independently of its involvement in telomere lengthening by telomerase in normal human cells [76].

Possible non-telomeric functions of hTR have been discussed in reviews [77, 78]. Numerous studies have provided evidence on the implication of hTR in protective mechanisms. Thus, hTR contains an open

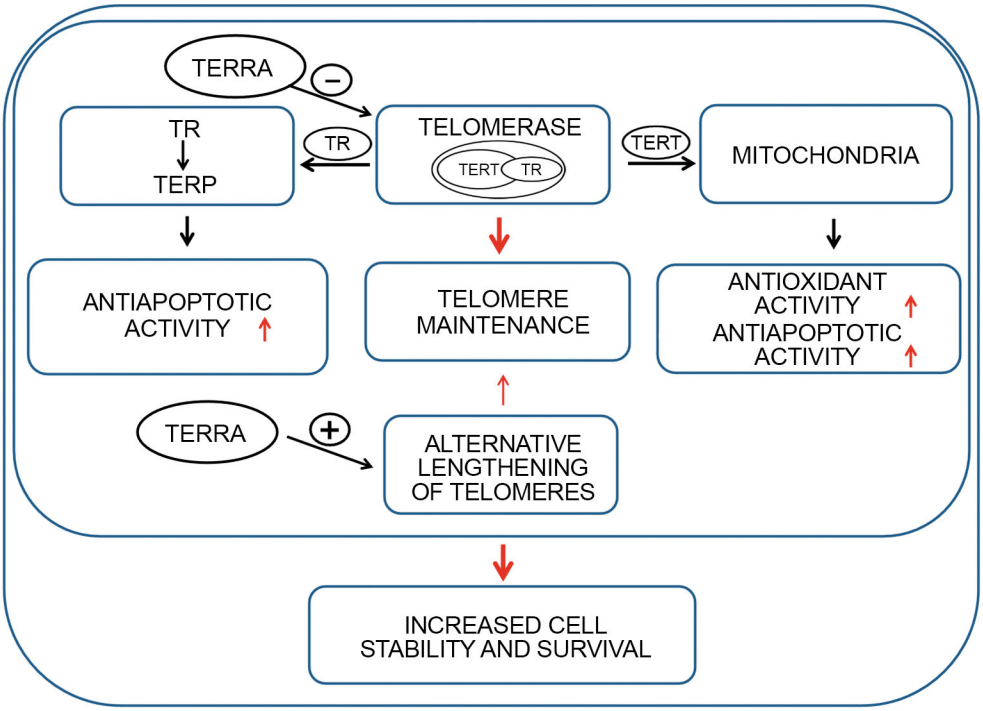

Fig. 3. Telomerase functions in the cell. The TL is maintained through the synthesis of telomeric DNA by telomerase or via the ALT pathway, which is necessary for the T-loop formation and chromosome preservation. Telomerase components TERT and TR possess the protective functions. TERT can migrate to the mitochondria and increase the level of antioxidant defense and anti-apoptotic activity in these organelles. TR can serve as a template for the synthesis of TERP protein, which exhibits the anti-apoptotic activity. TERRA reduces the TA and stimulates the ALT pathway to maintain the TL.

reading frame that starts at position 176 and codes for a 121-amino acid protein named hTERP. hTERP is encoded as an immature transcript. The existence of hTERP has been confirmed by several experimental approaches including mass spectrometry and immunodetection [79]. The overexpression of the wild-type hTR (but not its mutant version incapable of directing hTERP synthesis due to mutation in the start codon) protected HEK293T cells from the doxorubicin-induced apoptosis. hTERP may be involved in cell protection from stress and promotion of cell survival under the action of damaging factors and during adaptation to unfavorable conditions. This alternative activity of telomerase components may play an important role in ensuring cell survival under the influence of deleterious factors.

The known functions of telomerase ensuring cell stability are shown in Fig. 3.

Therefore, the maintenance of TL in proliferating cells by telomerase and CST–Polα/Prim or via the ALT mechanism, the antiapoptotic activity of telomerase components TERT and TR, and the antioxidant activity of TERT in the mitochondria provide cell resistance to the action of damaging factors.

Telomerase and telomere maintenance in tumors. Telomerase expression is suppressed in resting cells, but is reactivated upon their malignant transformation. High expression of telomerase gene and high TA have been detected in various types of malignant tumors [26, 52, 80]. Therefore, the presence of TA in a tissue extract can be used as a marker in the diagnostics of malignant tumors. Unlimited cell proliferation facilitated by telomerase reactivation is a hallmark of cancer, as it enables long-term proliferation of cells with short telomeres.

In 5-15% tumors, the TL is maintained by the ALT mechanism [26, 38, 81]. ALT is frequently found in tumors of mesenchymal origin: in 60% of soft tissue sarcomas, 100% of chondrosarcomas, 63% of undifferentiated pleomorphic sarcomas, 53% of undifferentiated leiomyosarcomas, and 14% of fibrosarcomas. In CNS tumors, ALT was found in 63% of diffuse and anaplastic astrocytomas and in 44% of glioblastoma multiforme in children (but only in 11% tumors in adults).

No association between the TL in peripheral blood leukocytes and risk of cancer development has been established for solid cancers [82]. At the same time, there is a correlation between the reduced TL in leukocytes and various hematological malignancies [83]. Therefore, the assessment of TL and TA for the diagnosis and prognostics of cancer should be performed only in tumor biopsies or surgically removed tumor tissues.

The high level of TA in malignant tumors may be due to mutations in the *TERT* gene promoter or genes coding for the shelterin and CST complex proteins; it can also be determined by specific expression patterns of numerous transcription factors that regulate the TA [84].

The use of TA analysis in the early diagnostics of bladder cancer seems particularly promising due to the possibility of non-invasive TA measurement in the urine. Therefore, the TA assay might become a gold standard for the non-invasive diagnostics of bladder cancer [85, 86].

A pilot study demonstrated a high value of the urine and tissue TA levels (measured by RT-TRAP-2PCR) in detection and monitoring of bladder cancer. An association was found between the tumor size (≥3 cm) and elevated TA levels in tissues and urine. However, no such correlation was observed between the higher TA levels and tumor grade/size at the advanced cancer stages [87, 88]. It should be noted that TA assays are available, but they have not yet been introduced into clinical practice because of the high cost and problems with the standardization of procedures for the sample collection and preparation. At the same time, preparation of samples for the currently used histological examination is less complicated and can be automated. A more promising approach for the diagnostics of malignant tumors and monitoring of the therapy efficacy is a rapidly developing analysis of specific marker mutations in liquid biopsies. For example, the C228T and C250T mutations in the *TERT* gene promoter play an important role in the malignant transformation of cells and can be used as biomarkers in many types of cancer, including glioblastoma multiforme, as they are detected in 80% of glioblastoma multiforme cases [89, 90].

To summarize the above, we should emphasize, in most proliferating cells the TL is maintained by telomerase and CST–Polα/Prim with the participation of DNA polymerases δ and ε. The TA is regulated at the transcriptional level, by post-transcriptional modifications, and epigenetically. Proteins of the shelterin and CST complexes, as well as numerous other proteins and regulatory factors involved in the TL maintenance, have been intensively explored. At the same time, the telomerase components hTERT and hTR perform a number of functions aimed at increasing cell resistance to the stress factors. In some cells and tumors, the TL is maintained through the ALT mechanism. Reactivation of telomerase during malignant cell transformation ensures the long-term proliferation of tumor cells with short telomeres. The presence of TA and mutations in the *hTERT* gene promoter (C228T and C250T) may be the hallmarks of malignant tumors, with the exception of sarcomas and some brain tumors.

TELOMERE SENSITIVITY TO IONIZING RADIATION

DNA damage and telomere DNA repair. Among the types of molecular damage caused by IR, formation of DSBs in DNA has the most severe consequences for the cells, because persistence of unrepaired DSBs leads to cell death. DSB repair occurs through the two main mechanisms: homologous recombination, which occurs only during the S and G2 phases of cell cycle, and classical non-homologous end joining (c-NHEJ) that takes place during all phases of cell cycle [91-93]. In mammalian cells, DSBs are repaired by both c-NHEJ and homologous recombination mechanisms. The cells can also use the alternative end-joining mechanism, which is associated with the loss of nucleotides, requires the presence of homologous sequences in the same chromosome, and leads to the appearance of errors in the DNA structure. Alternative end-joining is a less studied mechanism; however, it is known to involve PARP1, DNA polymerase θ, Lig1 ligase, and Lig3–XRCC1 complex [91-95]. It is employed when c-NHEJ or homologous recombination cannot function properly.

In addition to the above-mentioned mechanisms, DSBs can be repaired via the RAD51-independent homology-dependent single-strand sequence annealing, i.e., joining of the ends of two homologous 3′ single-stranded DNA fragments in the region of tandem sequences, which leads to the obligatory removal of DNA fragment between the repeats. This mechanism functions only during the S and G2 phases of cell cycle and is associated with the appearance of errors in DNA and increased frequency of mutations [93].

The development of DDR in the telomere region can lead to the disturbances in the telomere structure; however, reactions that are dangerous for the cell can be suppressed by the shelterin complex proteins (Table 1). Thus, TRF2 binds the ATM kinase and inhibits the ATM-dependent DDR [96]. It was found that TRF2 interacts with DDR factors not only at the telomeres, but also in other chromosomal regions. Phosphorylation of TRF2 at serine 20 reduced its binding affinity to the telomeres and promoted TRF2 relocation to non-telomeric DSBs, thus facilitating their repair [97].

Another protein of the shelterin complex, POT1, prevents activation of the ATR kinase involved in the single-stranded DNA repair, thus arresting DDR in the telomeres [98]. TRF2 and POT1 act independently to suppress the two DDR pathways. It was found that X-ray irradiation of fibroblasts was followed by a long-term presence of DDR markers at the telomeres, while in other chromosomal regions, such damage was quickly eliminated by DNA repair. No telomere shortening was observed [28, 99]. The prolonged presence of unrepaired DSBs in the telomeric DNA leads to the DDR persistence, arrest of proliferation,

and activation of cell senescence [27, 29]. There is evidence that TERRA is also involved in the regulation of DSB repair in DNA [100, 101].

The features of the telomere response to DNA damage have been discussed in detail in comprehensive reviews [12, 13, 15, 17]. As telomeres constitute only a small fraction of the human genome, the probability that photons or accelerated particles will directly cross the telomeric sequence is very low. Therefore, the IR-induced telomere damage likely occurs due to disruptions in the telomere maintenance mechanism.

Telomere damage as a result of ionizing radiation-induced oxidative stress. IR causes both direct damage to the cell macromolecules and indirect damage through the water radiolysis products [28, 102]. Irradiation of water results in the formation of ROS, reactive nitrogen species and other free radicals capable of damaging DNA and other macromolecules. The effects of irradiation can be manifested within several minutes to several hours after exposure. The most reactive species are superoxide anions, hydroxyl radicals, nitric oxide radicals, lipid radicals, and some other molecules with a high oxidizing potential. ROS production can also be a result of activation of some cellular metabolic processes, as well as inflammation, ischemia, and stress [103].

One of the most prevalent forms of oxidative DNA damage is 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-OxodG), a product of guanine oxidation. Its accumulation in DNA is considered a hallmark of chronic oxidative stress [104]. Mitochondrial DNA is more susceptible to the oxidative damage than nuclear DNA [105, 106]. Thus, an exposure to IR induced a multifold increase in the 8-OxodG content in mitochondrial DNA [107].

Densely IR is a more potent inducer of the long-lasting oxidative stress than sparsely IR (gamma- and X-rays) [104, 108, 109]. The oxidative damage to telomeric DNA is thought to cause premature senescence by accelerating telomere shortening. Barnes et al. [110] tested this model directly using a precision chemoptogenetic tool that generated 8-OxodG exclusively in the telomeres. It has been shown that a one-time induction of the 8-OxodG formation in the telomeres was sufficient to trigger the p53-dependent senescence. Formation of 8-OxodG activated the ATM and ATR signaling and resulted in telomere dysfunction in replicating cells [110]. Chronic selective induction of telomeric 8-OxodG shortened the telomeres and impaired cell proliferation over time. Accumulation of telomeric 8-OxodG in chronically exposed 7,8-dihydro-8-oxoguanine-DNA glycosylase-deficient cells triggered the replication stress, significantly increased the loss of telomeres, and generated chromosome fusions, leading to the formation of chromatin bridges and micronuclei during cell division [111]. Targeted oxidative stress directed toward either telomeres or mitochondria increased the ROS production, extent of telomere damage, mitochondrial dysfunction, and cell apoptosis [112]. Figure 4 illustrates the relationship between the development of disorders caused by the elevated levels of ROS and other free radicals and decrease in the TA and TL.

The damage and shortening of telomeres as a result of oxidative stress development occur throughout the lifetime of an organism against the background of normal metabolism and influence of various damaging factors and inflammation, which explains the decrease in the TL in human peripheral blood leukocytes with age [113-115]. The damaging factors causing telomere shortening include chemotherapeutic drugs and IR [116].

The effect of irradiation on the telomere–telomerase system. The IR-induced changes in the TL depend on the type of radiation, its duration, dose received, and time after exposure.

Analysis of TL and chromosomal instability in the prostate cancer patients subjected to the intensity-modulated radiotherapy (IMRT) showed individual response to the therapy: 11 patients with a relatively short telomeres at baseline showed a dramatic increase in the TL immediately post IMRT that was sustained for at least 3 months. In patients with initially longer telomeres, the TL dramatically decreased post IMRT [117].

The telomere–telomerase system was found to be highly sensitive to IR. Proteins of the shelterin complex play an important role in protecting telomeric DNA from the IR-induced damage. Thus, it was shown that the siRNA-mediated inactivation of TRF1, TRF2, or POT1 led to the impairment of telomere protective functions and the increase in the frequency of spontaneous and radiation-induced mutations in the heterozygous thymidine kinase locus in human lymphoblastoid WTK1 cells after exposure to γ-radiation and accelerated ^{56}Fe ions with an energy of 1 GeV/n at the doses of 1 and 2 Gy. The highest increase in the mutation rate was observed upon inactivation of POT1 [118].

The effect of IR on the TA varies depending on the cell type, dose, type of radiation, and TL [118]. For example, after exposure of human breast adenocarcinoma MCF-7 cells to IR, the TA increased within the first 48 h, but then decreased. These changes were accompanied by changes in the TL. Irradiation of cultured cells at a dose of 10 Gy caused a decrease in the average TL 5 days after the exposure, followed by the partial TL recovery after 10 days [118], indicating disappearance of short telomeres that had appeared after irradiation. However, it remained unclear whether this phenomenon was caused by the actual recovery of telomeres by telomerase or via the ALT mechanism or by the death of cells with short telomeres.

Along with the decrease in the TL, the post-irradiation disturbances in the TA-TL system included an increase in the proportion of senescent (β-galactosidase-positive) cells to 45% (MCF-10A cells) and 70% (MCF-7 cells) on day 10 after irradiation [118]. The results of this detailed study suggest a high sensitivity of the TA-TL system to the effects of IR in both normal and tumor cells.

An increase in the TA after irradiation has been shown in various tumor cells, such as glioma [119] and nasopharyngeal carcinoma cells [120], colon carcinoma and colorectal carcinoma cell lines [121], Ewing sarcoma SK-N-MC cells, breast cancer MCF-7 cells, chronic myelogenous leukemia K562 cells [122], etc. The upregulation of the TA after irradiation can be considered as a protective response leading to the increased radioresistance of tumor cells. In this regard, there is currently an active search for the efficient and selective TA inhibitors to increase the sensitivity of tumors to the radiation therapy.

Radiation damage to regional stem cells may be an important mechanism in radiation-induced carcinogenesis and some delayed post-irradiation effects. Stem cells are found in almost all tissues of an adult organism. They play an important role in the maintenance of cellular homeostasis and post-damage tissue regeneration. Since stem cells are slowly renewed throughout the lifetime, they can accumulate genetic damage, which can cause their malignant transformation, formation of cancer stem cells, and cancer development, in particular, emergence of secondary tumors after radiation therapy. Therefore, many studies of the mechanisms of radiation carcinogenesis are currently focused on the effects of different types of radiation on stem cells. In addition, a decrease in the stem cells pool due to the whole-body or regional exposure to radiation at the doses that cause the death of some stem cells can lead to the accelerated aging of organs and tissues and slow down regeneration processes.

γ-Irradiation at a dose of 0.1 Gy increased the TA in cultured mouse bone marrow mesenchymal stromal cells (MSCs) 2 months after irradiation, while γ-irradiation at a dose of 6 Gy and exposure to neutrons at the doses of 0.05, 0.5, and 2 Gy decreased the TA. At the same time, only γ-irradiation, but not exposure to neutrons decreased the TL in the irradiated vs. control MSCs [123]. The absence of telomere shortening in MSCs with a low TA suggests that either the existing TA was sufficient to maintain the TL or that the cells utilized both the TA and ALT to restore the TL.

In subsequent experiments, irradiated MSCs were cultured for 2 months and then transplanted to mice to induce tumors (sarcomas). Next, fibrosarcoma cell lines were prepared from these formed tumors. In cells of the fibrosarcoma lines, the TA was either absent (after γ-irradiation at a dose of 1 Gy and after neutron irradiation at a dose of 0.5 Gy) or remained at a very low level. Hence, malignant transformation of MSCs was accompanied by the decrease in the TA (up to its complete disappearance), which distinguishes sarcomas from other types of tumors, which are characterized, on the contrary, by telomerase reactivation [123].

TL was investigated as a marker of replicative aging of bone marrow and thymus cells after prolonged irradiation of mice with neutrons at the doses of 0.05-0.5 Gy [124]. It was found that the TL in the bone marrow cells and the thymus in control animals aged 4 and 16 months was similar and did not change during mice aging. In the irradiated animals, the TL in the bone marrow and thymus did not differ from the TL in the corresponding organs of the control mice 2 months after exposure; however, 14 months after the irradiation, the decrease in the TL was proportional to the radiation dose in both bone marrow and thymus cells [124]. Analysis of internal organs in the euthanized animals found that 2 out of 10 C57Bl/6 mice had uterine tumors (squamous cell keratinizing carcinomas) and 1 out of 10 CBA mice had uterine carcinosarcoma. One of the CBA mice irradiated at a dose of 50 mGy had invasive bronchoalveolar adenocarcinoma in the left lung. These data attest that neutron irradiation caused a delayed decrease in the TL in the bone marrow and thymus and increased the probability of tumor development. The appearance of cytogenetic abnormalities in bone marrow cells was observed at the irradiation dose of 10 mGy and above [125].

Telomeres are more sensitive to IR than chromosomal DNA. Analysis of the association between the TL and sensitivity of TK6 lymphoblasts to IR revealed no correlation between the radiosensitivity and mean TL; however, there was a positive correlation between the radiosensitivity and loss of telomeres, suggesting it is the irradiation-induced telomere loss, rather than changes in the TL itself, that plays an important role in the formation of radiosensitivity or radioresistance [126].

Experiments in telomerase-deficient mice generated by the deletion of the telomeric RNA gene (*Terc*), have shown the role of telomerase and telomeres in the response of cells and entire organism to IR. It was found that the telomere dysfunction in the late generations of $Terc^{-/-}$ mice has led to the development of the increased radiosensitivity syndrome associated with accelerated mortality, intensification of apoptosis in intestinal cells and thymocytes, and elevated radiosensitivity of embryonic fibroblasts. The radiosensitivity of these cells correlated with a decrease in the DDR rate, persistent chromosomal breaks, and cytogenetic profiles characterized by complex chromosomal aberrations [127].

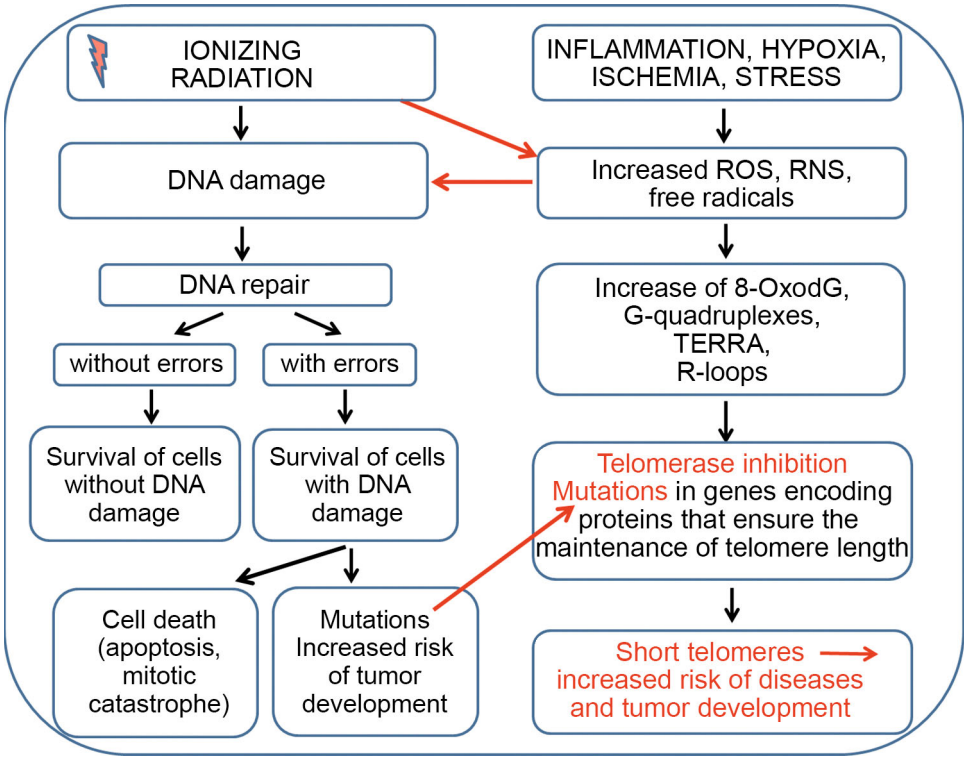

Fig. 4. Mechanisms of TA suppression and TL shortening in cells subjected to IR and oxidative stress. 8-OxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; R-loops, RNA–DNA hybrids formed by TERRA with the cytosine-rich sequences of telomere DNA that can cause displacement of DNA strand containing TTAGGG repeats.

DNA damage triggers the DDR response, which causes cell cycle arrest until the damage is repaired. The presence of unrepaired DNA damage induces persistent DDR activation, leading to the cell apoptosis and/or senescence. It is important to note that the repair of DNA damage in the telomere region is limited [28, 128], which determines a higher sensitivity of telomeres to IR compared to the rest of the genome. The mechanisms underlying this phenomenon require further study. Figure 4 shows the sequence of events leading to the TA decrease and TL shortening as a result of direct damage by IR and the action of ROS, reactive nitrogen species, and free radicals, as well as by the oxidative stress caused by ROS and reactive nitrogen species generated in pathological processes not associated with radiation.

Delayed effects of the radiation exposure on the telomere length. The biological effects of the long-term exposure to a low-dose IR are attributed to the direct DNA damage and indirect effect mediated by the oxidative changes in DNA. As specific nucleoprotein structures with a high guanine content, telomeres are more sensitive to oxidative damage and can be damaged and shortened more easily under the combined effect of radiation and oxidative stress caused by it.

In the study of Hiroshima atomic bomb survivors, Lustig et al. [129] showed that the TL in leukocytes 50 and 68 years after the exposure inversely correlated with the radiation dose. The results indicate a long-term negative effect of IR on the TL in leukocytes, which depended on both the irradiation dose and the age of subjects at the time of exposure [129]. A nonlinear relationship between the TL in T lymphocytes and radiation dose was also demonstrated in a study of 620 individuals who had survived the atomic explosion, which discovered the presence of longer telomeres in individuals who had received a lower radiation dose and a trend to the TL decrease in subjects who had received a dose higher than 0.5 Gy [130].

Scherthan et al. [131] examined 100 workers of the plutonium production plant at the Mayak Production Association, who had been chronically exposed to plutonium-239 α-radiation and/or γ-radiation, for the relative TL in leukocytes in comparison to the control group (51 local residents). The mean age of all participants was about 80 years. The authors found that the TL decreased with age. The workers irradiated at the lowest dose demonstrated a significant decrease in the TL (by ~20%) after being exposed to external γ-radiation (≤1 Gy) or internal α-radiation (≤0.05-0.1 Gy to red bone marrow). The relative TL in the workers exposed to a high-dose irradiation (>0.1 Gy for α-radiation and >1-1.5 Gy for γ-radiation) did not differ from that in the control group.

Stratification by sex revealed a significant (~30%) decrease in the TL in the males exposed to the low doses, but not in the females. The authors concluded that chronic systemic exposure to different radiation doses leads to different changes in the TL, which may be due to the selection against cells with a low TL [131].

The TL and telomere damage were also studied in the individuals working at the diagnostic radiology departments and occupationally exposing to low doses of X-ray radiation. These workers demonstrated increased levels of chromosomal aberrations and damaged telomeres in peripheral blood lymphocytes, as well as increased levels of lipid peroxidation products and 8-OxodG in the blood plasma. The latter two parameters correlated with the extent of telomere damage, suggesting that the chronic exposure to a low-dose radiation causes oxidative modification of guanine, leading to telomere damage [132].

These data confirm a delayed decrease in the TL after exposure to a low-dose (including chronic) irradiation, as well as significant contribution of oxidative stress to the telomere damage in individuals subjected to the chronic low-dose irradiation.

Telomere length and radiosensitivity. The TL is closely related to the cell radiosensitivity. For instance, it was demonstrated that the radiosensitivity of human larynx squamous cell carcinoma Hep-2 cells negatively correlated with the TL and positively correlated with the TA, which allowed the authors to conclude that both TL and TA can serve as markers for predicting the radiosensitivity of patients' cells [133]. These data have been confirmed in different types of tumor cells (eight hepatocellular carcinoma cell lines and five breast cancer cell lines [134]) and mice [135].

A similar dependence was found for the mouse cells defective in the telomerase gene. At the same time, telomerase-deficient mouse fibroblasts immortalized by transfection with the telomerase gene displayed no correlation between the TL and radiosensitivity (as demonstrated for three cell clones with the TL of 13, 10, and 4 kb, respectively) [136]. The authors concluded that the presence of TA causes stabilization of short telomeres, which prevents the increase in the radiosensitivity of cells.

A relationship between the TL and radiosensitivity has been well established. It was shown that the shortening of telomeres enhances cell sensitivity to IR, but the molecular mechanisms of this phenomenon remain unclear. To understand the relationship between the telomere shortening and radiation sensitivity, Drissi et al. [137] showed that the late-passage cells with short telomeres are characterized by an increased sensitivity to IR compared to the early-passage cells with longer telomeres. Before exposure to IR, late-passage cells had a higher baseline level of γH2AX, which reduced after irradiation. The rates of the appearance and disappearance of γH2AX foci in irradiated cells decreased, indicating a reduced level of DNA repair. Ectopic telomerase expression in the late-passage cells completely restored the kinetics of formation and resolution of γH2AX foci to the levels observed in the early-passage cells. These data demonstrate the role of TL in the DSB repair suppression in cells with short telomeres [137].

The phosphorylation kinetics of ATM kinase and p53 was similar in the early- and late-passage cells, but phosphorylation of the chromatin-bound SMC1 and NBS1 proteins (ATM targets) was reduced in the late-passage cells. The authors investigated the chromatin structure and showed that the late-passage chromatin was resistant to digestion with the micrococcal nuclease and had a reduced level of H3K9 acetylation and a higher level of H3K9 methylation, indicating its transition to heterochromatin. Such chromatin conversion to a more compact state may explain why phosphorylation and activation of the chromatin-associated DDR proteins (H2AX, SMC1, and NBS1) were reduced in the late-passage cells, while activation of the non-chromatin-associated proteins (p53 and ATM) remained unchanged. The authors concluded that short telomeres are associated with changes in the chromatin structure that limit the access of the activated ATM kinase to its targets present in the chromatin content and restrict the DDR, which might explain an increased radiosensitivity of cells with shortened telomeres [137].

To study the mechanisms underlying the radioresistance of tumors, Zhou et al. [138] generated a radioresistant cell line from the human larynx squamous cell carcinoma Hep-2 cells. The authors showed that the expression of some genes associated with the DNA repair, cell cycle, apoptosis, etc. (for example, genes for telomere proteins, such as POT1) was significantly altered in the radioresistant cells, which also had a higher TA and longer telomeres than the parental cells. The authors concluded that both TA and TL may be indicators of cell radioresistance [138].

Long telomeres and high TA have been commonly associated with the radioresistance of different cancers. The protection of telomeres, telomere function, and TL depend on the TA and proteins of the shelterin and CST complexes. Ferrandon et al. [139] evaluated the telomeric status of glioblastoma cells after the photon and carbon ion irradiation and found a significant correlation between the TL, basal POT1 expression, and photon radioresistance *in vitro*. The authors also observed a significant increase in the survival of patients with long telomeres or a high POT1 level. Expression of POT1 was a predictive indicator of the patients' response irrespectively of the TL. However, the observed correlations were absent *in vitro*

after the carbon ion irradiation. To identify radioresistant tumors in patients who would benefit from the carbon ion hadron therapy the authors proposed the assessment of the TL and POT1 expression in tumor biopsies [139].

As shown above, both TL and TA can be used as markers for monitoring the patients' condition during radiation therapy. Indeed, it has been shown that the stability of telomeres in peripheral blood lymphocytes correlates with the life expectancy of patients receiving radiation therapy, and that the telomere instability correlates with the development of toxic response long after the radiation therapy [140]. The implementation of this approach into clinical studies will help to analyze in more details the dynamics of this indicator in patients subjected to the radiation therapy.

The increase in the radiosensitivity associated with the TL decrease is not the only problem of cells and organisms with short telomeres. Another consequence of telomere shortening is an increased risk of chromosome aberrations and malignant transformation of cells [82, 128, 141].

The TL stability is currently considered as a promising marker of normal tissue radiosensitivity that can be used for predicting the development of complications in patients after radiation therapy [142].

The telomere–telomerase system in astronauts. Exposure to IR is the major health risk during future deep space missions. The keynote paper co-authored by 38 researchers representing 33 leading aerospace research institutes and agencies identified six processes that shape our current understanding of molecular changes occurring during space missions: oxidative stress, DNA damage, dysregulation of mitochondrial function, epigenetic changes (including regulation of gene activity), changes in the TL, and microbiome modification [143].

The TL in blood cells appears to be a relevant integrative biomarker for studying the impact of spaceflight factors on astronauts, because changes in this indicator reflect the combined effect of factors that astronauts encounter in extreme space conditions. The most striking discovery was the lengthening of telomeres during the spaceflight found in all crew members. The TL reduced rapidly upon return to Earth, so that eventually the crew members had significantly shorter telomeres after the spaceflight than before it.

Chronic exposure to the space radiation and associated development of DDR and other stress responses may promote activation of the TL maintenance pathways. Although the exact mechanisms and health effects of spaceflight-associated shifts in the TL dynamics remain to be elucidated, the existing findings have already highlighted the importance of monitoring the TL and genome stability in the cells of spacecraft crew members for the assessment of the overall health and risks of disease development and aging. These data should be taken into account in the creation of personalized aerospace medicine and protective measures for future astronauts.

More detailed results of the studies of the TL in astronauts were presented in the reports from the laboratory of S. M. Bailey [144, 145]. The authors found that the identical twins Scott and Mark Kelly had relatively similar telomeres before the spaceflight. The telomeres of Mark Kelly, who stayed on Earth, remained stable throughout the study, while the telomeres of Scott Kelly increased in length during the spaceflight and then shortened rapidly upon his return to Earth. Overall, the content of short telomeres increased after the spaceflight. The level of chromosome aberrations also increased during the spaceflight, and the elevated frequency of inversions persisted after the flight, indicating long-term genome instability in the astronaut's cells after returning to Earth.

During the spaceflight, all crew members experienced oxidative stress that correlated positively with the TL dynamics. The frequency of chromosomal inversions increased significantly during and after the spaceflight. The authors believed that it was an adaptive response of telomeres to chronic oxidative stress under extreme conditions, because the studied cells also demonstrated simultaneous ALT activation, as evidenced by the increase in the chromatid exchanges in the telomeres. The radiation doses received by the astronauts and calculated based on the level of chromosome inversions were 50-350 mGy, and the average effective dose over 6 months on the International Space Station was 80 mSv. The authors suggested that the observed damage was related to the oxidative stress (Fig. 4) rather than to the radiation exposure [145]. This may be true, since no changes in the TL were found in the residents of regions with a high natural background radiation (mean dose, 0.5 to 15 mSv/year) despite the presence of cytogenetic abnormalities [146].

During the long-term flight, the astronauts showed the signs of inflammation [144], which is always accompanied by the increase in the ROS content (oxidative stress).

It should be noted that similar changes in the TL were registered in the participants of the short-term "Inspiration 4" civilian spaceflight, namely, an increase in the TL during the flight and telomere shortening after returning to Earth [147]. Other detected changes had an adaptive nature.

The above data indicate the possibility of developing an adaptive response to various factors encountered during the spaceflight, including oxidative stress. The detected changes in the telomeres probably reflect a universal adaptive response to the damaging factors.

TELOMERE LENGTH AND TELOMERASE IN TELOMEROPATHIES

There are several pathological conditions associated with mutations in the genes involved in the mechanisms ensuring the telomere stability. They are accompanied by disturbances in hematopoiesis, development of pulmonary and liver fibrosis, worsening of nail condition, and changes in the TL. Such conditions are called telomeropathies, or telomere biology disorders [148, 149]. The TL analysis is of great importance for the diagnostics and monitoring of telomeropathies, while clarification of the mechanism of telomere dysfunction in such diseases is necessary for their diagnostics, genetic counseling, clinical management of patients, and choice of therapy.

Dyskeratosis congenita (DC) is a congenital syndrome of bone marrow failure and dysplasia of oral mucosa and skin, which is characterized by the classic triad of nail dystrophy, spotted hyperpigmentation of skin, and oral leukoplakia, as well as predisposition to cancer. Genetic analysis has shown that DC is associated with a defect in the gene located in the Xq28 locus. The *DKC1* gene encodes dyskerin [150], which is one of the proteins forming a complex with telomerase. It is found in the nucleolus, where it binds to RNA (including ribosomal RNA and hTERC) and influences many cellular functions.

Laboratory diagnostics of DC is difficult. Detection of very short telomeres in lymphocytes by flow cytometry and fluorescence *in situ* hybridization (FISH) in commercial laboratories can differentiate DC from other syndromes involving bone marrow failure. Genetic screening of DC patients has also revealed heterozygous mutations in the *TERC* gene, homozygous mutations in the *NOP10* and *NHP2* genes coding for proteins associated with the telomerase complex, and mutations in the *TERT* gene. The autosomal dominant form of DC is characterized by mutations in the *TINF2* gene. The loss of functionally active TIN2 in the shelterin complex leads to the cells with extremely short telomeres [151]. Another cause of DC is thymidylate synthase deficiency [152]. Mutations in the *CTC1*, *RTEL1*, *ACD*, *PARN*, *NAF1*, *ZCCHC8*, *NPM1*, *MDM4*, *RPA1*, *DCLRE1B*, *POT1*, and *TYMS-ENOSF1* genes were also found in DC and DC-like diseases. These mutations disrupt the functioning of telomerase and/or proteins involved in the telomere maintenance and lead to the appearance of short telomeres [153, 154].

Genetic testing of a large cohort of clinically diagnosed patients with DC and DC-like diseases has identified several new mutations in the known genetic loci, X-linked *POLA1* gene, and *POT1* and *ZCCHC8* genes. Functional characterization of the new *POLA1* and *POT1* variants revealed abnormal protein–protein interactions between primase and CST and shelterin complex proteins, which are critical for the TL maintenance [155]. Assessment of the TL allows to conduct differential diagnosis between the acquired aplastic anemia and DC [156] and control sickle cell anemia [157] and some other diseases of the hematopoietic system [158]. Modern genetic technologies make it possible to conduct such studies.

TECHNOLOGIES FOR THE ASSESSMENT OF TELOMERASE ACTIVITY AND TELOMERE LENGTH IN CLINICAL AND EPIDEMIOLOGICAL STUDIES

Analysis of published data on the TA-TL system dysfunction in the pathogenesis of diseases, including cancer and genetic disorders associated with telomeropathies, has demonstrated the relevance of TA assessment in the identification of the malignant nature of a tumor. The presence of short telomeres can be an important disease (telomeropathy) marker, an indicator of individual sensitivity to the damaging factors, and a prognostic marker for the development of complications during chemo- and radiation therapy of oncological diseases. The significance of disease diagnostics and monitoring has promoted the development of methods for the assessment of TA and TL in clinical and epidemiological studies.

The methods for measuring TA in cell and tissue extracts were described in detail in the review [159]. The authors examined the sensitivity, advantages, disadvantages, and implementation of existing methods for TA analysis, which they divided into two types: direct assessment of telomerase-synthesized DNA and methods using various signal amplification schemes to increase the sensitivity. There are numerous ongoing studies on the improvement of TA analysis methods [87, 160, 161].

In terms of sensitivity, cost, complexity of implementation, and equipment availability, the most available method for the TA assessment in tumor tissue in clinical trials is amplification of telomeric repeats followed by their fluorescent detection, called TRAP (telomeric repeat amplification protocol). The method is based on the elongation of oligonucleotide substrate by telomerase, PCR amplification of the synthesized telomeric repeats, separation of the amplified TRAP products by electrophoresis, their visualization by staining with the SYBR Gold dye, and gel documentation for subsequent processing of the results and evaluation of the relative TA vs. positive control sample [162]. An optimized protocol for this method was described in [163] that included a procedure for preparing the lysate from surgical specimens, as well as the protocol for the result analysis and quantification suitable for the use in diagnostic studies.

Given the above-mentioned difficulties associated with the standardization of sample preparation, it is possible that the TA assessment in biological materials will be eventually replaced by the analysis of C228T and C250T mutations in the *hTERT* gene promoter or other mutations that lead to the telomerase activation and malignant transformation [89]. A combination of modern DNA sequencing technologies used for the mutation identification and liquid or conventional biopsy might be a more promising approach compared to the routine TA assessment.

A wide range of methods have been developed for the TL assessment, including analysis of terminal restriction fragments (TRFs), quantitative PCR (qPCR), STELA (single telomere length analysis), MMQPCR (monochrome multiplex qPCR), Q-FISH (quantitative FISH of telomeres in metaphase chromosomes), and FlowFISH (determination of relative TL using flow cytofluorimetry). Until recently, FlowFISH had been the only method acceptable for clinical use, as it allows highly reproducible, simple, and rapid measurement of absolute TL [164]. The authors of [164] developed an algorithm for converting the flow cytometry data into the absolute TL expressed in kb, which makes it possible to compare results obtained in different laboratories, as well as to perform the retrospective conversion of previously obtained flow cytometry data. Flow-FISH and qPCR offer specific and highly sensitive measurement of normal-length telomeres. However, the sensitivity and specificity of qPCR in the detection of short telomeres are lower [165], although this method is suitable for TL measurement in epidemiological studies. The STELA method has the highest sensitivity for analyzing short telomeres, based on the fact that all telomeres have the GGG repeat at the 3′ end. This method has also been adapted for epidemiological studies, but it requires special equipment and reagents [166].

Hence, this brief review of methods used for TA and TL analysis allows us to conclude that the current array of molecular biological and genetic technologies allows to conduct research on the TA and TL in clinical and epidemiological studies, although these methods and software for data processing continue to improve.

CONCLUSION

Analysis of published data on the telomere biology and mechanisms of TL maintenance in human cells demonstrate that these topics still remain in the research focus. In recent years, new data have been obtained on the important role of CST–Polα/Prim in the TL maintenance. The primase forms a complex with CST, which ensures the high specificity of replication initiation for the complementary C-strand after completion of the G-chain synthesis by telomerase. In the case of low TA or its absence, the TL is maintained by the ALT mechanism based on homologous recombination. The regulation of TA by different mechanisms has been well studied, including the control of TA at the epigenetic level. TERRA, a long non-coding telomeric repeat-containing RNA transcribed by RNA polymerase II from the intrachromosomal telomeric repeats, likely plays an important role in telomerase regulation and heterochromatin organization in the telomere region.

TL is an important marker of cells well-being. The state of telomeres (length, structure, presence of oxidized guanine and/or G4s, quality of shelterin and CST complex proteins) is currently considered as an indicator of health. The accumulation of short telomeres indicates replicative aging of proliferating cells, transition to the senescent state, and increased sensitivity to damaging factors. Proteins of the shelterin complex are crucial for the telomere protection. The inactivation of shelterin binding to the telomere DNA leads to the appearance of unprotected telomeres, activation of different DDR pathways, and genomic instability. The alternative functions of telomerase associated with the anti-apoptotic activity of its components after their translocation to the nucleus (TR) or mitochondria (TERT) may be of great importance in the maintenance of cell stability.

A high TA can serve as a marker of neoplasm malignancy. Mutations in genes coding for proteins involved in the maintenance of telomere structure and length lead to the development of genetic diseases (telomeropathies) that can be diagnosed by the analysis of TL and TA.

IR damages telomeres via direct DNA damage and through the action of ROS generated by the radiolysis of intracellular water. A high guanine content makes telomeres highly sensitive to ROS, while formation of 8-OxodG in the telomere DNA inhibits telomerase. Similar lesions were detected after the long-term γ- and X-irradiation at low doses (mainly after a certain period of time) or after the chronic low-dose irradiation of occupationally exposed individuals.

According to the National Aeronautics and Space Administration, changes in the TL in astronauts represent one of the important biological responses to the effects of spaceflight factors. Both TL and TA are among the parameters that should be analyzed in astronauts before, during, and after completion of space mission.

Telomeres are dynamic structures that undergo significant changes under the influence of deleterious factors. The TL is determined by the cumulative impact of all endogenous and exogenous damaging factors acting throughout the lifetime, rather than by the chronological age.

The current state of biochemical and molecular genetic methods has made it possible to develop technologies for the TA assay and identification of mutations affecting TA that can be used for the diagnostics of malignant neoplasms, as well as the methods for TL assessment in order to monitor telomeropathies and health status in clinical practice. The introduction of these technologies into clinical practice and epidemiological studies will significantly expand the possibilities of medical and biological diagnostic studies, allow prediction of individual sensitivity of patients to the planned radiation and chemotherapeutic treatment, and facilitate the assessment of risks of developing unwanted late complications.

Abbreviations. 8-OxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; ALT, alternative lengthening of telomeres; CST–Polα/Prim, CST–Polymerase α/primase; DC, dyskeratosis congenita; DDR, DNA damage response; DSB, DNA double-strand break; G4s, G-quadruplexes; TERT, telomerase reverse transcriptase; TR (TERC), telomerase RNA component; IR, ionizing radiation; MSC, mesenchymal stromal cell; ROS, reactive oxygen species; TA, telomerase activity; TL, telomere length.

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