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Telomere G-Quadruplex as a Potential Target to Accelerate Telomere Shortening by Expanding the Incomplete End-Replication of Telomere DNA

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Abstract: Chromosomes in human cells are protected by telomeres. Telomere shortens during each round of cell division because of the DNA end-replication problem. Cancer cells maintain telomere length homeostasis by either telomerase or/and the alternative lengthening of telomere (ALT) mechanism to sustain their division potential. Telomeric DNA tends to form G-quadruplex preferentially at the extreme 3' end. This unique feature prevents the 3' end from being used as a substrate of telomerase and as a primer in the ALT. Therefore, stabilizing telomere G-quadruplex is expected to inhibit both pathways and limit the proliferation of cancer cells. Based on a mathematical modeling and experimental results, this mini-review proposes a hypothesis that the formation of G-quadruplex in telomere may constitute a significant contribution to the incomplete end-replication of telomere DNA by preventing the priming of DNA synthesis near the 3' end during telomere replication. According to this, stabilization of telomere G-quadruplex by chemical ligand may promise to accelerate telomere shortening in proliferating cells.

Keywords: Cancer, DNA replication, G-quadruplex, Proliferative potential, Telomere extension, Telomere shortening.

1. MATHEMATICS OF A MECHANISTIC MODEL OF INCOMPLETE TELOMERE DNA REPLICATION

Chromosomes in human cells are protected at each end by telomere, a linear DNA array of TTAGGG repeats with associated proteins [1]. Human telomere DNA stretches for several thousands to several tens of thousands of base pairs. Due to the semi-conservative nature of DNA replication, the 3' end of the guanine-rich telomere DNA strand can not be fully replicated, producing a single-stranded 3' overhang (Fig. **1A**) [2]. As a result, telomeres in human cells shorten by about 50 nucleotides in average at each round of cell division [3]. Continued telomere shortening, if not reimbursed, will eventually limit the division potential of a cell [4].

In human cells, DNA replication are initiated from short RNA primers that have a size of 9±1 nucleotides [5]. The guanine-rich strand of the telomere DNA is the lagging strand that is replicated by primers at short intervals, producing many short Okazaki fragments. The Okazaki fragments are later ligated to form an intact DNA strand [6]. According to the canonical model of telomere shortening at cell division, the region occupied by the primer at the very 3' end could not be filled after the removal of the primer, which, as a result, leads to an incomplete telomere replication (Fig. **1A**). However, the single-stranded telomere overhangs at the 3' end can be as long as several hundreds of nucleotides [7,8], far greater than that of a RNA primer. This fact implies that additional factors contributed to the incomplete 3' end replications, which are more profound than the size of the region occupied by the primer. Two hypotheses were put forward to explain this discrepancy. One hypothesis proposed that while the original incomplete replication can be small, a later exonucleolytic cleavage at the 5' to 3' direction may further expand the incomplete replication (Fig. **1B**) [7]. In another hypothesis, it was assumed that a primer is randomly placed on the guanine-rich template telomere strand such that it may not match to the very 3' end (Fig. **1C**) [8]. Under this condition, the nucleotides occupied and missed out by the primer both contributed to the 3' end overhang. Therefore, the incomplete replication in both hypotheses can be greater than the size of a primer.

To distinguish the two hypotheses, we developed in 2002 a mathematical model based on the random priming assumption [9]. We assumed that for a random priming event at the very 3' end, the 3' overhang size (d) was composed of two components given by

$$d = d_1 + d_2, \quad (1)$$

where d_1 and d_2 represented the number of nucleotides occupied and missed out by the primer, respectively (Fig. **2A**). We further assumed that the sizes of d_1 were uniformly

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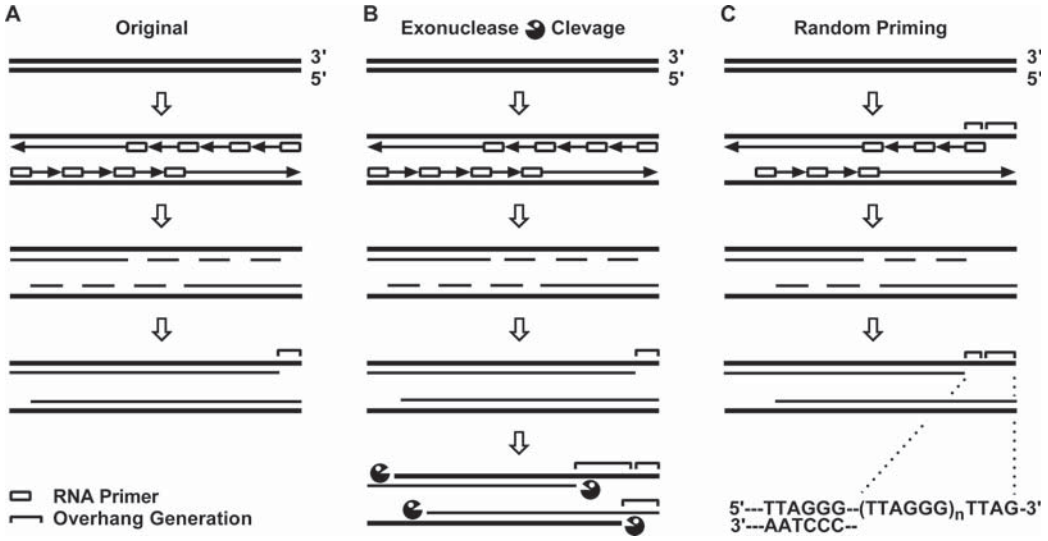


Fig. (1). Hypotheses of incomplete telomere end replication. During DNA replication, the syntheses of new DNA strands (thin line) are initiated from RNA primers (open rectangle) on a parental DNA template strand (thick line) and extended in the 5' to 3' direction (arrow). The lagging strand synthesis on the guanine-rich DNA strand produces Okazaki fragments (short thin line) that are subsequently ligated to form an intact strand. (**A**) The original end-replication model. The removal of the 3'-most primer yields a protruding single-stranded overhang at one 3' end of each chromosome. (**B**) The exonucleolytic model that counts for the generation of long 3' overhang by exonuclease cleavage at the 5' end of the newly synthesized cytosine-rich DNA strand in addition to primer removal. (**C**) The random priming model in which the 3'-most primer may not match to the very 3' end. The overhang size (brackets) in both (**B**) and (**C**) can be greater than that of a primer.

distributed over a fixed length between k_1 and k_2 nucleotides described by the following probability function

$$p_1(d_1) = \frac{1}{k_2 - k_1}, \qquad (k_1 \le d_1 \le k_2), \qquad (2)$$

and d_2 had their sizes followed an exponential decay distribution

$$p_2(d_2) = \frac{e^{-d_2/m}}{m}, \qquad (0 \le d_2 \le \infty). \qquad (3)$$

The mean size of d_1 and d_2 was $(k_1+k_2)/2$ and m, respectively. Combining equations (2) and (3) via the following integration

$$p(d) = \int_{k_1}^{d} p_1(x)p_2(d-x)dx, \qquad (k_1 \le d \le k_2), \qquad (4)$$

$$p(d) = \int_{k_1}^{k_2} p_1(x)p_2(d-x)dx, \qquad (k_2 \le d \le \infty), \qquad (5)$$

we obtained the following function that gives the probability of finding an overhang with a size of d nucleotides [9].

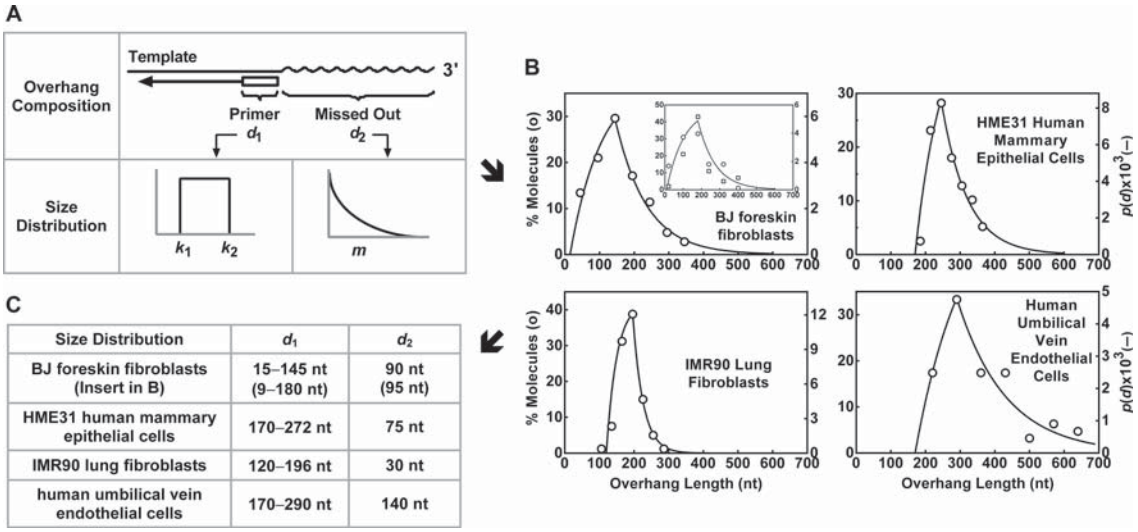
$$p(d) = \begin{cases} \dfrac{1}{k_2 - k_1}\left[1 - e^{-(d-k_1)/m}\right], & (k_1 \le d \le k_2), \\ \dfrac{1}{k_2 - k_1}\left(e^{k_2/m} - e^{k_1/m}\right)e^{-d/m}, & (k_2 \le d \le \infty), \end{cases} \qquad (6)$$

we fitted the equation to the experimental data available in literature [8,10] and found that it could perfectly describe the size distribution of the telomere overhangs in five different human cell strains (Fig. **2B**). These results suggest that the equation reflects the biological reality of the telomere end replication in human cells. However, the model parameters derived from the fittings (Fig. **2C**) revealed that the size of the two components are both far greater than the size of a primer [11]. This fact indicates that the original assumption needs modification.

To make the model compatible with the biological reality, we decomposed the d_1 component into two: a d_0 with a fixed size of k_0 and a d_1 with a size uniformly distributed between $(k_1\text{-}k_0)$ and $(k_2\text{-}k_0)$ (Fig. **3**). This modification does not affect equation (6) and its fittings to the experimental data (Fig. **2B**), but only the assignments of the model parameters. Under this condition, the incomplete replication was attributed to three components or events represented by d_0, d_1, and d_2, respectively (Fig. **3**). Among them, the d_0 was set to 9 nucleotides to represent the contribution of primer occupancy (Fig. **3**). Since the d_2 was assumed to represent the region missed out by the 3'-most primer, we speculated that the new d_1 might represent a region on the telomere DNA template that is blocked and inaccessible to primase for primer synthesis by an unknown event (Fig. (**3**) herein and Fig. (**4**) in reference [9]). We did not know what the biological nature of this event was.

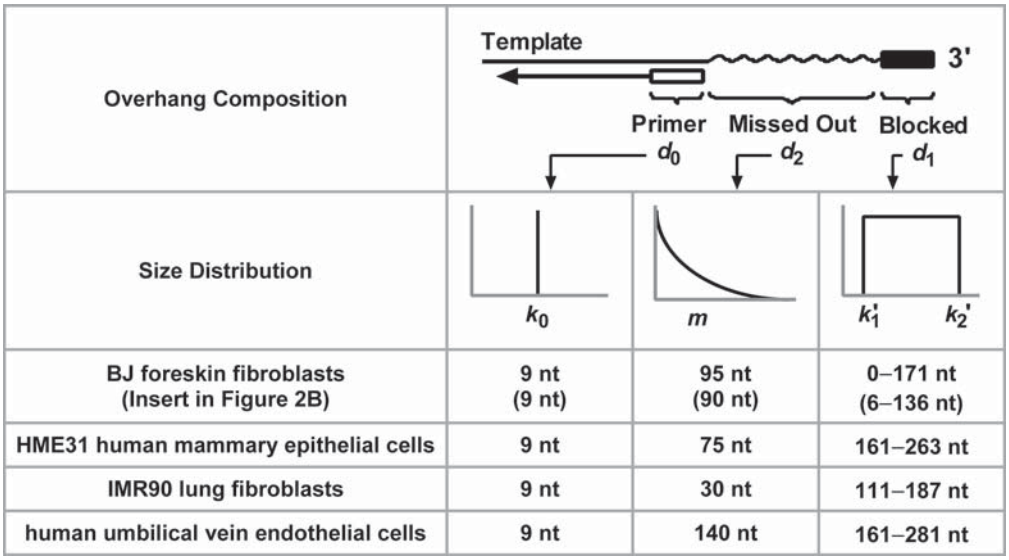
2. A POLARIZED FORMATION OF G-QUADRUPLEX AT THE 3' END OF TELOMERIC DNA

Guanine-rich nucleic acids with at least four consecutive guanine tracts can fold into a four-stranded structure termed G-quadruplex [12,13]. G-quadruplex formed by human telomeric DNA is the most extensively studied G-quadruplex structure because it is a potential target in anti-cancer therapy [14]. In 2008, we were able to find out that telomeric



Size Distribution	d_1	d_2
BJ foreskin fibroblasts (Insert in B)	15–145 nt (9–180 nt)	90 nt (95 nt)
HME31 human mammary epithelial cells	170–272 nt	75 nt
IMR90 lung fibroblasts	120–196 nt	30 nt
human umbilical vein endothelial cells	170–290 nt	140 nt

Fig. (2). The original mathematical modeling of the incomplete 3' telomere end replication based on the random priming hypothesis (adapted from Hao [9] and modified). (**A**) Assumed contributions of random priming in the incomplete replication at the 3' telomere end in reference [9]. The last RNA primer (open rectangle) may not be placed at the very 3' telomere end and, therefore, the un-replicated region is composed of the nucleotides occupied by the primer (d_1) and those missed out by the primer at its 3' side (d_2). (**B**) Fittings of the probability equation (6) of telomere overhang size distribution (—) with experimental data (○, □) from five different human cell strains. The equation was derived from the assumptions in (A). Experimental data were adopted from the Fig. (**2**) of reference [10] and Fig. (**3**) of reference [8] (insert), respectively. The (○, □) in the insert indicate measurements at two different population doubling levels, respectively. (**C**) Model parameters obtained from the fittings illustrated in (**B**).



BJ foreskin fibroblasts (Insert in Figure 2B)	9 nt (9 nt)	95 nt (90 nt)	0–171 nt (6–136 nt)
HME31 human mammary epithelial cells	9 nt	75 nt	161–263 nt
IMR90 lung fibroblasts	9 nt	30 nt	111–187 nt
human umbilical vein endothelial cells	9 nt	140 nt	161–281 nt

Fig. (3). Biological activities assigned to the model parameters in reference [9] (adapted from Hao [9] and modified). Because the d_1 and d_2 in Fig. (**2A**) are both greater than the size of a primer in human cells (9±1 nucleotides), the d_1 component was decomposed into d_0 and d_1. The d_0 was given a fixed size of 9 nucleotides to present the nucleotides occupied by the primer (open rectangle). The new d_1 then implies that a region on the DNA template was blocked by an unidentified event. Here $k_1' = (k_1\text{-}k_0)$ and $k_2' = (k_2\text{-}k_0)$, respectively. The k_1 and k_2 in equation (6) then equal (k_0+k_1') and (k_0+k_2'), respectively. The modifications do not alter function (6) and its fittings to the experimental data shown in Fig. (**2B**).

G-quadruplex preferentially forms at the 3' end when it can, in principle, form at multiple positions along a DNA. The formation probability followed an exponential decay function with respect to its distance to the 3' end [11]. This property was detected in a DNA oligonucleotide of seven TTAGGG repeats by both DMS footprinting and exonucleolytic cleavage with T4 polymerase. Long telomeric DNA forms multiple intramolecular G-quadruplexes possibly at each four consecutive guanine-tracts, which are connected by linkers like beads on a string [15]. While the seven TTAGGG repeat can only host one G-quadruplex at four possible positions, the polarized formation of G-quadruplex is also reserved in longer telomeric DNA. Here we determined the probability distribution of G-quadruplex formation in a single-stranded $(TTAGGG)_{12}$ DNA that is capable of accommodating a maximum of three G-quadruplexes (Appendix). A formation of G-quadruplex in this DNA can possibly occur at nine positions at an increment of 6 nucleotides counting from the 3' end. The result shows that it also followed an exponential decay function (Fig. **4**).

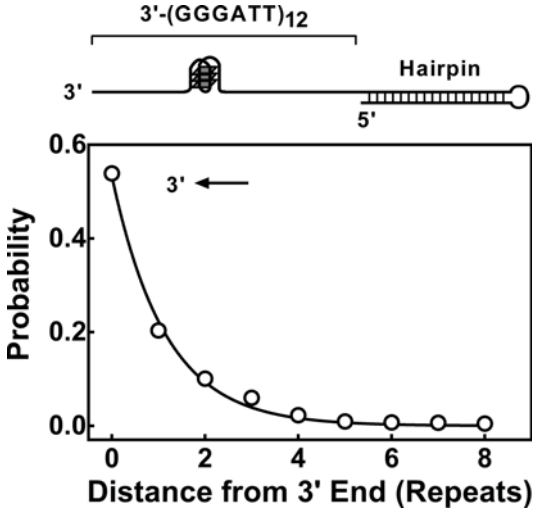


Fig. (4). Probability of G-quadruplex formation in a $(TTAGGG)_{12}$ telomere overhang attached to a hairpin detected by the 3' to 5' exonucleolytic activity of T4 polymerase. The data (open circles) were derived from the denaturing gel in the Fig. (**7**) of reference [11] using the method described in the Appendix and fitted to an exponential decay function (solid line). The original gel image was digitized to trace the peaks of stall signal. The peaks were then isolated and quantitated by fitting with the software fityk version 0.9.8 (http://fityk.nieto.pl). In a control DNA without G-quadruplex, 20% of the DNA remained un-cleaved in full-length and the rest was completely digested throughout the overhang. The full-length fragment remaining in the telomere sequence was calibrated for this incomplete cleavage.

The existence of G-quadruplex in human cells has recently been demonstrated by independent techniques [16,17]. Although a polarized formation of G-quadruplexes on telomere DNA with a preference towards the 3' end is clearly shown to occur under the *in vitro* condition (Fig. **4**), it is technically difficult to characterize such a feature inside cells. However, this feature is supported by our finding of a specific protein, hnRNA A2*, in mammalian cells that interacts with both telomere DNA and telomerase to facilitate the extension of telomere by telomerase [18]. One unique trait of hnRNP A2* is that it actively unfolds telomere G-quadruplex in a rate much faster than the spontaneous opening of telomere G-quadruplex. In particular, hnRNP A2* preferentially binds to the recognition unit (TAGGGTTAGG) at the very 3' end of the guanine-rich strand of telomeric DNA where multiple recognition units are available. Upon binding, hnRNP A2* exposes the last five nucleotides required to align with the RNA template in the telomerase to promote its extension. This binding polarity is in correlation with the preferential formation of G-quadruplex at the 3' telomere end and implies that a positional preference is adapted by the hnRNP A2* to coordinate with the positional preference of telomere G-quadruplex formation in cells.

3. THE MATHEMATICAL MODEL REVISITED. INVOLVEMENT OF G-QUADRUPLEX IN THE 3' TELOMERE END REPLICATION

Although our original mathematical model satisfactorily provides a quantitative description of the incomplete 3' telomere end replication in human cells, it seems that the parameter d_1 and d_2 in our previous work were improperly assigned to biological events due to the limited information available when the model was built [9]. As the first step to initiate the synthesis of nascent DNA, RNA priming requires a single-stranded template DNA strand. It is expected that a formation of G-quadruplexes on the guanine-rich telomere strand will prevent the synthesis of a RNA primer onto the template strand near the 3' end. Therefore, priming can only occur at the internal region free of G-quadruplex. Because of the discovery of the polarized G-quadruplex formation at the 3' telomere end [11], we feel that there is reason to modify the original assignments of the components d_1 and d_2 by simply switching their definitions. With this modification, the exponential decay component (d_2) is now attributed to the formation of G-quadruplex in telomere DNA during telomere DNA replication that follows an exponential decay function. On the other hand, the d_1 component now represents the region missed out by the 3'-most primer on the single-stranded region at its 3' side upstream of the G-quadruplexes. It could be imagined that if the size of d_1 is larger than that of an Okazaki fragment, another primer would be synthesized nearby to create a new initiating site for nascent DNA synthesis. In agreement with this, the size distribution of d_1 matches well with that of the Okazaki fragments (25-300 nucleotides) in eukaryotic cells [5].

With these modifications, now the three components in the mathematical model satisfactorily agree with the biological characteristics of the correspondent events in DNA replication (Fig. **5**). The mathematical model with the new parameter assignments now quantitatively describes the incomplete telomere 3' end replication by the contribution of three biochemical events (Fig. **5**). They are: 1) primer occupancy (d_0) that covers 9 nucleotides; 2) a region (d_1) missed out by the 3'-most primer due to the randomness of its synthesis onto the template DNA strand; and 3) a 3' terminal region (d_2) that is unable to serve as template because of the formation of G-quadruplexes. From the values of the parameters obtained by fitting the model to the experimental data, we can judge how much each individual event contributed to the incomplete telomere replication. From Fig. (**5**), it is seen that the sequence missed out by the 3'-most primer is the major source. We can also see that the formation of G-quadruplex at the 3' end also makes a significant contribution. G-quadruplexes preferentially blocks RNA priming at the 3' end. As the probability of G-quadruplex formation decreases when it goes inwards from the 3' end, the chance of RNA priming increases. As judged from the values of the correspondent parameter (m) for d_2 derived from the fittings, human cells loss 30-140 nucleotides due to the formation of G-quadruplex at the 3' telomere end (Fig. (**5**), parameters at the right-most column).

4. POSSIBILITY TO ACCELERATE TELOMERE SHORTENING BY STABILIZATION OF TELOMERE G-QUADRUPLEX

Telomere length homeostasis is essential for the continued proliferation of cancer cells. Cancer cells employ two pathways to maintain telomere length homeostasis. In approximately 85% of cancer cells, telomere shortening is compensated by telomerase that adds telomeric repeats to the 3' end of telomere DNA [19]. In the minority of cells,

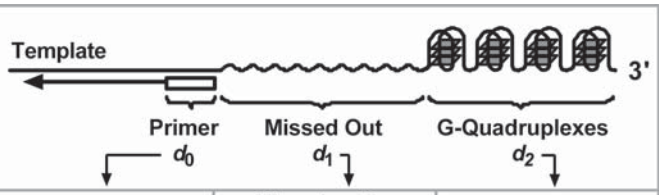
Overhang Composition			
Size Distribution	Occupied by Primer  k_0	Missed out by Primer  k_1' k_2'	Blocked by G-Quadruplexes  m
BJ foreskin fibroblasts (Insert in Figure 2B)	9 nt (9 nt)	0–171 nt (6–136 nt)	95 nt (90 nt)
HME31 human mammary epithelial cells	9 nt	161–263 nt	75 nt
IMR90 lung fibroblasts	9 nt	111–187 nt	30 nt
human umbilical vein endothelial cells	9 nt	161–281 nt	140 nt

Fig. (5). Events contributed to the incomplete 3' end replication of telomere DNA with the re-assigned model parameters. Formation of G-quadruplexes (d_2) at the 3' end follows an exponential decay function. It prevents the correspondent region (with an average size of m nucleotides) to serve as template for RNA priming. At the internal G-quadruplex-free region, RNA primer (d_0, open rectangle) is synthesized onto the guanine-rich telomere strand randomly and occupies a fixed size of 9±1 nucleotides. Because of its random positioning, it may not match to the very 3' end of the G-quadruplex-free region. The distance between the 3'-most primer to the 5' border of the G-quadruplexes (d_1) is expected to be roughly about the size of a Okazaki fragment, who's minimum and maximum is represented by $k_1' = (k_1\text{-}k_0)$ and $k_2' = (k_2\text{-}k_0)$, respectively. The k_1 and k_2 in equation (6) equal (k_0+k_1') and (k_0+k_2'), respectively. The re-assignment of the parameters does not alter equation (6) and its fittings to the experimental data shown in Fig. (**2B**).

telomere is elongated by an alternative lengthening of telomere (ALT) mechanism, in which the guanine-rich telomere 3' end invades into a telomere double helix and anneals with the cytosine-rich strand to prime telomere extension [20]. Because telomerase activity is expressed in most cancer cells but not in normal somatic cells, telomerase serves as a preferred target for preventing telomere elongation [21]. However, targeting telomerase may suffer from a possibility of selecting or inducing the ALT such that cancer cells may eventually survive and propagate without telomerase.

The preferential formation of G-quadruplex at the 3' end of telomere DNA is an important trait of telomere with respect to the control of telomere extension. Telomere extension by either telomerase or the ALT needs a free 3' tail in a single stranded form. We have demonstrated that a 3' tail of 8 nucleotides is required for telomerase to extend telomere. For the ALT, a tail of 12 nucleotides is needed. The preferential formation at the 3' ends minimizes the size of the 3' tail and, as a result, ensures inhibition of telomere extension by both telomerase and the ALT [22]. Therefore, stabilization of telomere G-quadruplex by chemical ligand may avoid the risk associated with the targeting of telomerase. The hnRNP A2* protein we found recently unfolds telomere G-quadruplex *in vitro* and promotes telomere extension under both *in vitro* and *in vivo* conditions. Decreasing in the expression of the protein leads to telomere shortening in rat cells, implying that telomere G-quadruplex in cells negatively impact telomere length homeostasis [18]. Telomere G-quadruplex may affect extension via telomerase and the ALT, and RNA priming near the 3' telomere ends as well (Fig. **5**).

In supporting of the above notion, an *in vivo* investigation showed that a G-quadruplex stabilizer, BMVC4, induced senescence in both telomerase-expressing and telomerase-negative ALT cancer cells [23]. In spite of this, study on the effect of chemical G-quadruplex-stabilizing ligands on telomere metabolism is a complicated issue. This is because sequences with potential to form G-quadruplexes, including the DNA:RNA hybrid G-quadruplexes we found recently that can form during transcription with as few as two G-tracts on the non-template DNA strand [24,25], are so prevalent and abundant in human genome [26,27]. They are present in almost all genes and are potential targets involved in various physiological processes. Clarification of the target-consequence relationship in such studies demands analyses in great details regarding the contribution from different sources. Among them, assessment of telomere shortening upon treatment of ligands and potential therapeutics requires more precise technology. The massive DNA sequencing technique, as it is becoming more and more popular, has recently been employed in quantitating telomere content [28]. For telomere quantitation, massive DNA sequencing may provide a promising and more reliable approach.

In this review, we collected evidences supporting the notion that the formation of G-quadruplexes near the 3' end of telomere DNA contributes to the incomplete telomere end replication. If this is true, then it provides an additional way to manipulate the telomere size homeostasis in cells besides targeting the telomerase and the ALT. When telomere extension is inhibited, the incomplete telomere end replication is the only replication-dependent mechanism to induce telomere shortening. Stabilization of telomere G-quadruplex is anticipated to expand the range of G-

quadruplex formation. Based on our hypothesis, we can expect that stabilization of telomere G-quadruplex by chemical ligand should accelerate telomere erosion during telomere replication. Clarification of the biological processes contributed to the telomere shortening is important for the interpretation of the mechanisms by which a drug functions. Knowledge on the involvement of telomere G-quadruplex formation in the incomplete telomere end replication should help seek more insight into the mechanism in such applications. Generally speaking, the mathematical model may be used to quantitatively evaluate the contribution of each individual biochemical event involved in the incomplete telomere replication and retrieve information on how a drug affects the relevant processes.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

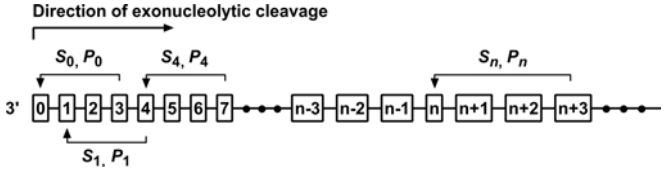
Declared none.

APPENDIX

Quantitation of G-quadruplex formation probability by 3' to 5' exonucleolytic digestion

T4 polymerase and certain exonuclease cleave nucleotide from the 3' end of single-stranded DNA and stalls when they meet a G-quadruplex [11,29]. This property has been used to analyze G-quadruplex formation at different positions on telomeric DNA [11]. Because an intramolecular G-quadruplex involves four guanine tracts (G-tract), when a G-quadruplex-forming DNA has 4-7 G-tracts, a single G-quadruplex can be formed at 1-4 different positions, respectively. In these cases, the probability of G-quadruplex formation at each forming site can be determined directly on a denaturing gel in which the hydrolytic DNA fragments are resolved [11]. When a DNA possesses eight or more G-tracts, however, it will require some calculation to obtain the probability of G-quadruplex formation because the DNA can host more than one G-quadruplexes. Under this condition, a G-quadruplex will shield those at its 5' side from the 3' exonucleolytic activity, preventing them from being detected on a gel. In the following, we describe how the problem can be resolved mathematically to obtain the probability of G-quadruplex formation at any positions.

Here we define S_n, and P_n (n=0, 1, 2, . . .) the probability of exonuclease stall and finding a G-quadruplex at position n, respectively (scheme below). The probability of no G-quadruplex formation at position n is then given by (1-P_n).



Each box in the above scheme represents a G-tract. When an exonuclease stalls at a G-quadruplex, it implies that there is no G-quadruplex in the sequence region starting from the 3' end all the way to the site before the stall position. Therefore, the probability of detecting a stall is given by

$$S_n = P_n, \quad (0 \le n \le 3), \tag{A1}$$

$$S_4 = P_4(1 - P_0), \tag{A2}$$

$$S_5 = P_5(1 - P_0)(1 - P_1), \tag{A3}$$

. . .

$$S_n = P_n \prod_{i=0}^{n-4} (1 - P_i), \quad (n = 4, 5, 6, \ldots). \tag{A4}$$

The probability of stalls (S_n) can be obtained from fluorescence or radioisotope signal at each stall band on a denaturing gel [11]. Then, the probability (P_n) of finding a G-quadruplex at position n can be derived by iterating through equations (A1)-(A4).

REFERENCES

[1] Savage, S. A. Human telomeres and telomere biology disorders. *Prog. Mol. Biol. Transl. Sci.,* **2014**, *125*, 41-66.

[2] Olovnikov, A.M. A theory of marginotomy. The incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon. *J. Theor. Biol*., **1973,** *41*, 181-90.

[3] Allsopp, R. C.; Chang, E.; Kashefi-Aazam, M.; Rogaev, E. I.; Piatyszek, M. A.; Shay, J. W.; Harley, C. B. Telomere shortening is associated with cell division *in vitro* and *in vivo*. *Exp. Cell Res.,* **1995,** *220,* 194-200.

[4] Morin, G. B.; Telomere control of replicative lifespan. *Exp. Gerontol.,* **1997**, *32*, 375-82.

[5] DePamphilis, M. L.; Wassarman, P. M. Replication of eukaryotic chromosomes: a close-up of the replication fork. *Annu. Rev. Biochem*., **1980,** *49*, 627-66.

[6] Shay, J. W.; Wright, W. E. Hayflick, his limit, and cellular ageing. *Nat. Rev. Mol. Cell Biol.,* **2000,** *1,* 72-6.

[7] Makarov, V. L.; Hirose, Y.; Langmore, J. P. Long G tails at both ends of human chromosomes suggest a C strand degradation mechanism for telomere shortening. *Cell.,* **1997,** *88,* 657-66.

[8] Wright, W. E.; Tesmer, V. M.; Huffman, K. E.; Levene, S. D.; Shay, J. W. Normal human chromosomes have long G-rich telomeric overhangs at one end. *Genes Dev.,* **1997,** *11,* 2801-9.

[9] Hao, Y. H.; Tan, Z. The generation of long telomere overhangs in human cells: A model and its implication. *Bioinformatics.,* **2002,** *18,* 666-71.

[10] Huffman, K. E.; Levene, S. D.; Tesmer, V. M.; Shay, J. W.; Wright, W. E. Telomere shortening is proportional to the size of the G-rich telomeric 3'-overhang. *J. Biol. Chem.,* **2000,** *275,* 19719-22.

[11] Tang, J.; Kan, Z. Y.; Yao, Y.; Wang, Q.; Hao, Y. H.; Tan, Z.: G-quadruplex preferentially forms at the very 3' end of vertebrate telomeric DNA. *Nucleic Acids Res.,* **2008,** *36*, 1200-8.

[12] Bryan, T. M.; Baumann, P.: G-quadruplexes: from guanine gels to chemotherapeutics. *Mol. Biotechnol.,* **2011**, *49,* 198-208.

[13] Lipps, H. J.; Rhodes, D.: G-quadruplex structures: *in vivo* evidence and function. *Trends Cell Biol.,* **2009,** *19,* 414-22.

[14] Neidle, S. Human telomeric G-quadruplex: the current status of telomeric G-quadruplexes as therapeutic targets in human cancer. *FEBS J.,* **2010,** *277,* 1118-25.

[15] Yu, H.; Gu, X.; Nakano, S.; Miyoshi, D.; Sugimoto, N. Beads-on-a-string structure of long telomeric DNAs under molecular crowding conditions. *J. Am. Chem. Soc.,* **2012,** *134,* 20060-9.

[16] Murat, P.; Balasubramanian, S. Existence and consequences of G-quadruplex structures in DNA. *Curr. Opin. Genet. Dev.,* **2014,** *25,* 22-9.

[17] Yuan, L.; Tian, T.; Chen, Y.; Yan, S.; Xing, X.; Zhang, Z.; Zhai, Q.; Xu, L.; Wang, S.; Weng, X.; Yuan, B.; Feng, Y.; Zhou, X.: Existence of G-quadruplex structures in promoter region of oncogenes confirmed by G-quadruplex DNA cross-linking strategy. *Sci. Rep.,* **2013**, *3,* 1811.

[18] Wang, F.; Tang, M. L.; Zeng, Z. X.; Wu, R. Y.; Xue, Y.; Hao, Y. H.; Pang, D. W.; Zhao, Y.; Tan, Z. Telomere- and telomerase-

interacting protein that unfolds telomere G-quadruplex and promotes telomere extension in mammalian cells. *Proc. Natl. Acad. Sci. USA.,* **2012,** *109,* 20413-*8.*

[19] Kim, N. W.; Piatyszek, M. A.; Prowse, K. R.; Harley, C. B.; West, M. D.; Ho, P. L.; Coviello, G. M.; Wright, W. E.; Weinrich, S. L.; Shay, J. W. Specific association of human telomerase activity with immortal cells and cancer. *Science.,* **1994,** *266,* 2011-5.

[20] Henson, J. D.; Neumann, A. A.; Yeager, T. R.; Reddel, R. R.: Alternative lengthening of telomeres in mammalian cells. *Oncogene.,* **2002,** *21,* 598-610.

[21] Sekaran, V.; Soares, J.; Jarstfer, M. B. Telomere maintenance as a target for drug discovery. *J. Med. Chem.,* **2014,** *57,* 521-38.

[22] Wang, Q.; Liu, J. Q.; Chen, Z.; Zheng, K. W.; Chen, C. Y.; Hao, Y. H.; Tan, Z.: G-quadruplex formation at the 3' end of telomere DNA inhibits its extension by telomerase, polymerase and unwinding by helicase. *Nucleic Acids Res.,* **2011,** *39,* 6229-37.

[23] Huang, F. C.; Chang, C. C.; Wang, J. M.; Chang, T. C.; Lin, J. J.: Induction of senescence in cancer cells by the G-quadruplex stabilizer, BMVC4, is independent of its telomerase inhibitory activity. *Br. J. Pharmacol.,* **2012**, *167,* 393-406.

[24] Wu, R. Y.; Zheng, K. W.; Zhang, J. Y.; Hao, Y. H.; Tan, Z. Formation of DNA:RNA Hybrid G-Quadruplex in Bacterial Cells and Its Dominance over the Intramolecular DNA G-Quadruplex in Mediating Transcription Termination. Angew. *Chem. Int. Ed. Engl*., **2015,** *54,* 2447-51.

[25] Zheng, K. W.; Xiao, S.; Liu, J. Q.; Zhang, J. Y.; Hao, Y. H.; Tan, Z. Co-transcriptional formation of DNA:RNA hybrid G-quadruplex and potential function as constitutional cis element for transcription control. *Nucleic Acids Res.,* **2013,** *41*, 5533-41.

[26] Huppert, J. L.; Balasubramanian, S. Prevalence of quadruplexes in the human genome. *Nucleic Acids Res.,* **2005**, *33,* 2908-16.

[27] Xiao, S.; Zhang, J. Y.; Zheng, K. W.; Hao, Y. H.; Tan, Z. Bioinformatic analysis reveals an evolutional selection for DNA:RNA hybrid G-quadruplex structures as putative transcription regulatory elements in warm-blooded animals. *Nucleic Acids Res.,* **2013,** *41,* 10379-90.

[28] Parker, M.; Chen, X.; Bahrami, A.; Dalton, J.; Rusch, M.; Wu, G.; Easton, J.; Cheung, N. K.; Dyer, M.; Mardis, E. R.; Wilson, R. K.; Mullighan, C.; Gilbertson, R.; Baker, S. J.; Zambetti, G.; Ellison, D. W.; Downing, J. R.; Zhang, J.; Pediatric Cancer Genome, P. Assessing telomeric DNA content in pediatric cancers using whole-genome sequencing data. *Genome Biol.,***2012**, *13,* R113.

[29] Yao, Y.; Wang, Q.; Hao, Y. H.; Tan, Z. An exonuclease I hydrolysis assay for evaluating G-quadruplex stabilization by small molecules. *Nucleic Acids Res.,* **2007,** *35*, e68.

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