Maintaining the telomere and its implication in cancer

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Genomic instability has been proposed as one of the emerging hallmarks of cancer. However, molecular mechanisms which cause genomic or chromosomal instability still elude us in various cancers. One of the causes for this genomic instability is dysfunction of the telomere. Telomere dysfunction is associated with an increased risk for different cancers, which results, in principle, due to telomere shortening and loss of telomeric proteins which caps and guard telomeres to distinguish it from double-stranded breaks. In this review, we highlight existing understanding of telomeres, telomerase and their role in cancer progression.

Keywords: Cancer progression, epigenetics, genomic instability, telomere dysfunction, telomerase.

A historic milieu to our current understanding

THE discovery of telomeres was first made by Barbara McClintock[1] in maize and Hermann Muller[2] in *Drosophila* in the late 1930s, where they put forward the idea that the chromosome ends play an important part in maintaining genomic stability. The term 'telomere' was coined by Muller, using the Greek words *telos*, which translates to 'end', and *mere*, which translates to 'part', i.e. the end part of a chromosome. McClintock's experiments with maize showed that these end structures were essential as they would otherwise lead to breakage–fusion–bridge cycle during mitosis, causing an inappropriate segregation and ultimately, will give way to genomic instability. Before 1960, the central dogma regarding cell culture was that all cultured cells had indefinite potency to replicate. This dogma was quickly changed after the discovery made by Hayflick[3], when he demonstrated the phenomenon of replicative senescence in which the cells stopped dividing after certain number of cell divisions. A decade later, Alexei Olovnikov[4] linked the reason for this replicative senescence to the way telomeres are replicated, which was later defined by Watson[5] as the end replication problem because of the unidirectional nature of DNA replication. Further, in the 1980s, the amalgamation of the breakthrough work of the Elizabeth Blackburn in the study of telomeres in *Tetrahymena* with that of the proficiency of Jack Szostak in yeast genetics, paved way for the understanding that telomeres protect chromosome ends[6]. It was a big finding as many believed this could open up new ventures in cancer and ageing research, and did set the stage for the discovery of telomerase, by Greider and Blackburn[7], who found telomerase to be responsible in preserving telomere length. After the discovery of telomerase, its role in cancer research was explored independently by Hastie *et al.*[8] and Lange *et al.*[9], where they showed telomere shortening in cancer cells compared to normal cells. Further, immortal cells and cancer were found to have telomerase activity[10]. In subsequent studies, telomerase knock-out mouse models validated that cancer cell division could be limited, thereby controlling tumour formation by inhibiting telomerase[11]. However, it was also observed in some cases that telomere dysfunction leads to genomic instability by means of chromosome rearrangements[12]. During the early 90s, a different method of elongating telomeres, even when telomerase enzyme was not present, was discovered, first by Lundbald and Blackburn[13] in yeast cells, and then by Bryan and Reddel[14] in immortalized human cells in the later half of the decade. These historic aspects led to a boom in the studies in the area of the biology of telomeres and specifically the vibrant areas of cellular senescence and cancer.

Telomere and telomerase

Telomere structure and function

Telomeres are heterochromatic, repetitive sequences that are found at the ends of linear chromosomes. In mammals, the telomere sequence is composed of tandem repeats of the hexanucleotide, TTAGGG, and ends with a 3′ G-rich overhang. This is the site for telomere extension by the enzyme telomerase. Eukaryotic cells have to face two types of problem – end replication problem[4,5] and end protection problem[15]. End replication problem arises because DNA polymerase is incapable of adding nucleotides at the end of the lagging strand. On the other hand, end protection problem arises due to the identification of open chromosome ends as double-stranded breaks (DSBs) by the repair machinery. As a result, it evokes DNA damage response (DDR), which leads to fusion of chromosome

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ends. To protect the cells from both these problems, telomeres had evolved and functions to protect the ends by distinctly differentiating itself from DSBs and also protect the ends of chromosomes from defective repair processes.

The single-stranded G-rich sequence which is approximately 100 bp long in humans, loops back and intercalates the double-stranded DNA. The loop back looks like a noose and is called as 't-loop'. Due to intercalation of the t-loop into the double-stranded DNA, one strand is displaced and is known as the 'displacement or D loop'[16,17]. This type of conformational change at the end of the chromosomes is crucial in tucking in the 3′-end of the telomere and thereby preventing the DDR pathway. Also, because of high G content, the single-stranded overhang forms a G-quadruplex, where each guanine base can act as both hydrogen donor and acceptor to form a G-tetrad. The telomeric G-quadruplexes have been associated with telomere end protection. It also act as a suppressor of recombination and inhibitor of telomerase[18].

The shelterin protein complex

In mammals, the telomeric sequences are bound with a complex of six proteins known as the shelterin complex. Of these, three proteins directly bind to the telomeric sequences; the telomere repeat binding factor 1 (TRF1/*TERF1*) and telomere repeat binding factor 2 (TRF2/*TERF2*) bind to the double-stranded sequence, whereas protection of telomeres (POT1/*POT1*) binds to the single-stranded 3′ overhang. The other three proteins are TERF1-interacting nuclear factor 2 (TIN2/*TINF2*), which associates itself with TRF1 and TRF2; POT1 and TINF2-intearcting protein (TPP1/*ACD*), which forms a heterodimer with POT1, and TERF2-interacting protein (RAP1/*TERF2IP*), which is deployed at telomeres by TRF2 (refs 15, 19). TRF1 was the first telomere binding protein to be identified[20] and negatively regulates telomere length[21]. TRF2 was identified by homology search to TRF1 (ref. 22), and also negatively regulates telomere length[23]. Deletion of both alleles of TRF1 and TRF2 in mice results in lethality to the developing embryo[24,25]. RAP1 does not have telomeric binding domain and localizes itself to the telomere by binding with TRF2 (ref. 26). The probable role of RAP1 is in inhibiting homologous recombination at telomeres[27]. RAP1 deleted mice are shown to survive; however, they are frail with an increased frequency of recombination at the telomere[28]. POT1 recognizes a 10-nucleotide sequence, TTAGGGTTAG, and has two oligosaccharide/oligonucleotide binding (OB) domains. The amino-terminal OB domain recognizes and binds with the initial six nucleotides of the 10-nucleotide sequence and the second OB domain attaches and secures the 3′-carboxy terminal of the single-stranded DNA[29]. The carboxy terminal region binds TPP1 and attaches POT1 to the shelterin complex[30].

Telomerase structure and function

Telomerase enzyme, when biologically active in an organism, is composed of a reverse transcriptase (TERT) telomerase RNA (TERC, in humans hTR) which provides the template for TERT and two accessory protein complexes made up of dyskerin, NHP2, NOP10 and GAR1. The accessory protein complexes further bind to two hairpin stem domains (H/ACA motif) of hTR and to TCAB1 (a WD40 domain protein)[31,32]. Biogenesis of the human telomerase holoenzyme occurs when nascent hTR transcript is co-transcriptionally assembled with accessory protein complex of dyskerin, NHP2, NOP10 and NAF1[33]. All H/ACA RNAs are bound to these accessory protein complexes, including small nucleolar RNAs that are intron-encoded or RNAs like Cajal body (CB) that guide modification of RNA[34]. hTR goes through 5′- and 3′-end maturation after the H/ACA ribonucleoprotein (RNP) assembly. Along with the end maturation there is a switch-over of NAF1 for GAR1, which generates the hTR H/ACA RNP[32]. Further, CB-localization factor, TCAB1, is recruited by the hTR 3′-hairpin CAB-box motif[35]. The final catalytically active telomerase RNP is created after TERT binds with two structurally autonomous domains of hTR[36]. Human telomerase differs from those of the unicellular model organisms, as their assembly is exceedingly chaperoned as H/ACA RNP along with a highly complicated subunit trafficking through various nuclear areas[32].

End replication problem leads to telomere shortening, following every cell division[4,5]. To overcome this, TERT uses an RNA moiety (TERC) as template and extends telomere repeats at the 3′-end of the chromosome[37,38]. However, the enzyme is active only in some types of cells, like adult stem cells, lymphocytes and germ cells and inactive in other cell types, probably to prevent progression of tumour, yet restricting renewal of tissue[39–41]. Also, ageing due to telomere shortening is not prevented in stem cells which have telomerase expression[42].

Alternative lengthening of telomeres

Any mechanism that does not involve telomerase in telomere lengthening is known as alternative lengthening of telomeres (ALT)[43]. Various studies that were conducted on immortalized cell lines and cancers which are negative for the human telomerase, have provided a lot of information on the ALT mechanism.

The basic premise for the ALT mechanism is dependent on homologous recombination. The first step involves the invasion of the single-stranded G-rich overhang on homologous DNA. This homologous DNA could be from sister chromatid or from other chromosomes. However, binding of POT1 to this G-rich overhang prevents binding of other proteins (RAD51) which are essential for carrying out the recombination process. To load RAD51,

first POT1 is removed and replaced with replication protein A (RPA). Further, using TERRA (telomeric repeat containing RNA), heterogeneous ribonucleoprotein A1 (hnRNPA1) and some other mediator proteins, RPA is replaced with RAD51 (ref. 44). The second step, which is elongation of the invaded strand, is carried out by either polymerase δ or ζ, as they are presumed to play a role in homologous recombination[45,46]. In the next step, transitional products of the homologous recombination, like the Holliday junctions are processed. This involves Holliday junction resolution and dissolution, engaging two different protein complexes[47]. The fourth step, which may involve either the extension of the under hang strand or the elongated strand, may be used for semi-conservative replication, but the template is still largely unknown.

Role of telomere and telomerase in cancer

Telomere and telomerase dysfunction in cancer

Telomere and telomerase dysfunction has been a widely acknowledged event in the process of carcinogenesis. In gastric cancer, telomere length is shown to undergo shortening in early-stage cancer and lengthening in advanced cancer, suggesting initiation of tumorigenesis in gastric cancer due to telomere shortening[48]. A meta-analysis of 18 studies in gastric cancer carried out to investigate the association between telomerase activity and clinical outcome of gastric cancer[49], reported the expression of high telomerase activity to be coupled with metastasis of lymph node, invasion depth, distant metastasis, size of tumour and TNM stage. This suggests that telomerase over-expression plays a vital role not only in the crucial initiation, but also supports invasion followed by metastasis of gastric cancer.

In case of lung cancer, patients with shortest telomere showed notably worse overall survival and disease-free survival[50]. Also, in this study, relationship between telomere length and survival outcome was more pronounced in squamous cell carcinoma compared to adenocarcinoma. With regard to telomerase, hTERT expression and overall activity of telomerase were found in 48–95% and 67–85% of lung tumours respectively[51,52]. Further, hTERT expression and overall telomerase activity in non-small cell lung cancer (NSLC) patients were found to be associated with disease-free survival and poor overall survival[52,53].

Telomere shortening has also been reported and is nearly a general finding in pancreatic intraepithelial neoplasia[54]. Telomere shortening was reported to be an early event in intraepithelial papillary mucinous neoplasm of the pancreas (IPMNs) and preceded the activation of telomerase, suggesting that telomere shortening and further activation of telomerase in later stages may be the decisive stage in the development of pancreatic cancer[55]. A meta-analysis involving 19 studies has shown that telomerase activity could act as a valuable biomarker for differential diagnosis of adenocarcinoma of pancreas as well as in benign pancreatic diseases[56].

In gallbladder cancer, Hansel *et al.*[57] reported uniform telomere length in inflamed tissues which had normal epithelium. However, metaplastic lesions, dysplastic epithelium and adenocarcinoma of the gallbladder had shorter telomere lengths. Also, high hTERT expression was seen in low- and high-grade dysplasia and gallbladder cancer[58]. Our recent data also report telomere attrition to be confined only to early-grade tumours, whereas an increase in telomere length could be seen in late-grade cancer[59], probably suggesting telomerase activation at later stages of the disease.

Telomere shortening has also been seen in acute myeloid leukaemia (AML), where telomere length in patients was found to be significantly reduced in comparison to matched controls[60]. In this study, shorter telomeres were seen in AML patients with an aberrant karyotype in comparison to AML patients having normal karyotype, whereas patients having multiple abnormalities in their karyotype had the shortest telomeres. Also, expression of hTERT correlated with chromosomal aberrations along with the detection of splice variants of functional hTERT[60].

Shelterin dysfunction in cancer

Shelterin proteins are known to regulate telomere length by inhibiting the binding of telomerase[61]. TRF1 was reported to show decreased expression in cancers of gastric and breast and also in tumours of astroglial brain[62–64], whereas its expression was found to be increased in hepatic and lung cancers[65,66]. In colorectal cancer patients, tumours that showed over-expression of TRF1 along with shorter telomeres had a better clinical course[67]. TIN2 expression was reported to have increased in hepatic cancer[65], while it was decreased in gastric cancer[62]. Likewise, in another report, POT1 expression was upregulated in later stages and downregulated in early stages of gastric cancer[68]. Also, in breast cancer, POT1 expression was found to be lowered[69]. Both TRF1 and POT1 bind directly to the telomere, whereas TIN2 other than binding to TRF1, also helps in binding the POT1/ACD complex to the remaining shelterin complex proteins[70]. Thus, these three proteins are important in maintaining the telomere structure and integrity.

Increased expressions of TPP1 were shown to shield the telomere from DNA damage and were also resistant to ionizing radiation in human colorectal cancer cells, suggesting the utility of TPP1 as a probable radiotherapy target in colorectal cancer[71]. High level of RAP1 (transcriptional repressor/activator protein) expression in NSLC tissues was found to be associated with a 53%

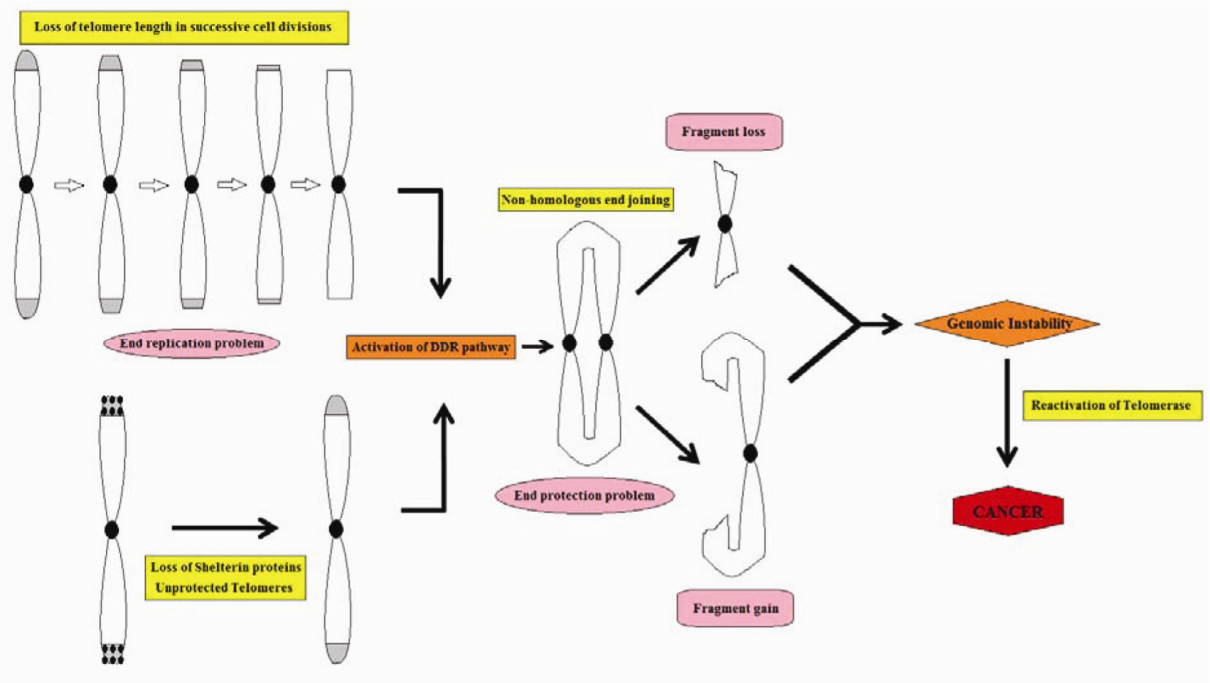

Figure 1. Role of telomere dysfunction in cancer. Loss of telomere length or shelterin proteins results in the uncapping of the telomeres. These uncapped telomeres activate the DNA damage repair (DDR) pathway and causes non-homologous end-joining of the chromosomal arms. Eventually, in the next cell division, breakage–fusion–bridge cycle occurs leading to genomic instability and finally to cancer.

decreased risk of death from cancer. Similarly, higher level of expression of TRF2 (telomere repeat-binding factor 2) correlated with lower tumour grade, implying that TRF2 might play an important role in protecting progression of lung cancer[72]. Our recent report on gallbladder cancer showed significantly decreased mRNA levels of *TERF1*, *POT1* and *TIN2* in inflamed tissues due to gallstone. However, these tissues showed telomere length similar to normal tissues, suggesting that the unloading of these proteins may result in telomere dysfunction during the stone-forming process and might possibly lead to gallbladder cancer[73]. All these observations suggest that telomere-associated proteins are important in managing telomere length, telomere stability and thereby integrity of the genome. Table 1 shows the expression levels of the shelterin proteins in different cancer types[73–83].

Epigenetic regulation of telomeres in cancer

A general characteristic feature seen in most of the cancers is global DNA hypomethylation at the level of chromosomes and specifically, hypomethylation of repetitive sequences; however, the hypomethylation frequencies differ in different cancers. They have been observed to act as an early episode in various cancers, including colon cancer, gastric cancer, breast cancer and recently, in gallbladder cancer[59,83–85]. However, how hypomethylation affects cancer, still remains unanswered. There are a number of reports suggesting that telomeres are regulated by changes in the chromatin structure[86–88]. CpG methylation does not occur at the telomeres because CpG sites are not present there; however, adjacent to the telomere, subtelomeric DNA repeats are profoundly methylated, both in humans and mice[89–91]. These two regions have also been shown to have loss of acetylation and trimethylation marks of histone 4 (ref. 89), which might be responsible for the loose chromatin structure. Recent studies suggest that epigenetic modification at subtelomeric regions are correlated with regulation of telomere length and the condition of the chromatin at these regions is important to control telomere length[89,91,92].

A recent report in human cancer cell lines has demonstrated a negative correlation between subtelomeric methylation and both telomere length and telomere recombination[93]. Many reports have also showed that different subtelomeric regions demonstrate varied methylation patterns, and thus, validate the fact that a particular methylation pattern is not followed at subtelomeric sequences and it also varies in different cancers[94,95]. Also, in tumour cells showing ALT mechanism, hypomethylation of subtelomeric sequences has been reported; however, this was shown not to be necessary for sister chromatid exchange at the telomere[96]. In another study carried out in hepatocarcinoma, subtelomeric methylation pattern showed dynamic changes, but only at some regions, and was related to either long or short telomeres. This implies that subtelomeric methylation and telomere length regulation are closely associated[97]. Lee *et al.*[98] also

reported different methylation patterns at different subtelomeric regions; however, no direct correlation was observed between telomere length and subtelomeric methylation. The conflicting patterns of methylation in subtelomeric regions, thus obscure their function in cancer pathogenesis. Further studies might offer some useful insights into the association of epigenetic modification and regulation of telomere length.

Perspective

The tremendous amount of work that has been carried out in the field of telomere biology has increased our understanding of numerous cellular mechanisms during carcinogenesis, particularly, the different players that are involved in maintaining the telomere integrity. Focus on these players, such as the telomere–shelterin complexes and telomerase interaction, and the pathways they regulate together, hold promise for targeted therapies. Future studies that look promising are the ones which are targeting telomerase for therapeutic purposes in cancer treatment. However, telomere maintenance by ALT mechanism is seen only in certain tumour types and not in others. An understanding as to why this happens could provide great scope to develop anticancer therapeutics. Identification of potential molecular changes in telomeres, such as epigenetic modification at the subtelomeric region and expression levels of the shelterin proteins in different stages of cancer is expected to throw some light in understanding the process of carcinogenesis.

Table 1. Expression levels of telomeric proteins in different cancers

Protein	Cancer type	Expression	Reference
TRF1	Gastric Carcinoma	High	74
	Hepatocarcinoma	High	65, 75
	Lung Adenocarcinoma	High	66
	Adrenal Cortical Cancer	High	76
	Colorectal Cancer	High	67
	Adult T-Cell Leukemia	High	77
	Gastric Cancer	Low	62
	Astroglial brain tumors	Low	64
	Breast tumors	Low	78
	B cell Non-Hodgkin lymphoma	High	79
TRF2	Hepatocarcinoma	High	65, 75
	Lung Adenocarcinoma	High	66
	Adult T-Cell Leukemia	High	77
	Oral Cancer	High	80
	Gastric cancer	Low	62
	Human Astroglial brain tumors	Low	64
	Breast tumors	Low	78
	B cell Non Hodgkin lymphoma	High	79
POT1	Stage III/IV Gastric Cancer	High	68
	Stage I/II Gastric Cancer	Low	
RAP1	Non-Small Cell Lung cancer	High	72
	Hepatocellular carcinoma cell line	Low	81
TIN2	Hepatocarcinoma	High	65, 75
	Gastric cancer	Low	62
	Gastric cancer	High	74
	Malignant hematopoietic cells	Low	82
	Adult T-Cell Leukemia	High	77
	Chronic lymphocytic leukemia	Low	83
TPP1	Colorectal Cancer	High	71
	Chronic lymphocytic leukemia	Low	83

TRF1, Telomere repeat binding factor 1; TRF2, Telomere repeat binding factor 2; POT1, Protection of telomeres; RAP1, TERF2-interacting protein; TIN2, TERF1-interacting nuclear factor 2; TPP1, TINF2-interacting protein.

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