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Telomeres and telomere dynamics: relevance to cancers of the gastrointestinal tract

Nivedita Basu, B.A.¹, Halcyon G. Skinner, Ph.D.², Kristin Litzelman, Ph.D.², Russell Vanderboom, PhD¹, Esha Baichoo, M.D.¹, and Lisa A. Boardman, M.D.¹

¹Division of Gastroenterology and Hepatology Department of Medicine Mayo Clinic 200 First Street SW Rochester, MN 55905 Tel: 507-266-4338; Fax: 507-266-0350;

²Department of Population Health Sciences School of Medicine and Public Health University of Wisconsin Madison, WI 53726 Tel: 608-265-4654

³Department of Biostatistics and Medical Informatics School of Medicine and Public Health University of Wisconsin Madison, WI 53726 Tel: 608-265-4654

Summary

Aberrations in telomere length and telomere maintenance contribute to cancer development. In this article, we review basic principles of telomere length in normal and tumor tissue and the presence of the two main telomere maintenance pathways as they pertain to GI tract cancer. Peripheral blood telomeres are shorter in patients with many types of GI tract cancers. Telomere length in tumor DNA also appears to shorten early in cancer development. Tumor telomere shortening is often accompanied by telomerase activation to protect genetically damaged DNA from normal cell senescence or apoptosis, allowing immortalized but damaged DNA to persist. Alternative lengthening of telomeres (ALT) is another mechanism used by cancer to maintain telomere length in cancer cells. Telomerase and ALT activators and inhibitors may become important chemopreventive or chemotherapeutic agents as our understanding of telomere biology, specific telomere related phenotypes, and its relationship to carcinogenesis increases.

Keywords

Telomeres; telomere maintenance genes; cancer; telomerase; TERT; alternative lengthening of telomeres (ALT); cancer risk

Telomere length and gastrointestinal tract cancers

The gastrointestinal (GI) tract encompasses the luminal organs (esophagus, stomach, small intestine and colon) and the solid organs (pancreas, liver, gallbladder and bile ducts), which connect and deliver to the luminal GI tract exocrine enzymes, bile salts and acids to aid digestion. Four of the ten most common cancers worldwide arise within the GI tract, with colorectal cancer being the third most frequent tumor, stomach cancer the fourth, liver cancer the sixth, and esophageal cancer the eighth worldwide. Though classified broadly as GI tract cancers, these are a diverse group of tumors, arising from organs with many

boardman.lisa@mayo.edu hgs Skinner@wisc.edu.

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different functions, and thus cancers of the GI tract have many distinguishing features. GI tract cancers originate from a variety of different cellular compartments including epithelial derived adenocarcinomas and squamous cell carcinomas, mesenchymal derived GISTs, neural cell derived carcinoids, and other neuroendocrine tumors. For many of the GI tract cancers of epithelial origin, a pre-malignant lesion has been identified that illustrates the steps through which a tumor transforms from non-neoplastic to malignant tissues (see Figure 1). Genetic aberrations in either germline or somatic DNA have been defined for all GI tract cancers, a subset of which may be shared by several different GI tract tumor types and a subset of which are unique to the specific location within the GI tract.

Telomeres are the caps of linear DNA and are made of nucleotides. However, they are not genes, since they do not encode proteins. These repetitive TTAGG caps on the ends of linear chromosomes shorten with successive rounds of DNA replication during sequential cell division [1] due to an inherent inability to replicate a portion of the telomere (i.e., the telomere overhang) during each cell division. In healthy cells, erosion of telomere length eventually leads to regulated cell senescence and apoptosis, serving as a means to clear DNA that may be more prone to mutation or altered expression that could lead to infection, cancer, or other disease (see Box 1). However, in abnormal cells, continued cell division after telomere depletion can lead to end-to-end fusion of chromosomes and chromosomal instability. Telomere shortening accompanies the process of aging [2], bio-environmental factors (Box 2), and psychosocial factors and to viral pathogen infection which may predispose our cells to malignant transformation [3,4].

Telomere length, biological marker of aging and carcinogenesis

Telomere length and telomere shortening has been long hypothesized to be a biological marker of aging at the cellular level and a potential mechanism of carcinogenesis [5,6]. However, there is considerable discussion over what criteria suitably define a biological marker of aging at the level of an organism, and whether a single omnibus marker of aging is likely to exist for humans [7-9]. Studies of the basic biology of telomere erosion and maintenance have established that 1) telomere shortening is a fundamental feature of dividing cells and directly related to the age of the cell lineage, and that 2) telomere crisis in the presence of defective cell-cycle control can lead to chromosomal instability and a malignant phenotype [6]. Because telomere erosion is a process that relates to aging at the cellular and tissue level, and is also implicated in carcinogenesis, it is an attractive candidate for studies that seek to explain the increase in incidence of many cancers with increasing age [10]. Other processes that may contribute to the increase in incidence seen with older age include mitochondrial dysfunction, and the accumulation of DNA damage in key regulatory genes [11].

Telomere length may be measured by Southern Blot [1,12], quantitative PCR [13], flow cytometry with fluorescence in situ hybridization (Flow-FISH) [14], quantitative FISH (Q-FISH) [15,16], and single or Universal single telomere length analysis (STELA) [17,18]. Southern blot and qPCR are the two techniques most commonly employed in epidemiological studies of the association of PBL telomere length with diseases such as cancer or with environmental exposures. Detection of terminal restriction fragments (TRFs) by Southern blot analysis begins with digestion of high quality DNA with restriction endonucleases that target sites throughout the chromosome and cut the DNA into tiny fragments. The select restriction enzymes, however, are unable to bind to telomeric and subtelomeric DNA, and thus leave the telomere and subtelomeres intact. Following digestion, DNA fragments are then separated on an electrophoresis gel. The DNA is next transferred to a membrane and finally the larger sized DNA telomere restriction fragments are identified by a chemiluminescent or radiolabeled probe for the terminal restriction site [1,12]. Southern blot analysis is considered the gold standard for telomere length

measurement because it is reproducible, and its results may be reported as the actual length of the telomere in terminal restriction fragment (TRF) units, allowing comparison of telomere length (actually) TRF between different studies. But Southern blot requires a fairly large amount of DNA (a minimum of 3 µg) and produces results that include the subtelomeric (non-canonical) regions of the telomere, which may overrepresent actual telomere length. Reproducibility may also vary depending on which restriction enzyme is used for the DNA digestion. The most frequently used technique to determine telomere length in cancer risk association studies is quantitative polymerase chain reaction (qPCR). This PCR-based assay uses a set of primers to the telomeric hexamer repeats, thus amplifying the telomere [13]. The average relative telomere length (RTL) for a sample is measured by determining the sample's telomere to single copy gene ratio in comparison to the telomere to single copy gene ratio of a reference DNA sample. qPCR assessment of relative telomere length developed has led to burgeoning in the field of telomere length studies because of its high throughput capabilities and requirement for very small amounts of DNA (e.g., 35 ng DNA) [13]. Relative telomere length qPCR is limited because the telomere length is relative to a standard DNA sample that differs for each laboratory, and therefore study-to-study comparison of the absolute telomere length cannot be made. A recent modification to relative telomere qPCR that utilizes an oligomer standard may solve this issue [19]. Reproducibility in reported studies remains an issue [20], although inter-assay variation (CV) may be improved with good quality control [21]. Inter-assay variability rates are >6% for qPCR, and >2% for Southern blot [20].

Both Flow FISH and Q-FISH utilize fluorescently labeled peptide nucleic acid probes to detect and ultimately measure the canonical telomere length not inclusive of the subtelomeric region-FISH [14-16]. Flow-FISH may be performed on the nuclei of intact cells that are in suspension or have been frozen and combines both in situ hybridization and flow cytometry which allows for assessment of the average telomere length across all chromosomes but of specific cell subsets; e.g., granulocytes and lymphocytes from a peripheral blood sample. Q-FISH requires actively dividing cells or a chromosome spread to assess metaphase and is able to identify telomere length for specific chromosome, rather than solely the average telomere length across all chromosomes. Inter-assay variability for Q-FISH has been reported to be >5% [20].

STELA combines DNA fragment separation via electrophoresis and Southern blotting hybridization followed by PCR amplification and has the ability to precisely measure even very short telomere segments single chromosome end. This technique is also able to measure chromosome specific telomere lengths from lower amounts of DNA than qPCR even at the single cell level but only for the chromosomes for which unique probes have been developed [17]. Universal STELA incorporates the precise measurement and ability to detect very short telomeres as an average measure across all chromosomes [18]. Both of these techniques measure more accurately canonical telomere length than qPCR but are more labor and time-intensive.

Telomeres, psychosocial and lifestyle factors

Psychosocial and lifestyle factors have also been associated with telomere length and telomere attrition. Several previous studies have provided evidence that greater psychological stress is associated with shorter telomeres [22-25]. In addition, childhood adversity and inter-partner violence, both substantial stressors, have been associated with shorter telomeres [26-28] as has low socioeconomic status [29-31] (although these results are somewhat conflicting; see Robertson et. al for a review [32]). While less research has examined other psychosocial and community factors such as neighborhood disorder [33] and lack of social support [34] both have also been correlated with shorter telomere length. Further, those who are married (sometimes considered a proxy for social support) have been

shown to have longer telomeres than unmarried individuals. Discrimination or racism may also contribute to telomere attrition, although this has yet to be explicitly studied.

Several physiologic pathways have been proposed to explain the associations between psychosocial factors and telomere length, including oxidative stress, inflammation, exposure to stress hormones, and allostasis [35-38]. Numerous lifestyle factors have also been associated with telomere length, including nutritional intake [39], physical activity [22,40,41], sleep duration or quality [42,43], smoking [24,44,45], and overall healthy lifestyle [45,46] (reviewed by Lin and colleagues [35]), and such factors may prove to mediate or moderate these associations [47]. Combinations of such factors may prove to be even more influential; for example, multisystem resilience (i.e., healthy emotion regulation, strong social connections, and health behaviors) was shown to moderate the association between depression and telomere length in one study [47].

Given this body of research, there is growing interest in the ability of psychosocial interventions to prevent telomere attrition and, potentially, subsequent cancer onset or progression. Several studies have associated stress reduction, mindful eating, meditation, or other alternative therapies (e.g. QiGong) with increased telomerase activity [25,48-50], telomere length [51], or expression of telomere maintenance genes [52] (see Epel *et al.* [53] for a review). In one longitudinal study, decreases in psychological distress following a psychosocial telephone counseling intervention were associated with increased monocyte telomere length [54]. Other interventions shown to reduce stress or improve coping, such as cognitive behavioral therapy, may also prove successful in preventing telomere attrition. In addition, statins have been shown to inhibit telomere shortening [55,56], suggesting that pharmacological interventions may also be feasible and effective in preventing telomere attrition. Additional translational research with larger, more generalizable populations is needed to develop and test the effect of psychosocial and pharmacological interventions on telomere length and subsequent cancer risk.

Telomeres and viral infection

While the focal point of this review examines gastrointestinal cancers, telomeres and TMM, the relationship between virus infections, viral structure and telomeres is significant. One-fifth of all cancers worldwide are attributed to human tumor viruses. Ten percent of all gastric cancers and seventy percent of all hepatocellular carcinomas are associated with viral infection.

Viruses leverage cellular telomeres during infection, and some viral infections are associated with progression to malignancies. Viruses deploy strategies to disable cellular innate immune responses that would normally lead to programmed cell death and senescence (reviewed by Bellon and Nicot, 2008; Deng *et al.*, 2012 [57,58]). Viruses target telomeres and employ multiple strategies to subvert normal telomerase function and facilitate viral transformation and carcinogenesis. Many viruses share structural homology to the terminal repeats of short nucleotide GC-rich elements of telomeres [59]. Thus, viral terminal repeats facilitate integration and localization into human host telomere repeats [60]. Endogenous telomere maintenance in those cells appears under control of the virus; telomerase is activated, telomere shortening is prevented, and the cells escape programmed senescence or apoptosis.

Viral pathogen members of the Herpesviridae family, including Human herpesvirus 6 (HHV6), and Epstein Barr virus (EBV) contain telomere-like terminal repeats within their genetic code and are associated with for the most part with non-gastrointestinal GI tract cancers. However, ten percent of gastric cancers are associated with EBV, a virus primarily associated with hematopoietic cancers [61].

Herpesvirus family members appear to target the telomere terminal repeat sequence for integration into the host cell genome. This targeted integration of the virus into human genome appears to be a product of homologous recombination, or, in some instances, direct transposition [62]. Viral infections restructure circuits controlling telomere maintenance mechanisms, including transcriptional activation of human telomerase reverse transcriptase (hTERT) [63]. Homologous recombination between telomeres is known to ensure telomere maintenance via an alternative telomere elongation (ALT) mechanism [64]. Kaufer et al. noted that in virally infected cells with recombinant terminal repeat replication, ALT may mediate recombination between host and viral telomeres [65].

Almost three quarters of hepatocellular carcinomas presenting worldwide are attributed to HBV or HCV infection [66]. RNA viral infections associated with hepatitis C virus (HCV) impact telomerase function and dislocation in hepatocellular carcinoma cell line [67]. Hepatitis B virus and HCV are associated with chronic liver disease and malignant transformation [68], and HBV hepatocarcinogenesis is associated with shortened telomeres and chromosomal instability. Tumor cells from HBV infected patients contained shortened telomeres in spite of strong telomerase activity. The HBV transcriptional transactivator oncoprotein HBV stimulates viral gene and cellular transformation of hepatocytes. HBV increases telomerase expression and activity in hepatoma cells [69]. Nonetheless, hepatocellular carcinomas have shortened telomeres.

Telomeres in tissues and blood

Telomeric biomarkers for aging and disease may present both in somatic cells or germline. Associations between tumor cell DNA and bloodline DNA telomere status are the focus of many research programs. Studies of cancer telomeric status probe both tumor tissue, normal somatic tissue, as well as associations of telomere length between organ site tumors and peripheral blood samples (PBL). Previous research has shown that the depletion of constitutional telomere structure end-sequences measured in the germline DNA of peripheral blood white blood cells is associated with an increased risk for some cancers, including head and neck, urinary bladder [70-72], renal [73], lung [74], and esophageal [75] cancers.

Many studies of the association of cancer risk with PBL telomere length utilize DNA from leukocytes, which are comprised of two main classes of white blood cells: granulocytes and lymphocytes. Granulocytes have longer telomere length than lymphocytes [76,77], and unless these distinct WBC types have been specifically separated out prior to DNA extraction, PBL telomere length in these studies typically refers to the telomere length from a mixture of WBC. PBL DNA is often extracted by one of three methods: column method, salting-out of the DNA, or organic extraction with Phenol/Chloroform [78]. However, the column method of DNA extraction has been shown to yield considerably shorter segments of DNA, indicating that the mechanism of column separation and extraction mechanically shears or disrupts telomeric DNA in a manner not truly representing the length of telomeres in blood, tissue or tumor samples. DNA extracted by a column-based method has been associated with significantly shorter PBL telomere lengths as measured by either Southern blot TRF analysis or qPCR than for DNA extracted by a salting-out or organic extraction method. Studies of telomere can be impacted by multiple factors including the sample size, tissue source for DNA, DNA quality, and tissue storage as well as DNA extraction method.

Telomere maintenance mechanisms

Telomere length is not solely determined by attrition resulting from cell division, and telomere maintenance mechanisms (TMM) can overcome and even reverse inherent end-replication shortening. The TERT (5p15.33) and TERC (3q21-q28) genes encode a complex

of proteins known together as telomerase. Reverse transcriptase telomerase (TERT) and telomerase RNA component (TERC) together comprise telomerase function to reconstruct eroded telomere DNA adding TTAGGG repeats to telomere ends. Normally, somatic cells do not activate telomerase to counter telomere shortening, but aberrant activation of telomerase in cancer cells can develop. Critical shortening of telomeres may lead to activation of telomerase in tumor cells and instead of triggering the expected response to terminate these cells, the activation of telomerase bypasses normal cell senescence. Thus, the malignant cell with its cancerous DNA escapes apoptosis. This is a particularly effective rescue mechanism that may contribute to the virulence of cancer. Telomerase function may be up or down-regulated by germline mutations in TERT, and less frequently by TERC. Telomerase may affect somatic tissue cells when activated from dormancy via epigenetic modulation of TERT expression through a variety of transcriptional mediators able to be regulated by exogenous exposures, including oxidative stressors like tobacco exposure that are known to be associated both with telomere length and risk for disease. hTERT expression directly correlates with telomerase activity, and has been suggested as a possible biomarker for the presence of colorectal cancer [79]. The detection of hTERT activity has also been suggested as a surveillance tool for at-risk patients, specifically those suffering from ulcerative colitis, with samples obtained from colonoscopic luminal washings showing high specificity and sensitivity [80,81]. In addition to being a potential marker of disease, telomerase levels have been proposed as a marker for progression and disease survival [82-85].

Alternative lengthening of telomeres (ALT) is another telomere maintenance pathway that protects cells from undergoing senescence and is a homologous recombination based mechanism that uses a DNA template to preserve telomere length [86]. Obligate telomere shortening may be overcome through ALT and this TMM is active in osteosarcoma, glioblastoma multiforme, and breast cancer.

The GI tract cancers, telomeres, and telomere maintenance mechanisms

Both shorter and longer PBL telomere lengths are associated with an increased risk for GI tract cancers. Tumor telomeres also may exhibit an intensified rate of shortening that is greatly accelerated compared to the normal tissue of origin. In cancers for which pre-neoplastic precursors have been identified, somatic telomere length changes compared to that of corresponding normal tissue have been reported (see Table 1).

Esophageal cancers

Esophageal cancer is subdivided according to the cellular component from which it originates. Esophageal squamous cell carcinoma (ESCC) arises from the flat cells lining the upper two-thirds of the esophagus, while esophageal adenocarcinoma (ACA) arises from mucus secreting glandular tissues of the distal esophagus. They are distinct cancers with different etiologies but are often clustered together when describing the incidence of cancers in the esophagus. However, they share several features, including a three times higher risk for esophageal cancer to develop in males and an older age of onset.

Squamous cell carcinoma of the esophagus

ESCC typically arises in the middle esophagus; tobacco and alcohol use are the two main modifiable risk factors for this disease. It is the predominant form of esophageal cancer in the developing world, compared to esophageal ACA which is more common and rising in incidence in developed countries. It is viewed as a more aggressive tumor than esophageal ACA.

Shorter PBL telomere length is associated with an increased risk for esophageal ESCC [87], and polymorphisms in the PBL telomere length related 1p34.2 rs621559 and 14q21 rs398652 SNPs are associated with an increased risk for ESCC [88].

At the tissue level, telomere shortening is detectable in DNA from the precursor lesion of ESCC- carcinoma in situ (CIS) [89]. Telomere lengths in normal epithelial cells that surround CIS are shorter than in normal epithelium from individuals who do not have CIS or ESCC [90], which is evidence that supports telomere attrition as an early initiating event for cancer.

ESCC has increased telomerase activity [91], but activation of telomerase is not related to prognosis in patients with ESCC [92].

A recent meta-analysis reported that 30% of ESCC are associated with human papilloma virus (HPV) infection [93]. Telomerase activation in response to tumor telomere attrition has been reported in HPV induced esophageal cell culture studies. However, in another HPV related tumor type, cervical cancer resulting from the HPV subtype E6 as the initiating factor for cancer exhibits telomerase activation while that associated with HPV subtype E7 utilizes alternative lengthening of telomeres [94]. Though the impact of telomerase inhibitors are not yet studied in ESCC, implications for therapies directed at telomerase inhibition may need to be tailored in patients with ESCC according to the predominant type of TMM of the tumor.

Barrett's esophagus and esophageal adenocarcinoma

Esophageal ACA is cancer of glandular tissues in the distal esophagus in which only 15% of people with the disease survive for five years, regardless of stage of cancer at presentation [95]. Barrett's esophagus (BE) is the precursor lesion for some cases of esophageal ACA and is comprised of metaplastic columnar epithelium in the distal esophagus that replaces the normal squamous epithelium damaged by chronic gastric acid reflux. Rates of transformation from benign BE to cancer occurs in up to 1% of people with known BE [96], leading to the practice of annual surveillance EGD with biopsies of BE and increased surveillance should dysplasia be detected. Ablation therapy and esophagectomy may be pursued in the setting of BE related dysplasia. However, for most individuals, there is no detectable premalignant lesion to follow and perform screening and surveillance to order to increase the chance for early detection, prophylactic ablation, or esophagectomy.

Individuals with BE and with shorter PBL telomeres have been reported to have an increased risk for Barrett's esophageal ACA (BEAC) of 20.6 fold (95% CI, 3.8-111.9) among people who were ever smokers, who did not use NSAIDs and had a low waist-to-hip ratio [75]. Environmental stressors that may increase oxidative damage have been associated with telomere shortening, and the increase in cancer risk itself attributable to telomere shortening appears to be potentiated by the effects of oxidative damage related to tobacco use, no use of anti-inflammatory agents and inflammation related to truncal obesity. The telomere length of BE is shorter than in normal esophagus epithelium and BE has reactivation of telomerase which is not expressed in the normal esophageal epithelium [97].

One study of BEAC targeted inhibition of telomerase activity as a treatment approach. In BEAC cell lines, a selective G-quadruplex intercalating telomerase inhibitor led to apoptosis and cell growth arrest via inhibition of telomerase activation that permitted progressive telomere shortening [97]. This agent demonstrates the potential for treatment of esophageal ACA, and possibly other cancers, since it is directed not at a genetic pathway that may be specific to a particular tumor type, but towards halting a generalized tumor rescue

mechanism of telomerase activation that allows damaged cellular DNA to bypass apoptosis and become immortalized.

Determining drug resistance based upon the TMM driving tumor immortality is another venue for treatment of esophageal ACA and is based on studies of esophageal ACA cancer cell lines showing increased responsiveness to cisplatin in the presence of telomerase activation but resistance to cisplatin in the presence of short tumor telomeres [94].

Gastric adenocarcinoma

Gastric adenocarcinoma (ACA) is an aggressive disease that has a 24% five-year survival, potentially related to the fact that patients present with later stage disease. Risk factors include male sex; African-American or Asian heritage; untreated *H. pylori* infection related inflammation; states that cause achlorhydria; tobacco use; alcohol use; intake of food preserved by pickling, drying, smoking or salting; decreased fresh fruit and vegetable intake; family history of a first degree relative with gastric cancer and other hereditary conditions including E-cadherin mutation related gastric cancer, Lynch syndrome, familial adenomatous polyposis, Peutz-Jeghers syndrome and SMAD4 related juvenile polyposis syndrome [98].

Gastric ACA risk is increased in people who had shorter telomeres (OR 2.04; 95% CI, 1.33-3.13), and this risk is intensified in people who had low risk for gastric cancer including *H. pylori* negative individuals (OR 5.45; 95% CI, 2.10-14.1), non-smokers (OR 3.07; 95% CI, 1.71- 5.51), and individuals with high fruit (OR 2.43; 95% CI, 1.46-4.05) and vegetable intake (OR 2.39; 95% CI, 1.51-3.81), as observed in a Polish population study [98]. Comparable results were found with a similar risk (OR 2.14; 95% CI, 1.52-2.93) though smoking potentiated rather than minimized the risk for gastric cancer in this Chinese Han study population [99].

Several types of GI tract cancers have microsatellite instability (MSI), which is the result of deficient DNA mismatch repair (dMMR). Intact mismatch repair mechanisms maintain genomic stability through correction of small base-pair errors that occur during replication and prevention of homologous recombination. A portion of gastric (8-23%) and colorectal cancer (20%) are MSI high (MSI-H) with dMMR [100-103], but the majority of these cancers are microsatellite stable (MSS) and have proficient mismatch repair (pMMR) [104]. Gastric cancers with dMMR utilize alternative lengthening of telomeres, although concomitant evidence of telomerase activation as a method of telomere elongation is still present in 48% of MSI-H gastric cancer. Tumor telomere lengths in MSS compared to MSI-H cancer are not significantly different [105].

Precursors of gastric cancer include chronic gastric atrophy, intestinal metaplasia, and dysplasia, but the picture of the direct stepwise progression is at a lower resolution. In gastric cancer not characterized by its DNA MMR status, increasing chromosomal instability, inactivation of p53 tumor suppression, and increasing tumor telomere shortening has been reported [106]. Another evaluation of gastric tumors reported that telomere length was shortest in early stage cancers and then lengthened with increasing stage [107]. In addition, telomere length was increased in the antral mucosa of patients successfully treated for *H. pylori* infection [108]. Up to 40% of gastric cancers may utilize ALT, which relies on homologous recombination to elongate telomere ends that far exceed telomere lengthening by telomerase [109].

Pancreatic intraepithelial neoplasia and pancreatic adenocarcinoma

Ductal adenocarcinoma (ACA) of the pancreas is a virulent tumor from which only 4% of individuals are alive five years after diagnosis. Lack of effective strategies for early detection may contribute to this abysmal survival rate. Tobacco use, alcohol use, decreased fruit and vegetable intake, and consumption of processed, nitrite fixed meats are associated with pancreatic ACA.

Short and extremely long PBL telomeres are associated with an increased risk for pancreatic ACA [110], and a prospective study of PBL telomere length confirmed an association of longer PBL telomere length and risk for pancreatic adenocarcinoma [111]. Germline mutations in TERT are associated with increased risk for pancreatic ACA [112].

Pancreatic ACA develops through a series of steps from normal pancreatic ductal epithelium to pancreatic intraepithelial neoplasia (PanIN) to frank malignancy (see Figure 1). PanIN-1A is histologically classified as flat without dysplasia, PanIN-1B as papillary without dysplasia, while PanIN-2 is papillary with dysplasia, and PanIN-3 is carcinoma in situ. Telomeres are shorter in all four grades of PanIN relative to that of normal pancreatic epithelial cell DNA, but the telomere length is not significantly different between PanIN-1A from that of PanIN-3 [113]. The shortest telomere length is found in pancreatic ACA [114].

Intraductal papillary mucinous neoplasms (IPMN) are typically slow-growing, mucus-producing intraductal tumors that may progress to invasive pancreatic cancer. In IPMNs that have not progressed to cancer, similar to the PanIN staging, the steps to carcinogenesis include IPMN with low to moderate to high grade dysplasia to IPMN with cancer. The association of IPMN with cancer or location of the tumor in the main pancreatic duct, rather than side branch involvement, correlates more with prognosis than does the degree of dysplasia present in the IPMN.

Tumor telomere shortening in intraductal papillary mucinous neoplasm (IPMN) of the pancreas is noted in earliest stages of malignant transformation from adenoma to borderline IPMN and telomere shortening in premalignant IPMN precedes activation of telomerase, which is not engaged until progression to carcinoma in situ and persists in invasive IPMN [115].

Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is a primary liver cancer that is thought to arise from hepatic stem cells. It typically develops in the setting of underlying chronic liver inflammation and cirrhosis which may result from excess alcohol exposure, Hepatitis B or C infection, nonalcoholic fatty liver disease (NAFLD), hemochromatosis, and aflatoxin exposure. Hepatitis B related HCC may develop in the absence of cirrhosis.

HCC is the third leading cause of cancer death worldwide. The incidence is highest in developing countries in which Hepatitis B and C infection are endemic, but is increasing in incidence in the U.S., where the main causative conditions include alcohol related cirrhosis, hemochromatosis, and the often obesity related NAFLD, though both Hepatitis B and C contribute to the U.S. burden of HCC. As is the case with many other GI tract cancers, early detection strategies are not clearly defined and may be limited, particularly if medical resources are limited. Liver nodules and cirrhosis may prompt HCC screening through imaging with ultrasound or magnetic resonance imaging. A portion of HCC will be predated dysplastic nodules that may have low or high grade dysplasia in the setting of underlying cirrhosis. Unlike colorectal cancer, there is no premalignant lesion, e.g., a polyp that can be removed in order to prevent progression to cancer.

HCC risk in the setting of Hepatitis B infection is increased in individuals with longer PBL telomeres [116], but for HCC in general, whether shorter and/or longer PBL telomeres might increase the risk for HCC remains controversial and largely unevaluated for the many other non-Hepatitis B related causes of HCC.

In general, tissue telomere length progressively shortens through the steps of carcinogenesis, from cirrhotic to low grade dysplastic nodules to high grade dysplastic nodules and HCC [68]. Subtelomeric methylation may prompt chromatin restructuring and remodeling that may influence the telomere length variation associated with hepatocarcinogenesis [117].

Longer telomere length measured in circulating serum DNA has been associated with an increased risk of HCC in patients with Hepatitis B, and this association is the strongest in patients with non-cirrhotic Hepatitis B related HCC, indicating the possibility that serum telomere length may be useful for risk stratification in individuals with Hepatitis B [118]. In patients with HCC, longer PBL [119] and longer tumor [120] telomere length have been associated with a poorer prognosis and shorter overall survival.

Biliary tract cancer

Biliary tract cancer is the second most common primary hepatobiliary neoplasm after hepatocellular carcinoma. The majority of primary biliary tract cancers are carcinomas or cholangiocarcinomas arising from intrahepatic bile ducts, extrahepatic bile ducts, or gallbladder epithelium [121]. Precursor lesions for invasive biliary tract carcinoma include dysplasia of the epithelium, i.e. flat *in situ* carcinoma and metaplastic lesions for gallbladder carcinoma [122].

Cholangiocarcinoma has an extremely poor survival rate, with only 32% of affected individuals with stage 1 disease surviving past five years and less than 10% with more advanced stages. Risk factors include Native and Hispanic American heritage, cholelithiasis [123], obesity and chronic infections of the gallbladder for gallbladder carcinoma [124], primary sclerosing cholangitis and choledochal cysts for carcinoma of the extrahepatic bile ducts [123], and infection with the parasites *Opisthorchis viverrini* [125] or *Clonorchis sinensis* [126].

Studies evaluating the relationship between telomere length and telomerase activity in cholangiocarcinoma are limited. Hansel, *et al.* described normal telomere lengths in inflamed but normal gallbladder epithelium, while shorter telomere lengths were observed in metaplastic lesions of the gallbladder, dysplastic epithelium of the gallbladder, and infiltrating adenocarcinoma of the gallbladder, intrahepatic, and extrahepatic bile ducts [123]. These findings demonstrated that telomere shortening occurs at an early stage of biliary tract cancer development. Moreover, increased TERT activity was found in tissue samples from patients with cholangiocarcinoma [127].

Small intestinal adenocarcinoma

Adenocarcinomas of the small intestine predominantly arise in the duodenum and jejunum. Small bowel neoplasms are extremely rare, comprising only 3% of all GI tract malignancies [128]. Most patients are diagnosed at advanced stages of disease due to vague symptomatology [129]. It is important to note that individuals with small intestinal adenocarcinoma have a higher risk of acquiring secondary malignancies of the colon, rectum, ampulla of Vater, endometrium, and ovary [130]. Conversely, colorectal cancer patients have an increased risk of developing small bowel adenocarcinoma [131], indicating that similar underlying environmental and genetic factors may exist for tumor development

in these locations. For example, Warth *et al.* showed that most small bowel adenocarcinomas display microsatellite and/or chromosomal instability [132].

Heritable conditions such as familial adenomatous polyposis [133], Lynch syndrome [134], and Peutz-Jeghers syndrome [135] have been proven to increase the risk for both small and large bowel adenocarcinomas. Other known risk factors for small intestinal adenocarcinoma include Crohn's disease [136], celiac disease [137], and dietary intake of alcohol, refined sugar [70], red meat, and smoked foods [138].

While research on telomere length in small intestinal adenocarcinoma is limited, a study showed upregulated telomerase activity in 79% of periampullary cancer tissue samples tested, including 100% of duodenal carcinoma tissue samples. Moreover, tumors larger than two centimeters and high-grade tumors exhibited significantly higher telomerase activity than smaller, well-differentiated tumors [139]. This finding suggests that telomerase activity may be a useful marker for prognosis in periampullary malignancy.

Adenomatous polyps and colorectal adenocarcinoma

Colorectal cancer (CRC) is an epithelial cell cancer that is the second leading cause of cancer death in the U.S. Median age of disease onset is 65 years old. Risk factors include tobacco use, sedentary lifestyle, obesity, type 2 diabetes mellitus, family history of CRC; and longstanding history of inflammatory bowel diseases including chronic ulcerative colitis (CUC), Crohn's disease, or other hereditary CRC syndromes (Lynch syndrome or familial adenomatous polyposis). CRC can arise at any location in the colon or rectum, and tumor site determines surgical, chemotherapy, and/or radiotherapy approaches. CRC site is classified as right-sided or proximal colon (cecum, ascending and transverse colon); left-sided or distal colon (descending, sigmoid and rectosigmoid colon); and rectum. Five-year survival for individuals with Stage I disease is 95%; Stage II between 75 to 90%; Stage III 50% and Stage IV disease less than 5%, which can still be curable in some cases. It is largely a preventable cancer given the extended pre-neoplastic phase of the adenomatous polyp prior to progression to cancer. The molecular genetic pathways delineating the stepwise progression from normal colonic epithelium to aberrant crypt foci to adenomatous polyp or sessile serrated adenoma (SSA), from low to high grade dysplasia to frank cancer categorizes CRC by at least two distinct methods related to the microsatellite instability status of the tumor. Germline DNA MMR defects account for approximately 1 to 3% of all CRC in hereditary Lynch syndrome. Depending on the age that CRC develops, up to 20% of sporadic CRC will have defective DNA MMR due to inactivation of the DNA MMR gene MLH1 by the epigenetic phenomenon of hypermethylation of the MLH1 promoter, and patients with MLH1 related CRC will have a better prognosis than those with microsatellite stable (MSS) CRC. The precursor lesion for this is the sessile serrated adenoma.

Adenomatous polyps, including sessile serrated adenoma, are detected in 30 to 50% of individuals at colonoscopy. A portion of these polyps are termed advanced adenoma, which is an important classification given the increased risk that an advanced adenoma will recur and be more likely to progress to malignancy. An adenoma is considered advanced if it has at least one of the features of being greater than or equal to one centimeter in size, has high grade dysplasia, and/or contains villous histology. Advanced adenoma require more frequent colonoscopic surveillance than polyps that are not advanced.

Riegert-Johnson *et al.* reported that individuals with advanced adenoma had significantly shorter PBL telomere length than age and gender matched individuals without polyps, proposing telomere length as a possible biomarker for advanced polyps [140]. Studies of PBL telomere length and CRC risk have yielded variable results (see Box 3). Shorter PBL [141] telomeres or longer PBL telomeres have been found to be associated with an increased

risk for CRC, while several studies have found no association between PBL telomere length and CRC risk [142,143]. Jones *et al.* reported that individuals with a TERC-associated SNP (telomerase RNA component) were found to be at higher risk for CRC and have longer PBL telomeres than controls [144].

Studies of telomere length in CRC tissue have yielded inconsistent results. Several investigators determined that early stage tumors had shorter telomeres than later stage disease [83,145], but this was not confirmed by either Garcia-Aranda *et al.* [82] or Raynaud *et al.* [146]. Higher grade (i.e. poorly differentiated) CRC or tumors located in the right colon were more likely to have shortened tumor telomeres than tumors that were well or moderately differentiated or located in the left colon or rectum [82]. Telomere attrition has been reported as an early event that peaks at the transition between high grade dysplasia to invasive cancer [147] but then plateaus once invasive cancer has been established.

Telomere length in healthy GI tract epithelial tissue may be at least partially under the control of the individual and modifiable by following good health behaviors. In obese men who experienced significant weight and body fat loss following calorie restriction, telomere length in their rectal epithelium became longer. The authors speculated that elongation of telomeres in the healthy rectal epithelium might decrease the risk of cancer by preventing critical shortening of telomeres that leads to genomic instability and early initiating events of carcinogenesis [147]. The paradigm of somatic telomere attrition in non-cancerous epithelium being the nidus for genetic instability and then cancer has been shown by O'Sullivan *et al.* in CUC related CRC [148]. Telomere attrition may be even more pronounced in inflamed CUC affected epithelium from an individual with CUC-related CRC [148,149].

Numerous studies have established an association between telomerase activity and progression of adenoma to CRC. Engelhardt *et al.* and Boldrini *et al.* observed telomerase positivity in dysplastic polyps and adenocarcinoma samples, with higher levels in cancerous tissues compared to dysplastic ones [145,150]. It has been theorized that multiple genetic alterations occur in a stepwise fashion, which leads to cell immortality and expression of telomerase in more advanced adenomas. Boldrini *et al.* also found that higher telomerase activity was associated with late-stage CRC and metastasis [150], supporting the notion that telomerase activity contributes to disease progression. Multiple findings prove the relation of telomerase activity to Dukes' criteria; that is, individuals at stage A and B exhibited a lower percentage of positivity than those at stages C and D [151]. Moreover, Malaska *et al.* and Okayasu *et al.* demonstrated a link between positive telomerase activity and lymph node metastasis [152,153]. A multivariate analysis by Sanz-Casla *et al.* examined the impact of telomerase activity in prognosis in 103 CRC patients undergoing surgery, which showed that by adjusting for tumor stage, telomerase activity could be utilized to predict the risk of death or recurrence of CRC [154]. Thus, quantification of telomerase activity may be a useful method of evaluating prognosis in patients with CRC [155].

A recent report by Bertorelle *et al.* found that a higher level of hTERT is an independent prognostic marker of shorter overall survival, and its negative prognostic value is independent of pathological stage. The authors also observed that in Stage II patients, a high hTERT level identified individuals at greater risk of disease recurrence and death [85]. Notably, no relationship between MSI status and hTERT levels was observed.

Anal intraepithelial neoplasia and anal squamous cell carcinoma

Anal squamous cell carcinoma represents less than 2.4% of all GI tract malignancies, with about 880 deaths per year in the U.S. [128]. Anal intraepithelial neoplasia (AIN) refers to dysplastic changes in the squamous epithelium of the anus and is thought to be a precursor to squamous cell carcinoma (SCC) [156]. High grade AIN (HGAIN) has been proven to be

more prevalent in at-risk groups, including HIV positive men who have sex with men and renal transplant patients [157]. Data for treatment options for AIN are relatively weak; current strategies including imiquimod cream administration or surgical ablation are dependent on provider preference and experience [156].

The most significant risk factor for anal SCC is HPV infection, associated with 65% to 89% of all anal SCCs and implicated as a cause of the disease [158,159]. Risk factors associated with HPV infection include number of lifetime sexual partners, sexual practices, female sex, and underlying HIV infection [160].

Research describing a clear relationship between telomere length, TMM and anal SCC still needs to be conducted. One such study analyzed clinical and biomarker data in thirty patients with anal carcinoma who had chemoradiation. Notably hTERT expression was not significantly associated with these samples [161].

Gastrointestinal stromal tumors

Gastrointestinal stromal tumors (GISTs) are rare malignancies of the GI tract that arise from the autonomic nervous system's Interstitial Cells of Cajal, which help regulate normal gastric and intestinal peristalsis. GISTs are found in the stomach in nearly 50% of cases, but can occur throughout the luminal GI tract. GIST tissues assessed for hTERT mRNA expression do not appear to activate telomerase [127].

Carcinoids and other neuroendocrine tumors

For carcinoids and other neuroendocrine tumors, few studies of tumor telomere length and/or TMM have been reported. Neither telomere length in blood, tumor DNA nor telomere mechanisms have been studied in GI tract carcinoid. In lung carcinoid, however, tumors did not show evidence of telomerase activation or ALT [162]. Pancreatic endocrine tumors (PETs) (acinar cell and mixed acinar-endocrine cell carcinoma) that had activation of telomerase have been reported to have a worse prognosis, though the majority of PETs including insulinomas, glucagonomas, gastrinomas, and VIPomas do not express telomerase [163].

Expert Commentary and Five-year View

Individualized medicine is the new paradigm for medical care. For the person with cancer, profiles of germline and tumor genetic, methylation, and transcriptional events are available today to guide the surgical, chemotherapeutic and surveillance choices offered.

Recommendations for preventive care and predictive genetic testing may be extended to at-risk family members based on these germline and tumor profiles. Molecular classification of a tumor's telomere length and TMM adds another facet to person centered healthcare, which is already under study in on-going clinical research trials of adjuvant cancer treatment with a telomerase inhibitor.

PBL telomere length measured at multiple time points to determine the rate of change of telomere length and/or activation of telomerase or ALT during an individual's lifetime may become a part of preventive health strategies to determine risk for and detect cancer and other diseases.

Future use of blood testing of telomere length as a marker for GI tract cancer is dependent on the development of clear-cut ranges of normal telomere length in healthy people for whom the many epidemiological and clinical features that may influence telomere length have been controlled. Utilization of the modified qPCR for measurement of absolute telomere length may facilitate development of large scale reference cohorts akin to Centre

Etude Polymorphism Human (CEPH) reference populations necessary for genotyping to allow comparison of study results originating from different laboratories.

However, telomere length measured in peripheral blood alone may not provide the complete telomere profile needed for the development and implementation of early detection, risk stratification, or prognostication for GI tract cancers in patients and may require the use of multiple telomere related features, including the peripheral blood and tumor telomere length and the genetic and epigenetic aberrations of the tumor's TMM. GI tract cancer telomere length and TMM may be routinely assessed in order to determine risk for cancer recurrence and guide chemotherapy and radiation therapy. Interval surveillance of telomere length and TMM profiles in patients with GI tract cancer may be used to determine responsiveness to treatment and to identify cancer recurrences.

Clarifying the interaction of telomere dynamics with bacterial and viral infections that are associated with an increased cancer risk will be important for management of several GI tract cancers that are associated with Hepatitis B and C, HPV, HIV and *H. pylori* infections.

Additionally, telomerase and ALT inhibitors will be more widely studied and chemotherapeutic treatment plans will also be directed based on the tumor's telomere and TMM used by the tumor. Telomerase activators and possibly ALT activators will become more affordable and more widely used as a preventive therapy to protect against aging related diseases.

Box 1

Telomere Length in the Body and Gastrointestinal Tract

Though for the most part all of the tissues in a person start off at birth with the same telomere length, telomere shortening occurs in parallel in these different tissues and is mainly dependent on rates of cell division. Since cell divisions differ between tissue types, telomere length will not be the same for all cell types in the body even in health. For example, humans begin life with roughly 13,000 base pairs of telomere in their lymphocytes, and as young adults, approximately 33 base pairs of the telomeres are lost annually. This annual loss intensifies to roughly 61 base pairs of telomere after 60 years of age [24].

Human normal colonic mucosa has an annual attrition rate of 44 base pairs given that normal colonic mucosa regenerates every 5 days; roughly 50 cell divisions occur each year [74,75]. Thus, for at least the first 50 years of life, telomeres in the normal colonic epithelium shorten more rapidly than in the lymphocyte DNA. This loss reaches its nadir for individuals in their 7th decade, while lymphocyte telomere length continues to decline linearly with aging [148].

Box 2

Telomeres and Environmental Exposures

Telomere length at birth is not the only factor affecting lifetime telomere lengths in constitutional DNA or any tissues. For example, though males and females at birth have similar average telomere lengths in their lymphocyte DNA, males appear to have more rapid telomere attrition manifested by shorter telomere length in age-matched healthy males than in females. Menopause may affect telomere length in association with other bio-environmental factors, including insulin resistance and inflammation [164].

Telomeres also appear to be susceptible to environmental stresses. Oxidative stress has

been one of the most carefully studied associations to be correlated with the accelerated shortening of telomeres [36]. Diabetes mellitus type I [165], insulin resistance [166], and smoking [44] are some of the biological or environmental insults that have been associated with acceleration of the mitotic time clock. Exposure to tobacco smoke appears to be inversely related to telomere length; that is, greater tobacco exposure correlates with shorter telomeres [167]. Firsthand tobacco exposure was associated with shorter telomeres in the normal colon epithelium of smokers compared to non-smokers [168]. Chronic inflammation may also accelerate telomere shortening [36,169].

Box 3

Telomeres and Colorectal Cancer

In 75% of colorectal cancer (CRC) cases, the telomere length of the corresponding cancer has been reported to be 3-5 kbp shorter than the telomeres of the adjacent normal colon epithelium, far outpacing the natural rate of telomere attrition expected in healthy tissues [145,170]. This discrepancy between the degree of change in the telomere length in adjacent normal epithelium and the CRC could be interpreted as an accelerated cell turnover equivalent to 1000 cell divisions associated with the transformation ongoing from normal epithelium to adenoma to CRC [171]. But the increased rate of telomere attrition in the cancer does not create a field defect, and the normal epithelium adjacent to adenomas or CRC does not show acceleration in telomere attrition equal to that in the cancer [148]. This may suggest that the rapid cell division ongoing in the cancer does not influence or accelerate the rate of telomere attrition in other noncancerous cells.

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201. Statistics for Specific Cancers. <http://www.cancer.gov/statistics/find#stat>
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Key citations of considerable interest

The following references are considered of particular interest within the context of our review of Telomeres, telomere maintenance mechanisms and gastrointestinal tract cancer.

We have included a line of explanation as to the significance of these published research articles in the development of our review.

1. **Ma H, Zhou Z, Wei S, Liu Z, Pooley KA, Dunning AM, et al. Shortened telomere length is associated with increased risk of cancer: a meta-analysis. PLoS One. 2011; 6(6):e20466. [PubMed: 21695195]
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6. *Aviv A, Hunt SC, Lin J, Cao X, Kimura M, Blackburn E. Impartial comparative analysis of measurement of leukocyte telomere length/DNA content by Southern blots and qPCR. Nucleic Acids Res. 2011; 39:e134. [PubMed: 21824912]
185. Head to head comparison of the specificity and reliability of the two most commonly used methodologies in cancer and telomere length association studies.

Key issues

- Four of the ten most common cancers worldwide arise within the GI tract
- Telomeres are the caps of linear DNA that shorten with successive rounds of DNA replication, eventually leading to regulated cell senescence and apoptosis
- Depletion of constitutional telomere structure end-sequences is associated with an increased risk for some cancers
- Critical shortening of telomeres may lead to activation of telomerase in tumor cells, thus evading apoptosis and becoming more virulent
- Variations in telomere length in both tumor and peripheral blood DNA have been studied in almost all cancers within the GI tract
- Shorter telomere length in peripheral blood DNA is associated with an increased risk for many GI tract cancers
- Telomere length attrition starts in pre-malignant cells in many GI tract cancers including Barrett's associated esophageal adenocarcinoma, and pancreatic, colorectal and hepatocellular cancers
- Ongoing study of telomere length and maintenance mechanisms may lead to the development of targeted chemotherapeutics

Figure 1. Models of carcinogenesis for various gastrointestinal tract cancers with proposed corresponding telomere length and telomerase activity. (PanIN = pancreatic intraepithelial neoplasia)

Table 1

Telomere length and maintenance mechanisms in gastrointestinal tract cancers

	Tissue of origin	Premalignant Lesion	PBL telomere length	Tumor telomere length*	Activation of telomerase in tumor	Annual new cases; deaths in U.S.**
Esophageal SCC	Epithelial	Squamous dysplasia	Shorter	Shorter	Yes	7,196; N/A
Esophageal ACA	Epithelial	Barrett's esophagus	Shorter	Shorter	Yes	10,794; N/A
Gastric ACA	Epithelial	Atrophic gastritis, intestinal metaplasia, dysplasia	Shorter	Shorter (early stages) Longer (later stages)	Yes (pMMR, dMMR) ALT (dMMR)	21,600; 10,990
Pancreatic ACA + IPMN	Epithelial	PanIN	Shorter	Shorter	Yes	45,220; 38,460
Hepatocellular carcinoma	Epithelial	High grade dysplastic nodules	Longer	Shorter	Yes	30,640; 21,670
Small intestinal ACA	Epithelial	Focal dysplasia (e.g. adenoma)	Not studied	Not studied	Yes	8,810; 1,170
Colorectal ACA	Epithelial	Adenomatous polyp	Shorter and longer	Shorter	Yes	142,820; 50,830
Anal SCC	Epithelial	AIN	Not studied	Not studied	Not studied	7,060; 880
GIST	Mesenchymal	None reported	Not studied	Not studied	No	4,000; N/A
Carcinoid	Neuroendocrine	None reported	Not studied	Not studied	Not studied	12,000; N/A
NET	Neuroendocrine	None reported	Not studied	Not studied	Yes (acinar, mixed acinar-endocrine cell)	8,000; N/A

SCC = squamous cell carcinoma; ACA = adenocarcinoma; IPMN = intraductal papillary mucinous neoplasm; GIST = gastrointestinal stromal tumor; NET = neuroendocrine tumor PBL = peripheral blood leukocyte; pMMR = proficient mismatch repair; dMMR = deficient mismatch repair; ALT = alternate lengthening of telomeres; PanIN = pancreatic intraepithelial neoplasia; AIN = anal intraepithelial neoplasia

* As compared to normal epithelium

** Data from [201-202]