An overview of the role of telomeres and telomerase in pre-neoplastic lesions (Review)

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Abstract. Telomeres are tandem repeats of DNA sequences protecting the end of linear chromosomes. Replicative senescence due to telomere attrition is considered a tumor-preventing mechanism in differentiated somatic cells. However, telomere shortening is associated with genome instability and several disease entities. During carcinogenesis, the development of a telomere maintenance mechanism, predominately through the activation of the telomerase enzyme, represents a hallmark of cancer, since it enables cancer cells to avert senescence and divide indefinitely. Although research of the involvement of telomeres and telomerase in various malignant neoplasms has gained a large amount of interest, the timing and relevance of their role in pre-neoplastic lesions remain to be determined. The present narrative review aims to summarize the evidence regarding the role of telomeres and telomerase in pre-neoplasia across different types of tissues.

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1. Introduction

Mammalian telomeres are repetitive sequences of TTAGGGs at the ends of linear chromosomes. They are associated with a set of proteins, forming the shelterin complex, which protects the chromosome ends from generating DNA repair responses. Telomere length (TL) is gradually shortened after each cell division due to the incomplete replication of the lagging strand from the DNA polymerase. When telomeres reach a critical length, the cells are introduced into a permanent growth arrest signaling process (replicative senescence) (1). Telomere attrition and senescence have traditionally been considered a tumor suppressor pathway, preventing somatic cells from indefinite replication.

Telomerase is an enzyme that adds nucleotides at the ends of the chromosomes and counteracts their shortening. It consists of two main components: Human telomerase reverse transcriptase (hTERT), which is the catalytic subunit and also the rate-limiting component of the protein's expression, and the human telomerase RNA component (hTERC, also known as hTR), which serves as a template for telomere replication. Human telomerase is expressed during embryonic development; however, it is later silenced in the majority of somatic cells upon differentiation and its expression is restricted to germline and progenitor cells (2).

Telomeres are critical contributors to genomic stability and shorter telomeres have been associated with various diseases, mostly involving premature aging phenotypes (1). Of note, shorter telomeres have been observed in cancerous compared to healthy tissues (3). On the other hand, the activation of a telomere maintenance mechanism represents a hallmark of cancer, either through the activation of telomerase or, less frequently, through alternative recombination-based mechanisms (alternative lengthening of telomeres) (4). Telomerase activation mechanisms may involve *hTERT* promoter mutations, *hTERT* gene rearrangements, DNA copy amplifications, or epigenetic alterations (3).

Therefore, it has been suggested that shorter telomeres may be associated with genomic instability and the development of pre-neoplastic lesions; on the other hand, there is a critical point during oncogenesis when telomerase is activated, enabling cancer cells to maintain replicative immortality (5). Although this general concept has been proposed, a critical

gap remains regarding the direct evidence and the timing of telomere dysfunction in human pre-neoplastic lesions and solid tumors. Furthermore, precursors and preinvasive lesions represent heterogeneous entities with significant variations among each human tissue. A better understanding of the natural history and molecular characteristics of pre-invasive lesions will aid in the resolution of diagnostic, prognostic and therapeutic challenges associated with them and their corresponding invasive neoplasms. To this end, the aim of the present narrative review was to summarize and critically discuss the evidence regarding the role of telomeres and telomerase across pre-neoplastic lesions.

2. *hTERT* gene and telomerase re-activation

The *hTERT* gene is located on chromosome 5p15.33. It is ~40 kb in length and is composed of 16 exons and 15 introns (6). Its promoter region is the most critical regulatory element of telomerase expression, and is located 330 bp upstream of the translational start site and 37 bp of exon 2 (6). The functional part for the transcriptional activation of *hTERT* in cancer cells is however, located at the 181-bp fragment upstream of the transcriptional start site. The *hTERT* promoter is a 5' regulatory region, abundant with CpG nucleotides and specificity protein 1 (Sp1) sites, and allows binding with either negative or positive gene regulators (7). Negative transcription factors include Mad1, p53, retinoblastoma (Rb) and E2F, while the positive ones include c-myc, Sp1, the human papillomavirus virus (HPV)16 protein E and steroid hormone receptors (6).

As aforementioned, the main telomere maintenance mechanism in cancer cells is the reactivation of telomerase due to *hTERT* promoter mutations, gene rearrangements, DNA copy amplifications or epigenetic alterations (8) (Fig. 1).

The most frequent *hTERT* promoter mutations are found in the -124 (C228T) and -146 (C250T), which are C>T transitions and can rarely co-exist; the exact location on the chromosome is chr5, 1,295,228 and chr5, 1,295,250, respectively (8,9). Both mutations upregulate *hTERT* expression by elongating the promoter by 11 bases 5'-CCCCTTCCGGG-3', that include the binding section GGAA for E twenty-six (ETS) transcriptional regulators in the complementary strand. The overexpression of *hTERT* is induced possibly due to the GA-binding protein alpha chain ETS factor, which is the only one to form multimeric complexes when driving gene expression (7-9). Other rare genetic events leading to *hTERT* upregulation include gene rearrangements and copy number amplifications (8). More specifically, a variety of structurally heterogeneous rearrangements of the *hTERT* gene have been reported in high-risk neuroblastomas, which all induce the massive transcriptional upregulation of the gene (10). In a large genetic study on several cancer types, *hTERT* was shown to be amplified in ~4% of the cases, particularly in ovarian, lung (predominantly in adenocarcinomas), esophageal and adrenocortical carcinomas (3).

Additionally, epigenetic alterations, namely DNA methylation, histone modifications and non-coding RNAs all play roles in the regulation of *hTERT* expression in neoplasia (11). As regards DNA methylation, it is known that the *hTERT* promoter region includes several GC motifs where methylation may take place and affect gene expression (12). While the promoter is largely hypomethylated in somatic cells, it is found hypermethylated or partially methylated in numerous cancer cells (13). From a mechanistic point of view, the hypermethylation of the *hTERT* promoter reduces the ability of the CCCTC-binding factor, that functions as a transcriptional repressor for binding the CCCTC binding region, thus preventing the inhibition of *hTERT* expression (14). Moreover, histone modifications in the *hTERT* promoter may lead to the upregulation of the gene, such as the H3K4me3 mark which is significantly enriched in cancer cells (12). Finally, several non-coding RNAs interact with *hTERT* by binding to recognition sites, such as the 3' untranslated regions or the open reading frames and regulate its activity in cancer cells (11).

3. Telomeres and telomerase across pre-neoplastic lesions

Esophagus. A gradually increased telomerase activity, assessed using the microdissection telomerase repeated amplification protocol (TRAP) and the measurement of mRNA *hTERT* expression, has been detected in the normal esophageal epithelium, dysplastic tissue carcinoma *in situ* (CIS) and esophageal squamous cell carcinoma (SCC) in two studies with clinical samples (15,16). There was a statistically significant difference between normal tissue and pre-neoplastic lesions ($P<0.01$), whereas no marked difference was found between pre-neoplasia and SCC ($P>0.05$) (15). Moreover, two studies investigated telomerase activity in iodine-non-reactive esophageal tissues. Those lesions, which remain unstained with Lugol's iodine staining, were related to inflammation, dysplasia and cancer development (17,18). By comparing telomerase activity between Lugol-stained and unstained epithelia using TRAP assay, it was concluded that the Lugol-unstained lesions presented a higher mean telomerase activity compared to the stained ones from the same patient. The unstained lesions included esophagitis, mild and severe dysplasia, and intramucosal and advanced SCC. Additionally, in the same study, the mRNA expression of *hTERT* was parallel to the increase of the atypia and malignant transformation (18).

Barret's esophagus (BE) is a pre-cancerous condition associated with esophageal adenocarcinoma (EAC). In a previous study, the methylation status of the promoters of the genes, *hTERT*, adenomatous polyposis coli *(APC)*, TIMP metallopeptidase inhibitor 3 *(TIMP3)*, cyclin-dependent kinase inhibitor 2A and secreted frizzled related protein 1, was compared between BE samples with EAC and those without EAC (19). The methylation rates for the first category were 92% for *hTERT*, 91% for *TIMP3* and 100% for *APC*, while the other promoters reported no methylation. In BE without EAC, the same promoters were methylated in 17, 23 and 36% of the samples, respectively ($P<0.0001$). Therefore, the promoter methylation of *hTERT* appears to be involved in the carcinogenetic process of adenocarcinoma with pre-existing BE (19).

Stomach. The mRNA expression of *hTERT* was assessed using polymerase chain reaction (PCR) in 60 cases of chronic gastritis, 15 of which presented intestinal metaplasia (IM) (20). The results revealed that hTERT mRNA expression was present in 23% of the cases, while the frequency for IM and non-IM samples was 47 and 16%, respectively ($P=0.03$) (20). Gastric carcinoma (GC) presented a *hTERT* positivity rate of 89%, with rates for well-differentiated and poorly differentiated

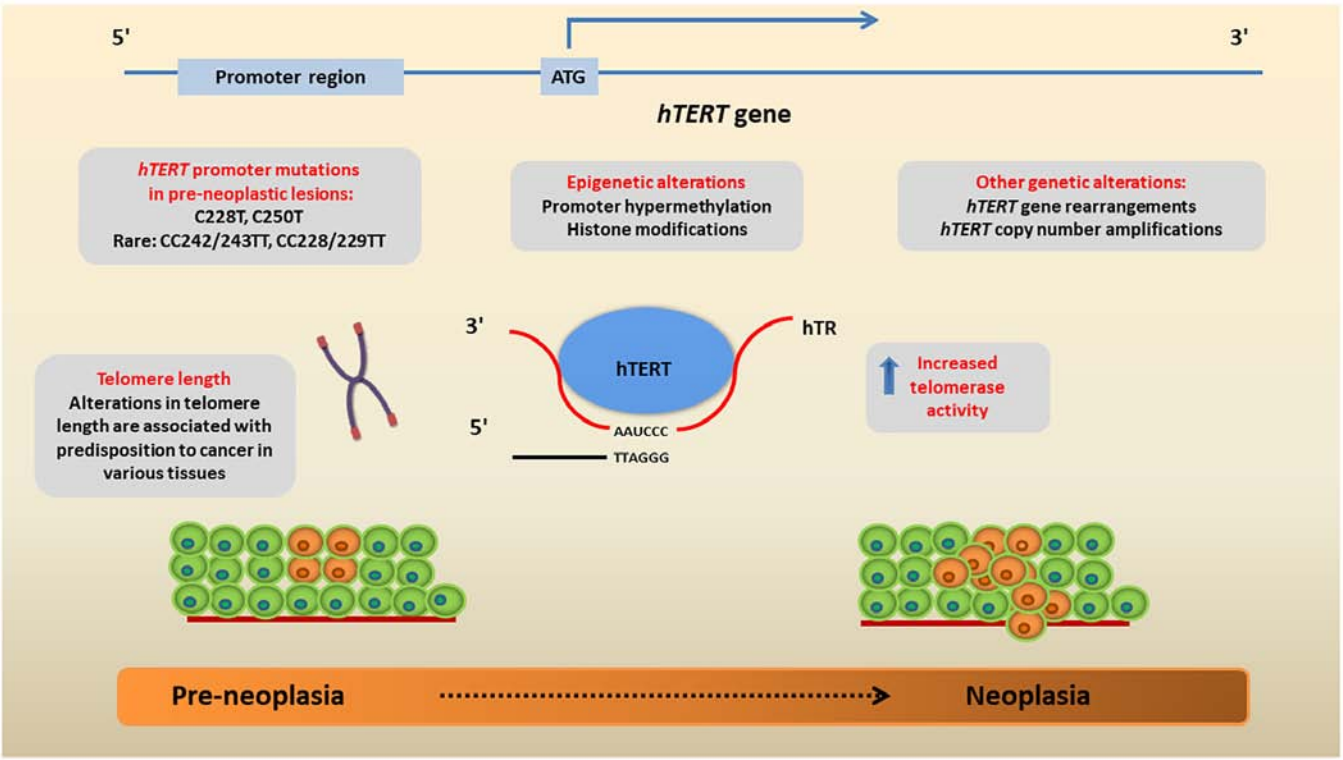

Figure 1. Telomere-related alterations are involved in the progression of pre-neoplastic to neoplastic lesions. The most frequent mechanism is the occurrence of *hTERT* promoter mutations, mainly C228T and C250T, leading to upregulation of telomerase. Other rarer alterations include *hTERT* gene rearrangements, copy number amplifications and epigenetic modifications. These alterations lead to increased expression of telomerase. In addition, abnormal telomere length (shorter or longer telomeres are observed in different cells and tissues) is found in pre-invasive lesions and appears to predispose to cancer development. The timing and importance of these alterations during carcinogenesis remain to be elucidated. hTERT, human telomerase reverse transcriptase; hTR, human telomerase RNA component.

tumors presenting 86 and 91%, respectively. *hTERT* expression was absent in the normal gastric mucosa (20). Wang *et al* (21) also found no *hTERT* expression in normal tissue samples, while the positivity rates for GC and pre-cancerous lesions were 87.5 and 47.4%, respectively (P<0.05). Finally, higher methylation rates of the *hTERT* promoter were observed in cancerous samples compared to the other groups (P<0.05), and there was no association between *hTERT* mRNA levels and *Helicobacter pylori* infection (20-22). Of note, gastric ulcer specimens presented an hTERT protein expression in 39% of the cases, while other studies reported no enzyme activity. In this case, *hTERT* upregulation could either contribute to the tissue's healing process or to its malignant transformation (22).

Gastric carcinogenesis and *hTERT* dysregulation are associated with the proto-oncogene *MYC* and the dysregulation of the *TP53* tumor suppressor gene. The immunoreactivity of these genes was previously found to be higher in IM compared to superficial and atrophic gastritis samples (23). Additionally, the expression of the telomeric proteins telomere repeat factor (TRF)1, TRF2 and TERF1-interacting nuclear factor 2, which regulate TL, were found to vary among normal mucosa, pre-neoplastic tissue and GC specimens. Pre-cancerous lesions, GC, and GC with lymph node metastasis presented significantly higher levels of these proteins compared to normal tissue (P<0.01), while they were significantly increased in GC samples compared to pre-cancerous lesions (P<0.01). Finally, the mean TL was inversely related to the expression levels of the studied proteins. It was significantly shorter in the GC and GC samples with lymph node metastasis compared to the pre-cancerous lesions and normal tissue (P<0.01) (24).

Colon. It has been suggested that telomerase activation occurs during the progression from low- to high-grade dysplasia in adenomas, and increases progressively with the degree of dysplasia and invasion during colorectal carcinogenesis (25). It has been found that *hTERT* mRNA expression is a feature of the late-stage development of colorectal cancer (25). In a previous study, the expression of hTERT in normal colon mucosa from patients with advanced colorectal adenoma was evaluated and compared to that of the controls. The results did not reveal any difference between the two groups of patients (26). In another study, the level of *hTERT* mRNA expression in colorectal adenocarcinomas was significantly higher than that in corresponding non-tumorous mucosa tissues (P=0.009), and the expression level in the adenocarcinomas was slightly higher than that of adenomas, although the difference was not statistically significant (27). Of note, a higher level of *hTERT* expression was often noted in the adenocarcinomas arising from the left colon and rectum compared to those from the right colon (P=0.029) (27).

Only a limited number of studies have evaluated the association between colorectal cancer precursor lesions and TL. A previous study suggested that individuals with a short leukocyte TL had an increased risk of developing advanced adenomas (28). Roger *et al* (29) reported, in an experimental setting, that extensive tissue telomere erosion could lead to

chromosomal instability and the initiation of colorectal cancer in polyps in patients with familial adenomatous polyposis. A recent study suggested that a short TL may be associated with an increased risk of developing colorectal polyps in both the adenoma-carcinoma and serrated pathways. That was the first study to report a statistically significant association between TL and serrated polyps, suggesting that telomeres may play an essential role along the entire serrated pathway (30).

Liver. In the study by Nault *et al* (31), the occurrence of *hTERT* promoter mutations during the malignant transformation of cirrhotic nodules into hepatocellular carcinoma (HCC) was evaluated. Their study included 58 patients with cirrhosis with HCC or pre-malignant lesions, including low-grade dysplastic nodules (LGDNs), high-grade dysplastic nodules (HGDNs), early HCC (eHCC), or small and progressed HCC (31). *hTERT* mutations were highly related to stepwise hepatocarcinogenesis, since they were identified in 6% of LGDNs, 19% of HGDNs, 61% of eHCCs, and 42% of small and progressed HCCs. There were 29 mutations which were detected in 96 nodules, including 25 cases mutated in the first hotspot at 2,124 base pairs (bp) before the ATG start (G>A substitution), and 4 cases mutated at the second known hotspot at 2146 bp before the ATG start (G>A substitution). These mutations were exclusive of each other. These *hTERT* promoter mutations were not found in the cirrhotic-matched tissues, indicating that they were somatic events (31).

In another study by the same research group, 401 liver samples from HCC, HCC lines, cirrhotic tissues, cirrhotic pre-neoplastic nodules and hepatocellular adenomas (HCAs) with or without malignant transformation were analyzed for *hTERT* promoter mutations (32). Mutations in hotspot regions were found in 179 cases (58%) of HCC and 15 cases (63%) of the HCC lines, indicating *hTERT* promoter mutations as the most frequent somatic genetic alterations in HCC. No mutation in the *hTERT* promoter was detected in the cirrhotic tissues. Among the 60 typical HCAs, no mutation in the *hTERT* promoter was detected. Of the 16 HCAs with malignant transformations, seven were positive for *hTERT* promoter mutations, indicating that *hTERT* promoter mutations were involved in the final step of the malignant transformation of HCA (32).

Pancreas. Matsuda *et al* (33) investigated TL between the normal pancreatic duct epithelium, pancreatic intraepithelial neoplasia (PanIN), and pancreatic cancer samples using *in situ* hybridization. PanIN types 1, 2 and 3, as well as cancer cells, exhibited weaker telomere signals in their nuclei and a decreased telomere centromere ratio (TCR) compared to the normal epithelium ($P<0.05$). Cancer cells also exhibited a lower TCR than the PanINs ($P<0.05$); however, it was unrelated to tumor grade. Atypical mitoses and anaphase bridges observed in PanIN and cancer cells were negatively associated with TCR (33). Based on the aforementioned findings, telomere shortening occurs early in pancreatic carcinogenesis and progresses as the malignant transformation develops (33).

Biliary tract and gallbladder. Pre-neoplastic conditions of the biliary tract include hepatolithiasis, biliary epithelial hyperplasia and biliary epithelial dysplasia. Dysplastic changes are related to cases of chronic cholangitis, e.g., hepatolithiasis and primary sclerosing cholangitis and are considered to be a progenitor of intrahepatic cholangiocarcinoma (ICC). hTERT has been shown to be expressed in dysplastic cells and ICC samples, but not in hyperplastic cells and the normal bile duct epithelium (34). Furthermore, another study demonstrated that samples of acute or chronic inflammation of the gallbladder epithelium presented normal TLs (35). On the contrary, metaplastic lesions with pyloric or intestinal metaplasia presented shorter telomeres compared to normal cells ($P<0.05$) in 63% of the cases, whereas dysplastic cells and CIS in 91% of the cases. Notably, two CIS cases presented heterogeneity in TL, with both short and long telomeres in the nuclei. Lastly, cholangiocarcinoma, infiltrating adenocarcinoma of the gallbladder and extrahepatic bile ducts presented shorter telomeres in 98% of the cases (35).

Congenital malformations of the biliary tree also contribute to carcinogenesis through inflammation. Congenital biliary dilation (CBD) causes the dilation of the extrahepatic bile ducts and pancreaticobiliary maljunction (PBM), resulting in chronic inflammation of the biliary tract and gallbladder epithelium. Patients with CBD also have higher rates of cancer development in these areas. Using the Q-FISH assay, Aoki *et al* (36) calculated and compared the normalized TCR between CBD, cholecystolithiasis and normal tissue samples. All three categories exhibited significant differences with each other ($P<0.001$), and the TCRs for each tissue sample were 1.24, 1.96 and 1.77 for CBD, cholecystolithiasis and normal tissue, respectively, indicating that CBD cells presented shorter TLs (36). Moreover, in another study, non-cancerous samples from PBM gallbladders including chronic epithelial inflammation presented telomerase activity, with a score of 3.06, 32.95 and 17.93 total product generated (TPG) units in three different cases. On the other hand, PBM gallbladder carcinoma samples scored 46.57, 85.18, and 206.14 TPG units in three different cases. It is thus suggested that telomerase is a catalytic factor in PBM carcinogenesis (37).

Respiratory system. The characterization of pre-neoplastic lesions of the lungs has been more comprehensively investigated in the case of squamous carcinoma, and to a lesser extent in adenocarcinoma and small-cell carcinoma. In the case of squamous carcinoma, it is generally considered that a stepwise accumulation of precursor phases occurs (38). The key pre-neoplastic lesions of the bronchial epithelium are atypical adenomatous hyperplasia (AAH), squamous dysplasia and CIS, as well as diffuse idiopathic pulmonary neuroendocrine cell hyperplasia. These are lesions of the bronchial epithelium and the precursors of lung adenocarcinoma, SCC and carcinoid tumors (39). Lantuejoul *et al* (40) performed an immunohistochemical and *in situ* hybridization study in pre-invasive and invasive bronchial lesions. They concluded that telomerase was increasingly expressed from the normal epithelium to squamous metaplasia, dysplasia and carcinoma *in situ*, and decreased in invasive carcinoma ($P<0.0001$), with a direct correlation between protein and mRNA levels of expression ($P<0.0001$). The expression of *hTERT* was also associated with resistance to apoptosis (40).

As regards TL, precancerous lung lesions present a shorter relative TL (RTL) than normal bronchial or alveolar tissue

and invasive tumors. Thus, telomere shortening is considered an early event in lung cancer development, which precedes p53/Rb pathway inhibition and results in DNA damage responses (40,41). This could be achieved by activating shelterin complex components, such as the TERF1 and TERF2 proteins. These molecules stabilize the telomeres; however, their mRNA expression is increased in pre-neoplastic lesions, such as AAH. Lantuejoul *et al* (41) investigated the RTL of premalignant lung tissue using FISH and concluded that mild dysplastic lesions presented a lower RTL (RTL=1.2; normal cells, RTL=2), while this number increased in severe dysplasia, CIS and SCC (RTL=2). However, these differences were not statistically significant due to the low number of specimens studied. Similar proportions were observed from AAH to advanced ADC; AHH presented RTL=1.83, which increased in stage I-II ADC and stage III-IV ADC (2 and 1.88, respectively, P=0.047) (41). In addition, comparative genomic hybridization studies in samples from early stages of non-small cell lung cancer have shown that the genomic region that harbors the *hTERT* gene, 5p15.33, is frequently amplified compared with normal tissues (42).

Breast. An analysis of 56 pre-neoplastic breast tissues, including atypical ductal or lobular hyperplasia and lobular *in situ* carcinoma and comparison with healthy tissue and invasive carcinomas revealed that the pre-neoplastic lesions were more likely (60%) to have telomere shortening than normal breast tissue (35%; P=0.0116) (43). As regards invasive carcinomas, TLs were increased in 38.9% and markedly decreased in 38.9% of breast carcinomas (P=0.0087 for comparisons with pre-neoplastic lesions) (43). The telomere DNA content and the number of sites of allelic imbalance were assessed in a set of pre-invasive, invasive and healthy breast tissue samples. It was observed that the level of genomic instability did not differ between ductal carcinoma *in situ* and invasive carcinomas (44). In another study, telomerase activity was evaluated in 27 fibrocystic and dysplastic tissue samples, and 28 fibroadenomas and phylloid tumors, and was reported to be significantly increased in the dysplastic tissue and fibroadenoma groups compared to normal tissues (45).

Cervix. A previous study with cervical samples from 100 patients revealed the overexpression of telomerase in 18.8% of the normal cervical samples, 32.0% of cervical intraepithelial neoplasia I (CIN I), 50.0% of CIN II, 60.0% of CIN III and 91.3% of invasive cervical cancer (46). Telomerase activity was significantly higher in patients with invasive cancer compared to those with CIN or a normal cervix (P<0.05), and its activity increased with the increasing CIN stage (46-48). Patients with benign lesions or CIN that exhibited TERC amplification relapsed or progressed to CIN II and CIN III more often than those without gene amplification (49-51). In addition, the levels of telomerase activity increased in parallel with the degree of CIN, with a significant increase during the transition to CIN3 (52).

Importantly, there is an association between HPV E6 proteins and the activation of *hTERT*, resulting in increased risk of oncogenesis in the cervix (53). The human papillomavirus E6 protein binds to and activates the *hTERT* promoter of polymerase, promoting the precancerous transformation of the cervix. As previously demonstrated, based on testing 29 types of the virus, it was found that the oncogenic types specifically activate the *hTERT* promoter, while the non-oncogenic types do not. (53). The amplification of *hTERT* has been used in combination with HPV testing and *cMYC* amplification in order to optimize screening for malignancy in cytological samples (54).

Endometrium. The normal human endometrium expresses significant telomerase activity in a menstrual phase-dependent manner (55). In a previous study, a total of 32 normal endometrial tissues at various stages of menstruation or postmenopausal conditions were tested for telomerase expression (56). The expression of *hTERT* mRNA was characteristic in the normal endometrium and was dependent on the menstrual cycle phase. In the intrauterine proliferative phase, there was a rapid increase in the expression of *hTERT*, although not in the secretory phase. In additional to its expression in the normal endometrium, *hTERT*, *hTR* and *TP1* were also found to be involved in the development of precancerous and cancerous lesions in the endometrium (56). Another study using a telomere-FISH assay to measure TLs compared chromosomal arm loss or gain in premalignant endometrial lesions with normal endometrium and reported TLs to be stable with the pathological transformation in endometrial hyperplasia and in endometrial carcinoma (57). A significantly increased number of telomere aggregates has been observed in atypical hyperplastic cells in mouse endometrial cancer models. It has been shown that alterations in the nuclear 3D telomere architecture are present in early proliferative lesions of mouse uterine tissues, indicative of endometrial cancer development (58).

Ovaries. In comparison with normal ovary/cystadenoma (32%), a previous study found a markedly higher frequency of moderate activity in low-malignant-potential tumors (67%) or invasive carcinomas (57%), suggesting a close association between the latter two categories (59). That study demonstrated a high prevalence of telomerase activity in low-malignant-potential tumors or invasive carcinomas, with the high telomerase activity associated exclusively with invasive ovarian carcinomas (59). In another study, telomerase activity assessed using TRAP assay was markedly increased in malignant compared to borderline tumors, benign tumors and normal ovaries (P<0.05) (60). The allelic discrimination analysis of primary and recurrent adult granulosa cell tumors has indicated that *hTERT* C228T promoter mutations are already present in some primary tumors; however, they may be late events that occur during adult granulosa cell tumor progression (61).

Urothelium. It has been suggested that *hTERT* promoter mutations represent the earlier onset of a clonal molecular process from which urothelial tumorigenesis may occur (62). *hTERT* promoter mutations were previously investigated among urothelial papilloma (UP) tumors and papillary urothelial neoplasms of low malignant potential (PUNLMPs). The results revealed that 46% of UPs and 43% of PUNLMPs carried a mutated *hTERT* promoter, and the activating *hTERT* promoter mutation C228T was detected in all mutant cases (63). These

findings suggest that these low-malignancy entities share a transformation path similar to aggressive urothelial carcinomas (63). Another study investigated the significance of the *hTERT* promoter mutation pathway in the pathogenesis of inverted UP (IUP), an entity whose neoplastic nature is debated (64). The results demonstrated that 15% of inverted papillomas, 58% of urothelial carcinomas with inverted growth, 63% of conventional urothelial carcinomas and none of the cystic glandular specimens harbored a *hTERT* promoter mutation. It was suggested that a subset of inverted papilloma shares a developmental pathway similar to the carcinogenesis pathway of urothelial carcinoma. It has to be mentioned that a female predominance was noted in *hTERT*-mutated inverted papillomas, taking into account the strong male predilection of inverted papillomas in the general population. All *hTERT* promoter mutations were C228T, apart from two C250T mutations observed in two invasive urothelial carcinoma cases (64). However, another study conducted a comprehensive genetic analysis for multiple oncogenic genes in a sample size of 11 UPs and 11 IUPs. No IUP tumors had a mutated *hTERT* promoter. One UP tumor harbored a *hTERT* promoter mutation and was found in a patient with recurrent non-invasive papillary urothelial carcinomas (65). The results of that study are in contrast to those of previous studies on activating *hTERT* promoter mutations in IUP and UP, which may be attributed to different methodologies (63,64).

In another study, *hTERT* promoter mutations in cases of *de novo* PUNLMP were associated with a risk of recurrence (66). Recurrence with or without progression was encountered in 13 of 30 (43%) cases of PUNLMP, which were included. More specifically, 31% of the cases recurred as PUNLMP, 69% exhibited progression (54% progressed to non-invasive low-grade papillary urothelial carcinoma, 8% to non-invasive high grade papillary urothelial carcinoma and 8% developed stage progression to invasive high-grade urothelial carcinoma) (66). Among the recurrent tumors, 80% harbored a *hTERT* promoter mutation, including C250T and C228T, in contrast to 53% among the cases that did not recur (66).

Moreover, the presence of *hTERT* promoter mutations was previously assessed in a morphological spectrum of microdissected urothelia from urinary bladder specimens with and without keratinizing squamous metaplasia (KSM) and non-KSM (NKSM), including cases of neurogenic lower urinary tract dysfunction (NLUTD), and urothelial and squamous carcinomas (67). The results demonstrated that 94% of cancer foci, 68% of KSM and 70% of NKSM foci were positive for *hTERT* promoter mutations. The authors of that study suggested an association between conditions with chronic urinary bladder injury (such as NLUTD) and a higher risk of developing bladder cancer (67). In a recent study, *hTERT* promoter mutations were examined in whole-organ bladder samples, including cancerous tissue and samples of the tumor-associated normal urothelium, non-invasive urothelial lesions, carcinoma *in situ* and muscle-invasive bladder cancers (68). That study demonstrated that *hTERT* mutations were detected in tumor-associated normal urothelium and non-invasive urothelial lesions. Therefore, mutated *hTERT* promoter regions within non-invasive urothelial lesions are insufficient to establish cancerous growth, indicating the contribution of other gene mutations as a requirement for tumor development (68).

Prostate gland. Pre-neoplastic lesions of the prostate gland include prostate intraepithelial neoplasia (PIN) and possibly atypical AAH, while benign conditions include benign prostate hyperplasia (BPH), which presents no risk for malignant transformation. Previously, when comparing BPH, PIN and prostate cancer telomeric fusion frequencies, the rates for each lesion were found to be similar and comparable: 65, 55 and 62%, respectively. The majority of normal prostatic epithelial tissue samples did not harbor telomeric fusions. As regards *hTERT*, all tissue samples presented detectable levels of its mRNA, with the rates being 69% in BPH, 60% in PIN and 94% in cancerous tissues. The normal adjacent epithelium also presented *hTERT* mRNA expression in 86% of the samples (69).

Variations are also evident in TL, as it appears that cancer telomeres are significantly shortened in comparison to normal tissue ($P<0.05$) (69). This characteristic is also shared by PIN, but not by BPH, as Southern blot analysis has revealed that normal epithelium and BPH have similar average TLs (6.6 and 6.4 kb, respectively) (70). Using Q-FISH assay, Cheng *et al* (71) compared TLs between normal epithelium, AAH, high-grade PIN and prostatic adenocarcinoma (PCA). Shortened telomeres were present in 20% of AAH, 68% of high-grade PIN and 83% of PCA samples. The reduction percentages for each lesion when compared with the normal epithelium were 86% ($P<0.001$), 72% ($P<0.001$) and 68% ($P<0.01$), respectively. TLs of these lesions differed significantly when compared with one another and the normal tissue ($P<0.001$). These findings, along with AMACR expression, suggest the premalignant nature of AAH and its role in prostate cancer development (71). Lastly, TL in BPH is associated with race, as African American males have been shown to exhibit significantly shorter telomeres compared to Caucasian males; longer telomeres have also been found to be associated with an increased risk of cancer (72).

Central nervous system (CNS). *hTERT* promoter mutations (C250T and C228T) are a key event during the carcinogenesis of CNS tumors (73). The frequency of these mutations varies among different tumors, such as primary glioma (80%), medulloblastoma (19.8%) and meningioma (7.4%). The WHO grade of primary gliomas is associated with the frequency of *hTERT* mutations. Similarly, it has been reported that glioblastomas (grade IV), oligodendrogliomas (grade II-III) and astrocytomas (grade I) present these mutations in 80, 60-70 and 30-40% of cases, respectively. The multi-sector sequencing of glioblastomas has revealed the clonal nature of the *hTERT* mutations in these tumors, indicating their essential role in the transformation from precancerous lesions to malignant tumors (73). As a result, increased TL and telomerase activation are significant risk factors for glioma and glioblastoma development. In addition, inherited mutations near *hTERT* and other telomere-related genes, namely *hTERC*, *RTEL1* and *POT1*, could increase the susceptibility of neural cells to oncogenesis (74).

Skin. Actinic keratosis (AK) and Bowen's disease (BD) are pre-invasive, *in situ* forms of cutaneous SCC (cSCC). Both AK and BD harbor *hTERT* promoter mutations: -146C>T or C250T, -124C>T or C228T, -138/-139CC>TT or CC242/243TT (genome location: chr.5.1295242_1295243CC>TT) and

-124/-125CC>TT or CC228/229TT (genome location: chr.5.1295228_1295229CC>TT), which gradually decrease following treatment (75). Consequently, telomerase activity in those lesions has been found to be increased. Moreover, TL has been shown to be associated with a greater tumor invasiveness, as the telomere centromere ratio values in cases of cSCC are lower than those in BD and AK. It is therefore understood that telomere shortening plays a crucial role in the invasive progression of cSCC from its precursors, as it precedes UV-induced *p53* mutations (76).

Benign, precancerous and malignant melanocytic proliferations exhibit different telomerase activity levels, which increase with tumor invasiveness. These findings have been confirmed by immunohistochemistry, as well as by the PCR-based TRAP assay. As expected, benign nevi, such as Spitz and acquired nevi exhibited lower enzyme activity levels than dysplastic nevi, which had similar scores with stage I melanoma (77,78). It should be noted that *hTERT* promoter mutations were initially described in familial melanoma and subsequently, in sporadic melanoma (79). Notably, a previous study identified *hTERT* promoter mutations in the early stages of melanoma. A total of 77% of areas of intermediate lesions and melanomas *in situ* harbored *hTERT* promoter mutations. This finding indicates that these mutations are selected at an unexpectedly early stage of the neoplastic progression (80).

Of note, telomerase activity has been reported to be higher in other non-malignant conditions, for instance, psoriatic lesioned skin, UV-damaged skin and poison ivy dermatitis (81).

Head and neck. Among head and neck squamous carcinomas, hTERT promoter mutations are frequent in cases which are derived from the oral cavity (82). As regards pre-invasive lesions, immunohistochemical analyses have demonstrated that oral epithelial dysplasia exhibit an increased hTERT expression compared to normal mucosa cells, while in oral SCC, the immunohistochemical expression of the protein has been found to be higher than in the dysplastic and normal tissue (83). Additionally, a sub-type of oral leucoplakia featuring ortho-keratotic dysplasia has been shown to exhibit a shorter TL than SCC *in situ* and the normal epithelium (84). Oral submucous fibrosis (OSMF) is a potential precursor of oral SCC. A study comparing telomerase activation (hTERT expression) between normal mucosa, OSMF and oral SCC found an increased enzyme activity in the latter two conditions. Finally, hTERT levels increased with the histological grading of the SCCs, which indicates that telomerase reactivation is critical during the malignant transformation of OSMF to SCC (85).

Thyroid gland. The presence of hotspot *hTERT* mutations in malignant thyroid tumors has been found to be associated with a worse prognosis and a poor response to treatment (86-89). Apart from *hTERT* promoter mutations, epigenetic alterations, *hTERT* gene copy number variations and alternative splicing are implicated in the pathogenesis of thyroid malignancies (90). Several studies have investigated premalignant and benign thyroid nodules as controls for identifying *hTERT* promoter mutations. The vast majority of the samples have not been found to harbor *hTERT* mutations (91-97).

A hotspot *hTERT* promoter C228T mutation was described in a case report of a 68-year-old female with a thyroid follicular adenoma (98). It was considered that *hTERT* promoter mutations comprised a potential early genetic event in the pathogenesis of follicular thyroid carcinoma (98). Moreover, a study including primary tumors from 58 patients with follicular adenoma, 18 with atypical follicular adenoma with uncertain malignant potential, 52 with follicular carcinoma and 20 negative controls from non-tumorous thyroids lesions revealed *hTERT* promoter hotspot mutations in one follicular adenoma (C228T), three atypical follicular adenomas (all C228T), nine follicular carcinomas (8 C228T and 1 C250T) and in none of the negative controls (99). The lesions that presented the mutations also tested positive for *hTERT* mRNA and telomerase activity. The C228T mutation was associated with *NRAS* gene mutations (P=0.16), the most common mutations in thyroid nodules. The TL was also examined in the follicular adenoma and atypical follicular adenoma specimens; however, no significant difference between the *hTERT* promoter mutation positive and negative was found (99). Another study examined the frequency of *hTERT* promoter mutations in 34 well-differentiated thyroid carcinomas, 29 follicular adenomas and 33 sporadic adenomas. *hTERT* promoter mutations were found in 6 patients with adenoma, although no *hTERT* promoter mutations were detected in the sporadic adenoma group (100).

As regards hTERT expression, a study found that 12 out of 33 follicular adenomas and 4 out of 31 multinodular goiters were positive for hTERT expression. The difference between them was significant (P=0.03); however, no significant difference was found between follicular adenomas and carcinomas (101). Of note, in another study, a positive *hTERT* mRNA expression among adenomas was associated with lymphocytic infiltration and thyroiditis rather than a worse prognosis, and it was suggested that since lymphocytes express *hTERT*, lymphocytic infiltration of the examined tissue may influence *hTERT* expression analysis (102).

A comprehensive investigation of *hTERT*-related divergence, namely mRNA expression, promoter mutations, promoter hypermethylation and gene copy number alterations, was explored in a study including 43 follicular adenomas and 33 follicular tumors of uncertain malignant potential (FT-UMP). *hTERT* mRNA was expressed in 6/43 (14%) adenomas and 9/23 (39%) of FT-UMPs (P=0.020). No *hTERT* promoter mutations were found in Fas, while 6/32 (19%) FT-UMPs were positive (P=0.005). No difference in median mutation frequency was observed between the FT-UMPs and follicular carcinomas (P=0.858). The promoter methylation intensity was higher in follicular carcinomas (13%) and FT-UMPs (11%) compared to FA (8%) (P<0.001 and P=0.045). *hTERT* gene copy number exhibited variations (gain or loss) in 5/19 FT-UMPs, similar to 11/77 follicular carcinomas (103).

4. Overview

Numerous studies have investigated the role of telomeres and telomerase during the development of pre-neoplasia and progression to cancer across a variety of tissues. The literature review revealed that the pre-neoplastic lesions and pre-invasive neoplasms generally express higher levels of hTERT compared to healthy tissues, as indicated by immunohistochemical and

PCR-based studies of clinical samples. Certain studies have reported an association between an increased hTERT expression with the histopathological progression of the pre-neoplastic lesion to cancer (47,52); however, this finding is not consistent in all studies; other studies have reported similar expression levels between pre-neoplastic lesions and corresponding carcinomas (26,27). As regards the occurrence of *hTERT* promoter mutations, which is a critical mechanism of telomerase reactivation in cancer cells, it appears that their role is crucial in certain pre-neoplastic lesions (and corresponding cancers), e.g., in liver tissue, CNS, urothelium and thyroid gland. TL is another parameter that appears to be tissue-dependent; in certain cases, the pre-neoplastic lesions are associated with shorter telomeres, e.g. in colorectal, pancreatic and biliary tract pre-invasive lesions (29,33,35), although in a few cases, such as in CNS lesions, longer telomeres have been observed (3). It should be noted that mutations and common variants that lead to longer telomeres have been associated with increased risk of melanoma and lung adenocarcinoma (104,105). Therefore, it appears that whether short or long telomeres predispose to cancer remains ambiguous and requires further investigation. Other alterations have been reported in pre-neoplastic lesions, including those affecting the additional proteins that comprise the telomerase holoenzyme complex.

Notably, the role of hTERT during carcinogenesis appears to be tissue-dependent and may reflect the differential dependence of tissue homeostasis to progenitor cell capacity (106). In particular, *hTERT* promoter mutations are frequently detected in specific types of cancers and not in other (107). It has been suggested that the occurrence of *hTERT* promoter mutations is essential in the early steps of cancer development in tissues with limited replicative potential, which do not continually self-renew, such as the nervous system and liver (107). Another explanation may be that these mutations can also result from environmental factors, such as ultraviolet radiation and chemical carcinogens, as suggested by their high frequency in melanoma and bladder cancer (97). The literature review revealed that this observation is also relevant in the case of pre-neoplastic lesions of the corresponding neoplasms.

Finally, although *hTERT* is the rate-limiting component of telomerase expression, whether *hTERT* expression translates directly to active telomerase activity remains unclear. Given that a large amount of research on this topic, mainly in older studies, is based on the investigation of hTERT expression, the results need to be interpreted with caution (2). Future research is required to incorporate novel fields, such as computational investigation, to recapitulate telomerase's function in different settings.

It has been proposed that the existence of *hTERT* promoter mutations in an intermediate pre-invasive phenotype, at least in some tumors, may translate into the development of a potentially useful diagnostic biomarker (8). A recent study reported that *hTERT* promoter mutations could be detected in urine samples up to 10 years prior to the diagnosis of bladder cancer, while they were not present among matched controls that did not develop cancer (108). Such findings indicate a slow tumorigenic process in certain cases, which could provide a window of opportunity for early molecular detection and intervention (108). Nevertheless, it should be noted that the timing and method of intervention in the pre-invasive phenotypes remain to be determined and require carefully designed randomized trials.

5. Conclusions

Pre-neoplastic lesions across different tissues are associated with the increased expression of *hTERT*, abnormal TL and the occurrence of *hTERT* mutations, indicating the critical involvement of telomerase reactivation during carcinogenesis. The timing and relevant importance of telomerase reactivation appear to be tissue-dependent and may be associated with the differential self-renewing features of the homeostasis of each tissue. Future research is required to focus on elucidating the role of telomerase activation in pre-neoplasia in order to address its diagnostic and therapeutic potential.

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EKa, AK and AA performed the literature review and wrote the original manuscript. EKa, AK, AA, EKo and GG wrote the revised manuscript and prepared the figure. EKo and GG supervised the work. Data authentication is not applicable. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

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Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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