Telomerase promoter mutations in cancer: an emerging molecular biomarker?

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Abstract

Cell immortalization has been considered for a long time as a classic hallmark of cancer cells. Besides telomerase reactivation, such immortalization could be due to telomere maintenance through the "alternative mechanism of telomere lengthening" (ALT) but the mechanisms underlying both forms of reactivation remained elusive. Mutations in the coding region of telomerase gene are very rare in the cancer setting, despite being associated with some degenerative diseases. Recently, mutations in telomerase (TERT) gene promoter were found in sporadic and familial melanoma and subsequently in several cancer models, notably in gliomas, thyroid cancer and bladder cancer. The importance of these findings has been reinforced by the association of TERT mutations in some cancer types with tumour aggressiveness and patient survival. In the first part of this review, we summarize the data on the biology of telomeres and telomerase, available methodological approaches and non-neoplastic diseases associated with telomere dysfunction. In the second part, we review the information on telomerase expression and genetic alterations in the most relevant types of cancer (skin, thyroid, bladder and central nervous system) on record, and discuss the value of telomerase as a new biomarker with impact on the prognosis and survival of the patients and as a putative therapeutic target.

Keywords

Telomerase . TERT . Promoter . Mutations . Biomarker . Cancer

Telomeres and telomerase in germinative and somatic tissues

Normal somatic cells are not immortal and dispose of a predetermined limited number of divisions, a phenomenon known as the Hayflick limit. In 1961, Leonard Hayflick demonstrated that in cell cultures, a population of normal human fetal cells could divide around 40 to 60 times before entering into senescence [44]. At the time, the limited replication potential of somatic cells was not fully understood. Years later, the pioneering research of Nobel Prize winners Elizabeth Blackburn, Jack Szostak and Carolyn Greider identified a refined mechanism by which telomeres are shortened at each round of cell division creating a replication limit [9, 36, 116]. Currently, it is well established that telomeres are nucleoprotein complexes at the ends of eukaryotic chromosomes consisting of several repeats of the DNA sequence TTAGGG. The main function of telomeres is to preserve chromosome integrity and genome stability by preventing the chromosome end from degradation [41, 85]. At each cell division, the telomeric DNA is diminished and telomeres become progressively shorter. Eventually, this loss leads to a

stop in cell division that forces cell senescence or cell death. This telomere-based mechanism has been proposed to serve as the “clock” that controls the number of times each cell can divide [41, 85]. In order to achieve immortalization, cells need to overcome the aforementioned barrier. There are two major pathways cells use for maintain telomere lengthening; they either reactivate telomerase, a ribonucleoprotein polymerase, which elongates telomeres by adding hexameric 5’-TTAGGG-3’ tandem repeats to the chromosomal ends at the ends, or take advantage of a non-telomerase-dependent (alternative) mechanism, known as ALT [21, 59]. Reactivation of telomerase is present in up to 90 % of human cancers, and it allows proliferative cancer cells to maintain telomere length [65]. The remaining 10 to 15 % of human cancers do not have detectable telomerase activity, and a subset of such cases maintain telomere length relying on the ALT mechanism [21]. Heaphy and colleagues performed a comprehensive survey on ALT phenotype in 6,110 primary tumours from 94 different cancer subtypes and observed the presence of ALT in 3.7 % of all tumour specimens but its absence in all benign neoplasms and normal tissues [46]. In this study, the ALT phenotype was identified for the first time in medulloblastoma, oligodendroglioma, schwannoma and glioblastoma [46]. Later on, Heaphy and colleagues demonstrated that ATRX or DAXX mutations are closely associated with the development of ALT in pancreatic endocrine tumours whereas ATRX mutations lead to ALT phenotype in cancers of the central nervous system [45].

Benign neoplasms and normal somatic cells apparently lack telomerase activity but a high level of telomerase activity can be detected in germ cells and in stem cells of selfrenewing tissues [41]. Some putative stem cells, such as the main cells of thyroid solid cell nests, also express telomerase, as we have previously reported [95, 101]. Most cells that need that to escape telomere shortening rely on the reactivation of telomerase. The telomerase complex comprises several components, the most important being the telomerase RNA component (TERC), the telomerase reverse transcriptase catalytic subunit (TERT) and dyskerin (DKC1 gene) [22, 83, 87]. It was shown in telomerase-negative cells, such as differentiated epithelial cells or human fibroblasts [33], that TERT is the only component necessary to restore the activity of the telomerase complex. The TERT gene is located on chromosome 5 and includes 16 exons that span a 35-kb region. The core promoter of telomerase includes 330 base pairs upstream of the start site, is located in a GC-rich region and contains transcript sites/consensus for transcription elements, indicating a high level of regulation by multiple factors [23] at transcriptional and/or post-transcriptional level [22].

Methods to evaluate telomeres length and telomerase activity

As mentioned above, telomere length is maintained and higher levels of telomerase activity can be detected in cancer cells than in normal somatic cells. The interest in the detection of telomerase activity and/or in telomere length measurement has been increasing since it can represent a powerful tool for the diagnosis of telomerase-related diseases as well as for the understanding of cancer etiopathogenesis and, hopefully, for improving cancer treatment. In order to evaluate the two aforementioned features, several methods and approaches to measure telomere length, telomerase messenger RNA (mRNA) expression and telomerase enzymatic activity have been developed [113]. Herein, we merely provide a summary of the methods available; for a more detailed review, the reader is referred to references [46] and [115]. The traditional telomere restriction fragment (TRF) analysis measures the average length of all telomeres present in a cell population and is the most used technique for

evaluating telomere length [4]. Based on Southern blotting, TRF involves the use of restriction enzymes to digest genomic DNA and a hybridization step with a telomeric probe [60]. Additional techniques available include: STELA (single telomere elongation length analysis), a very accurate method that can only be used on a sample with a limited number of cells; quantitative PCR, less time consuming but less accurate [7]; Q-FISH (quantitative fluorescent in situ hybridization) which allows specific measurement of chromosome ends with high resolution [20]; and, finally, Flow FISH, a very accurate method that evaluates cells in suspension [5]. The detection of telomerase is mainly based on assays that evaluate telomerase enzymatic activity [64, 129]. Refinement of these techniques led to a sensitive technique, the telomeric repeat amplification protocol (TRAP). TRAP assay includes the preparation of a protein extract by cell lysis and the addition of a labelled oligonucleotide substrate along with dNTPs, followed by PCR. In the first step of the reaction, if telomerase is active in the extract, it adds a number of telomeric repeats onto the 3' end of a labelled substrate oligonucleotide; in the second step, the extended products are amplified by PCR using primers, which generates a ladder of products with 6-base increments starting at 50 nucleotides: 50, 56, 62, 68, etc. [59]. TRAP is the most used technique to evaluate telomerase activity due to its high sensitivity but it also has some limitations: it is very time consuming and can generate false-negative results if the PCR step fails [64]. Recent improvements in the TRAP technique avoid the use of radioactive nucleotides [115]; other efforts have been made to refine the protocol in an attempt to still improve its sensitivity and to increase its reliability [113, 129].

Telomerase in degenerative diseases

Three human diseases—dyskeratosis congenita (DC), aplastic anaemia (AA) and idiopathic pulmonary fibrosis (IPF)—are associated to mutations in genes that code for the telomerase components, either TERC or TERT, as well for the following telomerase-associated proteins: DKC1, telomerase Cajal body protein 1, TCAB1 (WRP53 gene), NOLA2 protein (NHP2 gene) and NOP10 protein (NOLA3 gene) [79]. Additionally, one of the six proteins that compose the shelterin complex— TERF1-interacting nuclear factor 2 (TIN2 gene)—has also been associated with autosomal-dominant DC, Hoyeraal Hreidarsson syndrome, Revesz syndrome and AA. Furthermore, some alterations affect proteins which do not have a direct impact on telomerase but concern the telomere such the telomere maintenance complex component 1 protein (CTC1 gene) that is associated to Coats plus syndrome, which is a form of cerebroretinal microangiopathy with calcifications and cysts. Finally, mutations of the regulator of telomere elongation helicase 1 (RTEL1 gene) have been identified in patients with severe autosomal recessive DC [35] (Table 1). DC is a rare inherited disorder characterized by a typical triad of clinical manifestations: skin hyperpigmentation, oral leukoplakia and nail dystrophy [26]. The majority of cases (>80 %) occur in children and are diagnosed usually about the age of ten when the children start presenting bone marrow failure together with the previously described clinical triad. Other symptoms that include indicators of premature ageing, such as pulmonary diseases, dental abnormalities and alopecia, are present in 15–25 % of the cases [79]. Within the DC spectrum, there is the Hoyeraal Hreidarsson syndrome, a multisystemic disorder characterized by mental retardation, microcephaly, intrauterine growth retardation, cerebellar hypoplasia, immunodeficiency and AA [51]. The Revesz syndrome that is characterized by bilateral exudative retinopathy, bone-marrow hypoplasia, nail dystrophy, fine hair, cerebellar hypoplasia and growth retardation is also present in the DC disease

spectrum [108]. DC is a genetically heterogeneous disease; to date, there are nine genes associated with DC and all of them contribute to telomere maintenance/protection or telomerase function, thus explaining the excessively short telomeres of DC patients. The group of DC genes encompasses the core telomerase component TERT and TERC and the telomerase complex proteins coded by DKC1, WRAP53, NOP10 and NHP2 genes. Other genes include the shelterin complex TIN2 gene, CTC1 and RTEL1 genes [35] (Table 1). AA is a rare and severe bone marrow disorder characterized by hypocelullar bone marrow and low blood cell counts [109]. Similarly to DC, the cases of AA arise from scarcity of haematopoietic progenitor and stem cells [17]. Since patients have shorter telomeres than matched controls, telomerase components constitute an attractive target for genetic screening. Mutations have been detected in the coding sequence of telomerase core components TERT and TERC (Table 1). Occasionally, AA can develop slowly and appear as an atypical form of DC due to bone marrow failure over time [31]. IPF is a rapidly progressive disorder with an autosomal dominant pattern of inheritance and different degrees of penetrance. The symptoms that characterize the disease are chronic cough and shortness of breath due to fibrotic lesions and scarring of the lungs [40]. IPF can co-exist in patients with AA and DC [35]. Like in the aforementioned disorders, IPF patients also have shorter telomeres than age-matched controls [17]. TERT and TERC telomerase component mutations have been found in familial forms of IPF [107]. The human diseases associated with telomerase or telomere dysfunction encompass mainly the above-referred three disorders (and some related syndromes) but there are other rare diseases reported in the literature [35]. Most of them are haematological disorders, such as myelodysplatic syndrome and paroxysmal nocturnal haemoglobinuria [35]. In cases of myelodysplastic syndrome, mutations are considered an extremely rare event and were described in TERC coding region and its promoter [17]. The same mutation, which ablates a transcription factor binding site in the TERC promoter, has also been detected in a case of paroxysmal nocturnal haemoglobinuria [17]. Additional information regarding mutations in degenerative disorders is summarized in Table 1, and further information can be retrieved on the telomerase disease database (http://telomerase.asu.edu/).

Table 1 Genes reported to be altered and respective associated diseases thought to reflect telomerase complex dysfunction

Gene	Associated disease reported in the literature[a]
TERC	Aplastic anaemia
	Autosomal dominant dyskeratosis congenita
	Dyspnoea
	Hypoplastic myelodysplastic syndrome
	Idiopathic pulmonary fibrosis
	Leukaemia
	Menorrhagia
	Myelodysplasia
	Paroxysmal nocturnal hemoglobinuria
	Refractory anaemia
	Thrombocytopenia
TERT	Aplastic anaemia
	Autosomal dominant dyskeratosis congenita
	Autosomal recessive dyskeratosis congenita
	Hoyeraal Hreidarsson syndrome
	Idiopathic pulmonary fibrosis
	Severe pancytopenia
DKC1	Hoyeraal Hreidarsson syndrome
	X-linked recessive dyskeratosis congenita
Nola2 *Nola3* *WRD79*	Autosomal recessive dyskeratosis congenita
TINF2	Aplastic anaemia
	Autosomal dominant dyskeratosis congenita
	Hoyeraal Hreidarsson syndrome
	Revesz syndrome

[a] Adapted from http://telomerase.asu.edu/

Telomerase promoter mutations and cancer

It has been known for 20 years that high levels of telomerase activity can be detected in cancer cells [59]; this contrasts with the fact that mutations affecting the telomerase coding region appear to be very uncommon in cancer [4]. A rare example of neoplasia presenting mutations in the coding region of telomerase is acute myeloid leukaemia in which few TERT mutations have been identified [17]. However, it should be taken into account that this form of leukaemia can arise from AA and myelodysplatic syndromes in which TERT mutations have been detected [17]. Published simultaneously in the beginning of 2013, two different studies reported mutations in the promoter of the telomerase gene in melanoma [50, 53]. We and others reported the presence of recurrent somatic mutations in the telomerase promoter in cancers of the central nervous system (43–51 %), bladder (59–66 %), hepatocellular carcinoma (59 %), thyroid (follicular cell-derived tumours) (10 %), skin (melanoma, 29– 73 %) and tumours originated from tissues with relatively low rates of self-renewal [58, 75, 88, 121]. Additionally, other studies reported the association of telomerase promoter mutations to other types of tumours, including atypical fibroxantoma (93 %), pleomorphic dermal sarcoma (76 %) [39], bladder cancer (65 %)

[1, 54], basal cell carcinoma (78 %), squamous cell carcinoma of the skin (50 %) [110] and clear cell carcinoma of the ovary [124]. In Tables 2 and 3, we summarize the frequency of TERT promoter mutations in human cancers with a high percentage of mutations and in human cancers with absent or low frequency of TERT promoter mutations, respectively. The in vitro biological assessment of the functional consequence of these mutations, studied by promoter luciferase assay, revealed that their presence results in a two to fourfold increase in telomerase expression [50, 53]. Since previously published studies reported high levels of TERT expression in the set of tumours with TERT promoter mutations [70, 78, 111], it is likely that such alterations may represent one of the missing links between telomerase gene regulation/ reactivation.

Telomerase promoter mutations in skin cancers

Telomerase activity has been reported in normal skin by some authors [43, 118, 119] while other authors suggest that in normal skin, it is a rare event [52, 91]. Its activation in the epidermis may be related with the need for cell proliferation and damage repair [11]. The shortening of telomeres, on the other hand, is believed to provide a barrier for epidermal cell proliferation (i.e. cancer) [11]. Telomerase activity has been reported in cutaneous melanomas, using the TRAP assay, with increasing values from normal skin to benign nevi and to dysplastic nevi and finally to melanoma [29]. An association between increased telomerase activity and worse prognostic features, namely, ulceration, vascular invasion, mitotic rate and Breslow thickness has been described in melanoma [18, 32, 81, 91, 100]. Furthermore, higher telomerase activity has also been associated with higher proliferation rate and early metastasis [100, 104]. The suppression of telomerase activity in melanoma cell lines induced cellular differentiation and reduced the metastatic ability [6, 30]. Longer telomere length has been linked with a higher number of nevi per patient and an increased risk for cutaneous melanoma development [2, 8, 42, 86]. It was proposed that shorter telomere length in nevi limits proliferation and promotes senescence, protecting against malignant transformation [41, 86]. At variance with the aforementioned reports, Burke and colleagues suggested that telomere length can also be influenced by CDKN2A mutational status (a high-risk melanoma susceptibility gene), sun exposure and pigmentation phenotype and therefore cannot be considered a biomarker to predict melanoma risk per se [12]. Two seminal papers reported high frequency of TERT promoter mutations in familial and sporadic melanoma [50, 53]. In the study from Horn and colleagues, a melanomaprone family was investigated through linkage and NGS and a germ-line disease-segregating mutation was identified in the telomerase promoter [50]. Further confirmation was obtained from the same group in a series of cell lines derived from metastatic melanomas, respective metastases and matched primary melanomas that revealed a higher frequency of the mutations in the metastases (74, 85 and 33 %, respectively) [50]. Huang and colleagues took a different approach, data mining of whole genome sequencing data, publicly available. They detected the presence of promoter mutations in 89 % of melanoma cases [53]. The mutations clustered mostly, but not exclusively, in two hotspots that are located at −146 and −124 bps distance upstream of the start site ATG [53]. The detected mutations were cytidine to thymidine transitions at a dipyrimidine motif indicating a putative ultraviolet lightinduced damage signature. These mutations generate a new binding consensus for ETS/TCFs transcription factors (CCGG AA) [50, 53]. Moreover, it was demonstrated in vitro by luciferase assay that the presence of these mutations lead to a two to fourfold increase of the TERT promoter activity [54]. TERT promoter mutations were not detected in nevi [121] but in 13 % of mucosal melanomas [27]. In primary cutaneous melanomas, TERT promoter mutations were

found to be associated with BRAF V600E mutations, worse prognostic features and shorter disease free and overall survival [94, 121]. In ocular melanomas, TERT promoter mutations were described in 0 to 32 % of conjunctival melanomas [25, 121]. Mutations were not detected in uveal melanomas [121]. At variance with the aforementioned data, Dono and colleagues observed a case of uveal melanoma harbouring a TERT promoter mutation that co-existed with GNA11 and EIF1AX mutations [25].

Table 2 Human cancers with high frequency (>5 %) of *TERT* promoter mutations

Cancer type	Number of mutations (%)	Range (%)	References
Nervous system			
Astrocytoma (*n*=597)	96 (16)	(7.7–25)	[3, 57, 58, 63, 121]
Glioblastoma (*n*=1103)	733 (66)	(28–84)	[3, 10, 57, 58, 77, 89, 121]
Medulloblastoma (*n*=700)	154 (22)	(19–42)	[58, 63, 71, 102]
Oligoastrocytoma (*n*=263)	116 (44)	(25–53)	[3, 57, 58, 63]
Oligodendroglioma (*n*=318)	233 (73)	(45–79)	[3, 57, 58, 63, 121]
Other tumours (*n*=792)	45 (5.7)	(0–28)	[34, 58, 63]
Digestive system			
Gallbladder carcinoma (*n*=164)	14 (8.5)	(0–9.1)	[58, 98]
Hepatocellular carcinoma (*n*=366)	206 (56)	(44–59)	[58, 88]
Endocrine System			
Thyroid cancer			
Follicular carcinoma (*n*=207)	39 (19)	(14–36)	[74–76, 80, 121]
Papillary carcinoma (*n*=1128)	132 (12)	(7.5–25)	[66, 74–76, 80, 121]
Poorly differentiated carcinomas (*n*=97)	42 (43)	(29–52)	[66, 75, 80, 121]
Anaplastic carcinoma (*n*=130)	57 (44)	(33–50)	[66, 74, 75, 80, 121]
Hürthle cell carcinoma (*n*=61)	4 (6.6)	(0–16)	[66, 80, 121]
Eye			
Conjunctival melanoma (*n*=42)	12 (29)	(0–32)	[38, 121]
Head and neck			
Laryngeal carcinoma (*n*=235)	64 (27)	-	[97]
Reproductive system			
Endometrial carcinoma (*n*=19)	2 (11)		[58]
Ovarian cancer			
Clear cell carcinoma (*n*=245)	39 (16)	(16–17)	[58, 124]
Skin			
Basal cell carcinoma (*n*=270)	125 (46)	(39–74)	[37, 94, 110]
Cutaneous melanoma (*n*=591)	215 (36)	(12–71)	[27, 47, 50, 53, 69, 94, 121]
Mucosal melanoma (*n*=53)	7 (13)		[27]
Metastatic melanoma (*n*=92)	72 (78)	(67–85)	[27, 50]
Squamous cell carcinoma (*n*=76)	32 (42)	(9.1–50)	[37, 58, 110]
Soft tissue and pleura			
Atypical fibroxanthomas (*n*=27)	25 (93)		[39]
Chondrosarcoma (*n*=2)	1 (50)		[58]
Fibrosarcoma (*n*=3)	1 (33)		[58]
Malignant peripheral nerve sheath tumour (*n*=38)	2(5.3)	(0–6)	[58, 62]
Malignant pleural mesothelioma (*n*=71)	8 (11)		[117]
Myxoid liposarcoma (*n*=63)	48 (76)	(74–79)	[58, 62]
Pleomorphic dermal sarcoma (*n*=34)	26 (76)		[39]
Solitary fibrous tumour (*n*=41)	6 (15)	(13–20)	[58, 62]
Kidney and urinary tract			
Bladder carcinoma (*n*=1447)	1028 (71)	(47–85)	[1, 54, 58, 77, 99, 121, 125]
Renal cell carcinoma (*n*=159)	12(7.5)	(0–9.2)	[121, 123, 125]
Renal pelvic carcinoma (*n*=16)	10 (63)	(60–64)	[123, 125]
Transitional carcinoma of the ureter (*n*=9)	1(11)		[123]

Table 3 Human cancers with absent or very low frequency of *TERT* promoter mutations

Cancer type	Number of mutations (%)	Range (%)	References
Breast			
Breast carcinoma (*n*=88)	0	–	[58]
Nervous system			
Spinal ependymoma (*n*=9)	0	–	[58]
Digestive system			
Colorectal adenocarcinoma (*n*=22)	0	–	[58]
Fibrolamellar hepatocellular carcinoma (*n*=12)	0	–	[58]
Gastrointestinal stromal tumour (*n*=45)	0	–	[58, 121]
Gastric carcinoma (*n*=468)	2 (0.43)	–	[73, 98]
Hepatoblastoma (*n*=3)	0	–	[58]
Pancreatic acinar carcinoma (*n*=25)	0	–	[58]
Pancreatic ductal adenocarcinoma (*n*=24)	0	–	[58]
Pancreatic endocrine tumour (*n*=68)	0	–	[58]
Endocrine system			
Medullary thyroid carcinoma (*n*=158)	0	–	[58, 74, 75, 80, 121]
Phaeochromocytoma (*n*=17)	0	–	[121]
Eye			
Ocular melanoma (*n*=25)	0	–	[121]
Uveal melanoma (*n*=118)	1 (0.85)	(0–2.0)	[25, 38, 121]
Head and neck			
Esophageal adenocarcinoma (*n*=90)	0	–	[120]
Esthesioneuroblastoma (*n*=11)	0	–	[58]
Squamous cell carcinoma (*n*=405)	17 (4.2)	(0–17)	[50, 58, 128]
Haematopoietic system			
Acute myeloid leukaemia (*n*=48)	0	–	[58]
Chronic lymphoid leukaemia (*n*=15)	0	–	[58]
Chronic myeloid leukaemia (*n*=6)	0	–	[58]
Reproductive system			
Ovarian cancer			
Endometrioid carcinoma (*n*=43)	0	–	[124]
High-grade serous carcinoma (*n*=80)	0	–	[124]
Low-grade serous carcinoma (*n*=41)	2 (4.9)	(3.3–13)	[58, 124]
Prostate carcinoma (*n*=47)	0	–	[58, 125]
Testicular carcinoma (*n*=17)	0	–	[125]
Uterine cervix cancer			
Endocervical adenocarcinoma (*n*=25)	0	–	[124]
Squamous cell carcinoma (*n*=75)	3 (4.0)	(3.7–4.5)	[58, 124]
Uterine corpus cancer			
Endometrioid carcinoma (*n*=24)	0	–	[124]
Leiomyosarcoma (*n*=22)	0	–	[124]
Serous carcinoma (*n*=12)	0	–	[124]
Soft tissues and pleura			
Alveolar rhabdomyosarcoma (*n*=7)	0	–	[58]
Alveolar soft part sarcoma (*n*=6)	0	–	[62]
Angiosarcoma (*n*=9)	0	–	[62]
Cholangiosarcoma (*n*=28)	0	–	[58]
Clear cell sarcoma (*n*=5)	0	–	[62]
Central/conventional chondrosarcoma (*n*=9)	0	–	[58]
Dedifferentiated liposarcoma (*n*=61)	0	–	[62]

Table 3 (continued)

Cancer type	Number of mutations (%)	Range (%)	References
Dermatofibrosarcoma protuberans (n=10)	0	–	[62]
Embryonal rhabdomyosarcoma (n=8)	0	–	[58]
Epithelioid sarcoma (n=4)	0	–	[62]
Extraskeletal myxoid chondrosarcoma (n=11)	0	–	[58, 62]
Leiomyosarcoma (n=30)	0	–	[58, 62]
Low-grade fibromyxoid sarcoma (n=18)	0	–	[58, 62]
Mesothelioma (n=4)	0	–	[58]
Myxofibrosarcoma (n=27)	0	–	[62]
Osteosarcoma (n=23)	1 (4.3)	–	[58]
Pleomorphic liposarcoma (n=15)	0	–	[62]
Synovial sarcoma (n=41)	1(2.4)	(0–4.0)	[58, 62]
Undifferentiated pleomorphic sarcoma (n=50)	0	–	[58, 62]
Well-differentiated liposarcoma (n=10)	0	–	[58]

TERT promoter mutations are frequent in non-melanoma skin cancer, ranging from 39 to 74 % in sporadic basal cell carcinomas (BCC) [37, 94, 110] and present in up to 50 % of cases of squamous cell carcinoma (SCC) [3 7, 110].Telomerase activity has been detected in BCC using TRAP assay both in tumour and tumour-free margins, varying between 20 and 100 %, with less activity in the latter [29]. In the tumour-free margins, telomerase activity was found to be more prevalent in sun-exposed skin [105, 119]. In SCC, the data are scarce: Ueda and colleagues found telomerase activity in 100 % of the cases of a small series (n=8) [119]. Few studies have examined the association between telomere length and skin cancer [2]. Some studies found no significant association between telomere length in peripheral blood leukocytes (PBL) and risk of non-melanoma skin cancer, either in BCC (two independent sets) [68] or in SCC [42, 68]. In contrast, other authors found that longer telomeres in PBL are protective for BCC [2, 86] and SCC [2]. Telomere length has also been evaluated by fluorescent in situ hybridization (FISH) showing that higher telomere length in BCC is significantly higher than in SCC [93].

Telomerase promoter mutations in thyroid carcinomas

Thyroid tissue is a conditionally renewing tissue that proliferates rarely in adult life. In line with this, telomerase activity in normal thyroid samples is almost absent, being detected in less than 7 % of cases [16, 114]. On the other hand, telomerase activity was consistently reported in a specific population of thyroid cells—the solid cell nests (SCNs) which are considered to represent embryonic remnants of the ultimobranchial body [95, 101]. Thyroid carcinomas apparently display less frequent telomerase activation than most human carcinomas. A wide range of frequencies have been reported [16]; in average, it seems that two thirds of thyroid carcinomas display telomerase activation that is more frequent in the undifferentiated (anaplastic) than in differentiated carcinomas [16]. When the results obtained by several authors are combined, telomerase activity occurs in 48 % of papillary thyroid carcinomas (PTC) and 71 % of follicular thyroid carcinomas (FTC). A TERT copy number gain was described in familial PTC [14], but this finding was not confirmed in another series [55]. A recent study by Capezzone and colleagues reported telomerase activity in most sporadic and familial malignant thyroid tumours as well as in some adenomas [15]. Telomerase activity was not observed in hyperplastic nodules or in normal thyroid tissue from patients with sporadic PTC [15]. In summary, the aforementioned findings suggest that telomerase activity may be associated with a more aggressive clinical behaviour of thyroid tumours. Recently, somatic mutations in the promoter region of TERT were

reported in thyroid tumours [66, 74, 75, 121]. In a large series of 469 follicular cell-derived thyroid carcinomas (FCDTC), TERT promoter mutations were found in 7.5 % of PTC, 17.1 % of FTC, 29.0 % of poorly differentiated thyroid carcinomas (PDTC) and 33.0 % of anaplastic thyroid carcinomas (ATC) [80]. This stepwise increase in the frequency of TERT promoter mutations from well to poorly differentiated and undifferentiated carcinomas was also reported in other studies [66, 74] (Table 2). TERT promoter mutations were not detected in normal thyroid tissue, benign lesions or medullary thyroid carcinoma (MTC). Moreover, very few tumours with oncocytic features harbouring TERT promoter mutations have been reported, and no mutations were detected in a small series of papillary thyroid microcarcinoma nor in tumours from individuals exposed to the Chernobyl accident [58, 77, 121]. The majority (about 80 %) of mutated cases presented the −124G>A mutation. In PTC, TERT promoter mutations were significantly more frequent in BRAF-mutated tumours than in BRAF wild-type tumours [74, 75, 80, 121]. The TERT promoter mutations were associated with increased mRNA expression, and this increase was particularly pronounced in tumours harbouring both BRAF and TERT promoter mutations [121]. Two studies analysed the relationship between TERT promoter mutations, clinico-pathological features and outcome. TERT promoter mutations were significantly associated with older age at diagnosis [74, 80], larger tumour size and higher stage [80]. TERT promoter mutations were also found to be an independent predictor of distant metastases and disease persistence at the end of follow-up in differentiated thyroid carcinomas (DTC) [80]. Patients with TERT promoter-mutated tumours were submitted to more radioiodine treatments with higher doses as well as to other treatment modalities including surgery, external beam irradiation and/or treatment with tyrosine-kinase inhibitors [80]. TERT promoter mutations were significantly associated with disease-specific mortality in the whole FCDTC group; this association held true if the subgroups of patients with DTC, PTC or FTC were independently considered [80]. In DTC, the prognostic value of TERT promoter mutations for disease-specific mortality was independent of age and gender [80]. Altogether, the aforementioned findings indicate that TERT promoter mutations are a major indicator of poor outcome in DTC. The two studies on record on MTC [59, 123] did not reveal TERT promoter mutations in this subtype of thyroid carcinoma.

Telomerase promoter mutations in bladder carcinomas

The putative role of telomerase in bladder carcinoma (BC) has been a matter of interest in the last two decades. Using TRAP assay, telomerase activity has been evaluated in BC; telomerase activity was detected in the majority of the studied tumours in contrast to the absence of activity in the respective normal counterpart samples [70, 90]. In some series, telomerase activity was associated with lower grade and lower stage BC [84, 90]. Other studies pointed out that both telomerase activity [70] and telomerase expression [126] are associated with higher stage and higher grade [70, 90]. Preliminary evidence obtained in cell lines suggest that BC might have TERT promoter mutations [53]. These early results motivated us and others to search for similar events in bladder tumour samples. Similar to cell lines, the same TERT promoter mutations were detected frequently in BC, with a prevalence ranging from 47 to 85 % (Table 2) [1, 54, 61, 77, 99, 121, 125]. These results rank TERT promoter mutations as one of the most frequent genomic events, possibly the most frequent, in BC [1, 54, 61, 77, 99, 121, 125]. TERT mutations were significantly more frequent among FGFR3 mutant tumours [1]. Wu and colleagues reported a significant co-occurrence of TERT promoter mutations and TP53/RB1 inactivating somatic mutations [125] indicating that both mutations may cooperatively contribute to the progression of BC [125]. Conflicting results have been reported on the association between TERT promoter

mutations and clinical stage and/or grade of bladder tumours. Wu and colleagues found that TERT promoter mutations are more prevalent in muscle invasive (MI) than in non-muscle invasive (NMI) tumours and also more prevalent in BC patients with advanced tumour stages (T2–4) than in those with low stage tumours (Ta or T1) [125]. At variance with this, another report found no association between mutation status and stage or grade of BC [54]. Similar results were reported by Allory and colleagues who did not find any differences between NMI and MI BC in two independent sets of tumours [1]. Similarly to stage and grade, diverging results were obtained on the association between TERT promoter mutation and prognosis. One group reported that the survival rate of patients with TERT mutations was significantly lower than that of patients without mutations [125], whereas another group found no association between clinical outcome and mutation status [1]. An interesting observation was reported by Rachakonda and colleagues who proposed that a common polymorphism, rs2853669 within a pre-existing Ets2 binding site in the TERT promoter, acts as a modifier of the effect of the mutations on survival and tumour recurrence [99]. The patients with the mutation presented poorer survival in the absence than in the presence of the polymorphism. The mutation in the absence of the variant allele was highly associated with disease recurrence in patients with Tis, Ta and T1 tumours [99]. These results may help to explain some of the divergence reported in studies relating TERT promoter mutations and prognosis of patients with BC. As it was previously noticed, several observations support a model in which TERT somatic mutations are an early event in urothelial carcinogenesis, including their occurrence in a small fraction of subjects with precursor lesions, their presence in tumours of both papillary and invasive features and their low level of intraindividual heterogeneity when analysing multiple tumour regions [1, 58]. TERT promoter mutations may potentially be used as urinary biomarker; several studies have already performed preliminary evaluations of the feasibility, sensibility and specificity of such procedure [1, 54]. Prospective studies based upon series are necessary to further assess the clinical utility of the detection of TERT promoter mutations in urine.

Telomerase promoter mutations in central nervous system tumours

Central nervous system (CNS) often have TERT promoter mutations competing favourably in this aspect with most other types of human cancer [58, 121]. Among CNS tumours, gliomas are those displaying by far the highest frequency of TERT mutations which can also be detected at lower frequencies in medulloblastoma and meningioma [63]. Within gliomas, the percentage of cases with TERT promoter mutations differs according to the histopathological type of tumour. TERT promoter mutations are detected in the majority of cases of glioblastoma multiforme (GBM) [World Health Organization (WHO) Grade IV] which is the most frequent and aggressive form of glioma and in oligodendrogliomas (WHO Grade II and III), in contrast to astrocytoma (WHO Grades I, II and III) and ependymoma (WHO Grades I, II and III), in which only a small percentage of the tumours harbour such mutations (Table 3) [63, 121]. Furthermore, the percentage of TERT promoter mutations in oligoastrocytomas, gliomas with a mixed origin, is intermediate between that of oligodendrogliomas and astrocytomas [58]. These findings fit with the reported data on telomerase activity in gliomas which is considerably higher in GBM (50– 89 %) and oligodendrogliomas (75–100 %), than in astrocytomas (0–45 %) [49, 67, 106]. The low frequency of TERT promoter mutations and telomerase activity in grades II and III astrocytomas can be explained by the high prevalence of ATRX mutations, one of the most frequent mutations in this type of glioma [56]. It is known that ATRX mutations trigger ALT in astrocytoma cells and it has been shown that this alternative mechanism is frequently activated in astrocytomas, allowing

telomere maintenance without the need for telomerase reactivation [48]. In line with this, the frequency of TERT promoter mutations in secondary GBMs (that arise from the progression of lower grade astrocytomas) is considerably lower than in primary GBMs (that appear de novo) [89]. TERT promoter mutations are rare in paediatric tumours of the CNS [63]. In medulloblastomas that typically develop in children, TERT promoter mutations are mainly detected in tumours of the group of older patients and are associated with sonic hedgehog and WNT mutations [102]. Upregulation of TERT expression in paediatric brain tumours was associated with hypermethylation of the TERT promoter, rather than with TERT promoter mutations [19]. These findings are consistent with the fact that the cells, from which paediatric CNS tumours are thought to originate, still have activated telomerase which obviates the need for activation of TERT through promoter mutation. Although ATRX and TERT promoter mutations provide an explanation for the maintenance of telomere length in most gliomas, TERT upregulation was also reported to occur in a subset of gliomas without TERT promoter mutations or ATRX mutations through an as yet unidentified mechanism [58]. Finally, it is worth noting that, previous to the discovery of TERT promoter mutations in gliomas, some studies had reported an association between SNPs in the TERT gene and an increased risk of glioma development [112, 127].

Telomerase promoter mutations in other tumour types

In Tables 2 and 3, we have summarized the data on record on the frequency of TERT promoter mutations in tumours from almost every site. For the sake of simplicity, we divided the tumours into those with a high frequency of mutations (>5 %, Table 2) and tumours with no mutations or with a very low frequency of TERT promoter mutations (described by Killela and colleagues, TERT promoter mutations can be relevant in tissues with relatively low rates of selfrenewal [58], an association that fits with the findings in follicular cell-derived thyroid cancer and gliomas. In these two settings (thyroid cancer and gliomas), TERT promoter mutations are associated with a guarded prognosis of the patients harbouring the tumours and probably represent late events of the oncogenic process. On the other hand, TERT promoter mutations can also result from environmental factors such as ultraviolet radiation and chemical carcinogens as suggested by their high frequency in melanoma, basal cell carcinoma and bladder and tongue carcinomas. In this second setting, TERT promoter mutations appear to be an early tumorigenic event and do not carry major prognostic value, with the exception of melanoma. Why clear cut differences exist in the frequency of TERT promoter mutations in tumours of the same system (e.g. hepatocellular carcinoma versus pancreatic carcinoma) remains to be clarified, although there is enough evidence to claim that the high or low prevalence of the mutations appears to be histotype- rather than site-associated. For instance, the high frequency in transitional carcinoma of the bladder and renal pelvis is in contrast to low frequency/absence in kidney carcinoma and the extremely low frequency/absence in adenocarcinomas of every organ of the gastrointestinal tract (Tables 2 and 3).

Telomerase as a therapeutic target

Several therapy strategies have been suggested to control TERT expression in tumours, mainly using small molecule inhibitors, gene therapy approaches and immunotherapy (reviewed in [82]). Inhibition of enzymatic activity with small synthetic molecules allows the disruption of the replicative capacity of cancer cells; in this way, it is though that normal somatic cells will not be

affected due to the absence of TERT activity. In vitro studies showed that BIBR1532, a noncompetitive inhibitor of both TERT and TERC [92], leads to cellular senescence reducing proliferation and telomere length [24] and is cytotoxic in high doses [28]. Additionally, a marked reduction of the tumorigenic potential of tumour cells treated with BIBR1532 was observed in a mouse xenograft model [24], with no adverse side effects and uncomplicated oral administration of the drug. BIBR1532 is one of the most promising TERT specific-inhibitors to date. Other small synthetic molecules—G-quadruplex ligands, such as BRACO19, RHSP4 and telomestatin—are promising drugs that can be used for TERT targeting therapies [103]. However, clinical testing of some of these molecules has been hampered due to the toxic characteristics of the compounds [82]. Cancer cells with TERT activity can be directly targeted by introducing suicide genes or oncolytic viruses driven by the TERT or TERC promoters, or the inhibition of TERT or TERC activity targeting their RNAs. In the latter strategy, antisense oligonucleotides, small interfering RNAs and ribozymes can be applied for inhibition of TERT activity. GRN163L (also known as imetelstat) is the most studied antisense oligonucleotide that causes TERT inhibition and telomere shortening in cancer cell lines derived from different organs [13]. This compound leads to apoptosis of cells and to inhibition of tumour growth, and it is being used in clinical trials of several cancer types [13]. DNA vaccines (immunotherapy) have been used to generate protective immunity against tumours in several models [96]. The presence of TERT activity in many human cancers turns TERT a tumour-associated antigen suitable for cancer immunotherapy. Contrary to other target antigens, as carcinoembryonic antigen (CEA) and melanoma-associated antigen, TERT-based immunotherapy may be applied to a wide range of malignancies due to the highly frequent TERT-altered expression [122]. In vitro and in vivo studies showed tumour regression using TERT-based vaccination approaches (reviewed in [72]). Different peptides have been used to induce anti-TERT immune response [13] and vaccination using the I540–548 peptide showed anti-tumour responses in cancer [122]. Several preclinical studies using TERT peptides are being conducted (reviewed in [103]). GV-1001, GRNVAC 1 and Vx-001 are the most promising vaccines available to date.

Future perspectives

The implication of telomerase in human diseases has been studied for a long time and firmly established in a few models of degenerative diseases. In cancer, telomerase dysfunction has been perceived as a potential mechanism for carcinogenesis although the underlying mechanisms remained elusive. The recent identification of telomerase promoter mutations in several types of neoplasia fostered the respective research, and in less than a year, numerous studies have been published reporting similar alterations in many cancer models (Tables 2 and 3). In several relevant cancer types, telomerase promoter mutations seem to constitute a new biomarker for prognosis with potential applications in pre-surgical diagnosis and in the follow-up of the patients. Low-grade bladder cancers represent a good example on how such finding can represent an added value from a clinical standpoint. Up to 70 % of lowgrade non-invasive bladder tumours recur, and long-term cystoscopic surveillance is the current standard of care. This procedure is expensive and time consuming and carries significant morbidity. The non-invasive evaluation of telomerase promoter mutations in urine may provide diagnostic information, independent of routine cytology, and most importantly, may identify low-grade tumours, which are difficult to identify by cytological examination alone. Whenever dealing with a recurrence, a non-invasive diagnostic test that also serves as a surveillance method will probably represent an attractive alternative for patients, taking into consideration the

limitations of the technique. A preliminary evaluation of the diagnostic usefulness of the detection of TERT promoter mutations was already performed in urine samples, and the results indicate that such detection may serve as a biomarker of early disease and recurrence [1, 54]. Moving to a trendier subject, it seems extremely interesting to evaluate whether or not TERT promoter mutations can be detected in tumour-circulating DNA from cell-free fragments in body fluids. In the affirmative case, this process may represent a major advance in the follow-up of cancer patients. Despite the large amount of information collected in these recent years, more questions than answers remain at present with regard to the role of telomerase involvement in carcinogenesis. A novel mechanism for telomerase re-activation and/ or re-expression was discovered; this mechanism, together with ALT, represents the two major pathways for telomere length maintenance. Besides them, other mechanisms may modulate telomerase expression, such as novel forms of transcriptional regulation or epigenetic alterations. We think it is the appropriate time to study large series with robust clinicopathological data and to search for correlations that may establish or rule out the prognostic value of TERT promoter mutations in the various types of human cancer. Last but not least, cell and molecular biology studies are mandatory to understand the role(s) of telomerase in cancer cells that appear to go beyond the increased replicative potential (immortalization) and have impact also in metastatic capacities.

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