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Review

The levels of telomere-binding proteins in human tumours and therapeutic implications

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ABSTRACT

Tumours require telomeric integrity to maintain viability, conferred by adequate length of telomeric DNA replenished by telomerase, and binding of telomere-binding proteins (TBPs), thus telomeres have received attention as an anticancer target. Levels of TBPs in tumour tissue may have implications for drug development if they render some cancers relatively more sensitive or resistant to telomere targeted agents. This review gives an overview of the studies examining the levels of TBPs in tumours and discusses possible reasons for differences in the findings, given the interplay between various factors determining telomere stability. Whether cancers with lower levels of TBPs will be more susceptible to therapies targeting telomere maintenance will require clinical trials of these novel therapies.

1. Introduction

1.1. *Telomere maintenance as cancer target*

Telomeres are the specialised DNA–protein complexes found at the tips of linear eukaryotic chromosomes. In humans they consist of several kilobases of the repeat sequence 5′-GGTTAG.[1] For most of its length it is double-stranded, but terminates in a 3′ overhang of 100–200 bases of the G-rich strand. Associated with the telomeric DNA are three sequence-specific DNA binding proteins: TRF1, TRF2 and POT1, plus three bridging or adaptor proteins: RAP1, TIN2 and TPP1. The telomere-associated proteins ('shelterin')[2] or 'telosome'[3] masks the chromosome terminus, preventing it from being mistaken for a double-strand break, while the telomeric DNA itself serves as a buffer of non-coding DNA that in normal cells is gradually eroded over the cycles of cell division due to the end replication problem.[4] To preserve genomic integrity, once the telomeres are shortened below a critical length, normal cells are signalled to cease replication. This signal is believed to be mediated through the release of shelterin proteins from the shrinking telomere ('uncapping').

Since a capacity for limitless replication is a hallmark of cancer cells,[5] it is clear that they must activate a mechanism to overcome the process of telomere erosion. In contrast to normal somatic cells, in 85–90% of tumour cells the enzyme telomerase is over-expressed and activated.[6] Telomerase is a specialised reverse transcriptase that can add multiple telomeric repeats (5′-GTTAGG) onto the 3′-end of a telomere, and so counter the process of replication-associated telomere shortening. In the remaining minority of tumours, an alternative recombination-based mechanism (ALT) maintains telomere length.[7] Either way, the maintenance of competent telomeres is crucial to the survival of tumour cells, and so the possibility of disrupting this process represents an exciting

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new approach to therapy – before examining this further, the key components involved will be briefly described.

1.2. Telomerase

Telomerase is a ribonucleoprotein and specialised reverse transcriptase, which consists of a 127 kDa protein, hTERT and a stably associated ~450 base RNA, hTR.[8] Embedded within the hTR sequence is the template used to add telomeric repeats. The structure and functions of telomerase have been recently reviewed, and the structure of the catalytic component was more fully elucidated.[9]

A large number of proteins have been identified that appear to interact with telomerase, the functional significance of many of which is unclear. *In vitro*, telomerase can function without any protein partners and it has recently been shown that the active entity *in vivo* appears to be just two copies each of hTERT, hTR and dyskerin.[10] This discussion will be restricted to identifying certain candidates that may directly mediate telomerase recruitment to the telomere (see Fig. 1a). The KU complex binds to telomeric DNA, TRF1 and TRF2, and interaction with both hTERT and hTR has also been demonstrated. Interestingly, KU and other DNA damage response proteins are required for telomere maintenance.[11] hEST1A is a human homologue of yeast EST1P, which interacts with both hTR[12] and CDC13P,[13] the yeast homologue of POT1, and is essential for telomere maintenance. hEST1A plays a similar functional role in man though whether this is through a similar mechanism remains to be established; it has been shown to uncap chromosome ends when over-expressed, manifested in anaphase bridges due to chromosome end fusions with persistence of telomeric DNA at the fusion points.[14] PINX1 is a TRF1-binding protein[15] that also binds to hTR.[16] Over-expression of PINX1 reduces telomerase activity and telomere length.

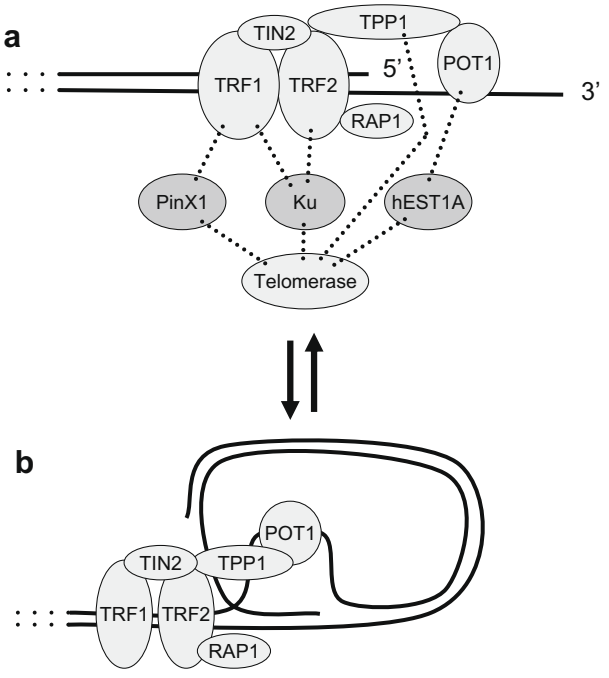

Fig. 1 – Organisation and interactions in the shelterin complex. (a) The putative 'open' form of the telomere terminus and the direct and indirect interactions between shelterin components and telomerase. (b) The topology of the 'closed' form which may involve significant reorganisation of protein interactions.

1.3. Telomere-associated proteins

TRF1 and TRF2 are homologous ~60 kDA proteins that are found associated with the full length of the double-stranded portion of the telomere. Both contain a C-terminal DNA binding domain homologous to the *Myb* proto-oncogene that recognises the sequence TAGGGT and a large central dimerisation domain.[17] Despite their homology, they are distinct enough in their dimerisation domains that only homo-, not heterodimers appear to be formed. They differ particularly in their N-terminal regions where TRF1 contains an acidic motif while TRF2 is rich in basic amino acids. TRF1 appears to play a particular role in regulating telomere extension; over-expression leads to telomere shortening while ablation results in telomere lengthening.[18] In contrast TRF2 is more involved in maintaining telomere structural integrity and can delay the senescence set-point; cells transfected with dominant negative TRF2 show end-to-end chromosome fusions.[19] Depletion of TRF2 activates ATM and other DNA damage response factors, including 53BP1, gamma-H2AX, RAD17, ATM and MRE11.[20,21]

POT1 consists of an N-terminal domain comprising two oligosaccharide/oligonucleotide (OB) folds that bind strongly and selectively to the G-rich single-strand of telomeric DNA, plus a C-terminal domain that can vary amongst splice variants and which is involved in protein–protein interactions, as well as considerably modulating the DNA binding affinity of the N-terminal domain.[22] Experiments examining the effect of POT1 over-expression illustrate the complexities involved. Thus, POT1 has been reported to facilitate telomere elongation by telomerase,[23] but conversely to act as a negative regulator of telomere length possibly though DNA binding and effects on substrate access by telomerase.[24] Removal of POT1 results in an ATR-dependent DNA damage response.[25]

TIN2 and TPP1 (PTOP/PIP1/TINT1) are the key adaptor/bridging proteins. TIN2 binds to TRF1, TRF2 and TPP1,[26] while TPP1 additionally bridges to POT1; TPP1 and POT1 can form a complex with telomeric DNA that has recently been shown to increase the activity and processivity of telomerase.[3] In contrast RAP1 interacts only with TRF2[27] though it also contains an apparently non-functional *Myb* domain.

In addition to shelterin, the six core-proteins of the telomere protection complex, the 'telosome' additionally includes HP1 and SUV39H1/H2 (chromatin regulators), the MRE11/NBS1/RAD50 complex, ATM, WRN, BLM, ERCC1/XPF, RAD54, RAD51D and XRCC3 (DNA repair proteins) plus TANK1, TANK2, DKC1 (associated with the hTR) and hSnm1B/Appolo exonuclease that directly binds TRF2.[28]

1.4. Telomere structure and dynamics

Information about the various DNA–protein and protein–protein interactions between the components of the telomere, combined with electron microscopy data, has led to a model

for telomere structure and dynamics.[2,29] Under normal conditions a 'closed' state is adopted, in which the single-stranded 3′-terminus loops back and strand invades an upstream double-stranded region of the telomere (see Fig. 1b). The loop thus formed (the t-loop) may be very variable in size. The displaced single-strand also forms a loop (the d-loop). TRF1 is proposed to be associated primarily with the more regular double-stranded regions of this structure, while TRF2, plus RAP1, localises to the non-canonical regions. POT1 binds to the displaced d-loop DNA, and TIN2 and TPP1 then act to 'glue' this complex together. However, this state is clearly incompatible with telomere extension by telomerase, or replication in general, so an alternative 'open' structure for the telomere must exist at least some of the time in such cells (Fig. 1a). A key element of this remodelling is unlooping of the telomeric DNA, and the relocation of POT1 to the single-stranded terminus where, in association with TPP1, it recruits and promotes telomerase activity. The different roles played by POT1 in the open and closed states of the telomere could help to explain the apparently contradictory observations regarding the effects of POT1 over-expression mentioned above (Section 1.3).

1.5. *Summary of possible therapeutic approaches targeting telomere maintenance/integrity*

Telomere maintenance presents a wide variety of possible opportunities for therapeutic intervention in cancer (for recent reviews see [29,30]). Broadly speaking these fall into two categories: those based on inhibiting telomerase and those based on destabilising the telomere, though in practice the distinction is less clear-cut. Attempts have been made to find small molecule inhibitors of the reverse transcriptase activity of telomerase, e.g. the nucleoside analogue AZT,[31] non-nucleoside natural products, e.g. EGCG[32] and novel chemicals, e.g. BIBR1532.[33] Antisense oligonucleotides with modified backbone chemistry have been targeted to the RNA component of telomerase, acting as telomere substrate antagonists; a short lipidated version, GRN163L is in clinical trial.[30] Indirect approaches targeting telomerase activity include the inhibition of the chaperone protein Hsp90 for which hTERT is a client protein, e.g. by novobiocin,[34] or interference with post-translational regulation of telomerase activity, e.g. by bisindolylmaleimide, a protein kinase C inhibitor.[35]

Alternative approaches to inhibiting telomerase action target the substrate – i.e. the 3′ overhang. The aim of these is to convert the terminus into a form where it can no longer serve as a primer for extension. Hybridisation with complementary peptide nucleic acids (PNAs) has been tried,[36] and there are also a variety of 'telomerase inhibitors' whose mechanism of action involves sequestering the overhang into a quadruplex form, e.g. BRACO19, RHPS4, 12459 (reviewed in [37]). It has been the study of this last type of approach that has revealed telomere disruption as the target. It may be appreciated that agents targeted to single-stranded telomeric DNA may have quite different effects on the 'closed' structure of the telomere. Indeed, both the quadruplex targeting agents telomestatin and RHPS4 cause the displacement of POT1 and TRF2 from the telomere and DNA damage events. Clearly, many other opportunities to target specific DNA–protein and protein–protein interactions within the shelterin complex exist.

1.6. *Why levels of TBPs are of interest*

Recent studies on G-quadruplex ligands have demonstrated the induction of DNA damage selectively at telomeres and the potential clinical utility of this mechanism.[38,25] Moreover, the displacement of the telomere-binding proteins POT1 and/or TRF2 was key to the anticancer properties of the ligands and their over-expression led to treatment resistance in mice.[25] Thus to understand the likelihood of innate tumour sensitivity or resistance to agents targeting telomere integrity, the basal level of telomere proteins may be revealing: levels of TBPs in tumour tissue may have implications for drug development if they render some cancers relatively more sensitive or resistant to telomere targeted agents. Several articles have now been published examining the levels of telomere-binding proteins in clinical samples and comparing to normal tissue or cancers at various stages of development; however, no clear trend seems yet to be emerging.

1.7. *Aims of this review*

This review aims to gather an understanding of the studies that have investigated how TBP levels vary between tumours and how levels compare to those in normal cells, on various tumour types, indicating the n value for the study and a brief description of methodology. Some studies which dealt only with tumour subtypes but not with normal tissue are also included to give an insight into how readily and widely the levels of TBPs can alter in tumours and other factors involved. Some studies are considerably stronger than others from a statistical point of view and some examined TBP levels alongside telomerase activity and telomere length, aiding in the interpretation of the findings. Findings will be focussed on and classified according to the trends observed for TRF2 and POT1; but the effects on TRF1 and other TBPs where available in these same studies will also be covered for completeness. Where several studies exist for a particular tumour type/group of related tumours, these will be considered together. Studies under the broad classification of tumours of haematopoietic origin appear to be the most abundant with several study results available for comparison; perhaps due to the greater accessibility of tissue samples as the need for invasive tumour biopsies are avoided. Having an understanding of how TBP levels vary in tumours may help to predict in which circumstances treatment with agents targeting telomeric integrity may be appropriate.

2. Review of data

2.1. *Studies showing up-regulation of TBP levels in tumour tissue compared to those in normal tissue*

2.1.1. *Leukaemias/ tumours of haematopoietic origin*

Up to ~1.5-fold higher expression of TRF2 mRNA in adult T-cell leukemia (ATL) patients ($n = 6$) compared to that in human T-cell leukaemia virus type 1 (HTLV-1)-infected asymptomatic carriers ($n = 4$) or resting peripheral blood mononuclear cells (PBMCs) from an HTLV-1 negative donor ($n = 1$) ($p < 0.011$)

(uncultured samples) was detected by RT-PCR, normalised to GADPH expression.[39] TRF1 and TIN2 mRNA were also over-expressed (~2.5-fold, $p < 0.0004$ and ~2.5–3-fold, $p < 0.0001$, respectively) but POT1 did not show this pattern ($p < 0.104$). Up-regulation of TRF2 was confirmed at the protein level (visually) in 2 of 3 ATL patients compared to PBMCs ($n = 1$) and in 3 of 3 ATL patients for TRF1 and TIN2. Considerably higher telomerase activity was detected in all the ATL patients when compared to the HTLV-1 infected AS carriers; control resting PBMCs from one healthy volunteer (uninfected) showed no activity. HTLV-I-infected cells from 5 ATL patients also had significantly shorter telomeres compared to non-infected PBMCs from the same patients. *In vitro* studies revealed consistent levels of expression of TRF1, TRF2 and POT1 mRNA between 2 HTLV-1 negative human leukaemic cell lines and 7 HTLV-1 transformed or immortalised cell lines, again by RT-PCR. Subsequent analysis of mRNA extracted from one of the HTLV-1 transformed lines compared to PBMCs did suggest the over-expression of POT1 (~2.5–3-fold, $p < 0.0002$), TRF2 (~1.5-fold, $p < 0.002$), TRF1 (~1.5–1.75-fold, $p < 0.002$) and TIN2 (<1.5-fold, $p < 0.003$) in human leukaemic cells and HTLV-1 expressing cells; this was confirmed at the protein level in 3 of the HTLV-1 cell lines compared to the PBMCs of one HTLV-1 negative donor.

Four different types of B-cell non-Hodgkin lymphomas (NHLs) (mantle cell lymphoma, follicular lymphoma, diffuse large B-cell lymphoma and Burkitt lymphoma, 7 or 8 samples of each subtype, specimens obtained during surgery) and 8 lymph nodes with benign unspecific lymphadenitis as control were examined for TRF2 mRNA levels (measured by real-time RT-PCR).[40] Levels were highest in Burkitt's lymphoma (approximately twice the level of that of the reactive lymph nodes); low levels, with only minor, non-significant differences were detected between benign lymph nodes and the other lymphomas. Parallel findings were made for hPif1 – also a telomeric DNA-associated protein (and DNA helicase) which acts as an inhibitor of telomerase-mediated telomere lengthening. No significant difference was detected for the expression of TRF1 and tankyrase. Telomerase activity was also highest in Burkitt's lymphoma and it was suggested that tumours over-expressing TRF2, such as Burkitt's lymphoma, might show greater resistance to telomerase inhibition. Another possible insight arising from the results of this study was the suggestion that the down-regulation of negative telomere length regulators (e.g. hPif1) in lymphomas with high proliferation and low telomerase activity, could balance telomere loss and synthesis.

In a study of chronic myeloid leukemia (CML) ,[41] $CD34^+$ leukaemic blast cells compared to the $CD34^+$ cell population from bone marrow of healthy individuals were compared for TRF2 mRNA levels, examined by quantitative real-time RT-PCR. TRF2 was raised in the 'majority' of chronic phase patients (CP) and accelerated phase patients (AP) compared to controls or post-imatinib treated CP patients in cytogenetic remission; these data are presented in Table 1. *p*-Values were not stated. Blast crisis (BC) levels were in the range of controls in the majority of patients. TRF2 mRNA appeared to be raised in CP and AP but then decrease with disease progression to BC, correlating with tankyrase at all stages ($r = 0.859$, $p < 0.0001$); it was postulated that the raised TRF2 and tankyrase may contribute to telomere maintenance in CML. TRF1 was also increased in the majority of CP and AP relative to normal controls (CP $p = 0.0015$), reduced in BC (see Table 1).

In this study, hTERT expression was actually reduced in the majority of CML patients (all disease stages) and progressively decreased with disease progression, although a non-statistically significant increase in the functional over the inactive splice variant transcripts was observed. Although telomere length was not measured in this particular study it was suggested that the reduced hTERT expression reported could be responsible for the shortened telomeres reported in CML (Ref. within [41]).

2.1.2. *Gastric tumours*

In a study of 20 primary gastric carcinomas,[42] 12 (60%) expressed TRF2 mRNA levels at least three times greater than the corresponding non-neoplastic mucosa (measured relative to β-actin by RT-PCR). Statistical significance was not reported. Using the data from that paper and applying the reciprocal threshold to examine the incidence of under-expression in tumours relative to normal tissue, 2 of the 20 carcinomas (10%) expressed lower levels of TRF2 mRNA than the corresponding non-neoplastic mucosa, leaving 30% of tumours with levels not markedly different from matched normal tissue. An advantage for TRF2 elevation is suggested by virtue of its frequency (60%) albeit without a measure of statistical significance. Levels of TRF2 did not correlate with tumour staging by histological classification, suggesting that if TRF2 over-expression is advantageous in gastric carcinoma, this is the case only at a very early stage of carcinogenesis.

In the same study, tumours expressing higher levels of TRF2 also expressed significantly higher levels of other TBPs – tankyrase ($p = 0.0012$) and TIN2 ($p = 0.041$). TRF1 and tankyrase were over-expressed in 50% of tumour tissues, TIN2 in 30%. Whereas tumours with short telomeres (<2 kbp) possessed significantly higher telomerase activity (~3.5-fold, $p = 0.039$) and TRF1 levels (0.5 cf. 0.2, $p = 0.033$), clear trends with TRF2 were not evident. The authors proposed that tumours with shorter telomeres may require relatively higher levels of both telomerase activity and TBPs, to maintain telomere function and therefore chromosomal stability.

In another study,[43] POT1 mRNA levels, measured relative to β-actin by quantitative RT-PCR, were raised in the majority of gastric cancer cell lines ($n = 23$) compared to 5 normal human cell lines ($p \leqslant 0.05$) with mean levels in all cancer cell lines studied ($n = 56$) of 171 ± 129.6 (range 27–819), cf. 56 ± 17.7 (range 31–76) in the normal cell lines; data are reproduced in Table 2. Messenger RNA levels of the POT1 interacting protein TPP1 were also measured and shown to weakly correlate with POT1 ($p = 0.055$); neither protein message correlated with telomere or 3′ overhang length but both correlated with hTERT levels ($p \leqslant 0.001$). An indirect role in regulating telomere and/or 3′ overhang length was suggested via an effect on telomerase processivity.

2.1.3. *Lung – NSCLC*

TRF2 mRNA was found to be increased in atypical adenomatous hyperplasia (AAH), a precancerous lesion, and in bronchioloalveolar carcinoma (BAC) as compared to normal lung tissues.[44] No TRF2 mRNA staining was detected in normal lung alveolar cells and staining was detected only focally as

Table 1 – Levels of TRF2 and TRF1 mRNA in CML (Data from Ref. [41]).

Gene	Disease status[a]	N	Range	Median[b]
TRF2	Control	8	0.82–1.43	0.97
	CP	12	0.89–4.46	1.70
	AP	3[c]	1.43–1.93	1.81
	BC	7[c]	0.20–1.80	1.13
	Post-imatinib tx	7[d]	0.77–2.12	1.26
TRF1	Control	8	0.65–1.53	0.99
	CP	12	0.78–6.45	2.67 ($p = 0.0015$[e])
	AP	3[c]	1.12–4.49	3.79
	BC	7[c]	0.20–1.22	0.80
	Post-imatinib tx	7[d]	0.68–1.54	1.06

a CP, chronic phase; AP, accelerated phase; BC, blast crisis; Post-imatinib tx, post-imatinib-treated CP patients in cytogenetic remission; and control, $CD34^+$ cell population of healthy individuals.
b Median mRNA levels, determined by real-time quantitative PCR, normalised to the internal reference gene *beta-2-microglobulin* and expressed relative to average control normalised level.
c One patient was followed through from CP to AP and BC.
d Two patients in this group were originally from the CP group.
e No other *p*-values were given suggesting that this was the only statistically significant difference.

weak to moderate staining in serous and ductal cells within bronchial glands and in non-ciliated bronchiolar cells. There was no statistical difference in TRF2 mRNA expression between low-grade AAH (9/11, 82%), high-grade AAH (15/17, 83%) and BAC (35/40, 88%). This study was performed on specimens obtained from 28 AAH lesions of the lung resected from 21 patients and 40 peripherally located BACs of the lung resected from 40 patients. The incidence of positive expression of TRF2 mRNA assessed visually using *in situ* hybridisation: tumours in which the stained tumour cells made up >10% of the tumour were graded as positive. The incidence of positive TRF1 mRNA expression also increased in cancerous tissue compared to normal lung but additionally increased progressively throughout grade (36% in low-grade AAH to 65% in high-grade AAH, $p = 0.0004$, to 88% in BAC, $p = 0.046$). Expression of TRF1 in low-grade AAH was lower than TRF2, but still higher than normal cells, which showed only a weak, focal TRF1 staining similar pattern to TRF2.

The authors point out an inconsistency that when RT-PCR was used, TRF1 and TRF2 mRNA were detected in all samples available for analysis by this technique, which included the total RNA samples from 6 normal lung tissues, 1 high-grade AAH and 6 BACs (albeit at variable strengths of expression) and suggest greater sensitivity of primers for RT-PCR as a possible explanation for this discrepancy.

The high rate of TRF2 expression (detected by *in situ* hybridisation) in precursor lesions compared to normal tissue implicates TRF2 in the early steps of (lung) carcinogenesis, a role which may differ from a possible regulation of telomerase by TRF1 inferred from the correlation of TRF1 with hTR and hTERT staining scores ($p < 0.0001$) where TRF2 did not.

An *in vitro* study showed raised levels of POT1 mRNA in 5 lung cancer cell lines compared to 5 normal human cell lines ($p \leqslant 0.05$) (see Section 2.1.2 and Table 2).[43]

2.1.4. *Breast tumours*

An *in vitro* study[45] showed that the levels of TRF2 protein (relative to β-actin signal intensity on immunoblots) were at least 2-fold higher in 11/15 breast tumour cell lines compared to a primary culture of normal breast tissue (finite lifespan human mammary epithelial cells). Furthermore, five independently derived immortal HMEC lines displayed markedly increased levels (10–15 times) of TRF2 protein over the carcinogen-treated extended life precursor strain. Levels of TIN2, hRAP1 and TRF1 showed little differences in the same cultures.

Results suggested that elevated TRF2 levels frequently occur during the transformation of breast tumour cells suggestive of a role contributing to the infinite lifespan. However, differences in TRF2 mRNA levels (measured relative to 18S rRNA signal intensity by northern blot) were less marked and did not correlate with the differences in protein levels: indeed the results were strongly suggestive of variations in post-transcriptional regulation of TRF2 levels existing among finite lifespan and immortalised HMEC and highlight the importance of examining protein as well as mRNA levels before drawing conclusions.

A further *in vitro* study showed raised levels of POT1 mRNA in 8 breast cancer cell lines compared to 5 normal human cell lines ($p \leqslant 0.05$) (see Section 2.1.2 and Table 2).[43]

2.1.5. *Hepatocellular carcinoma*

2.1.5.1. Hepatocellular carcinoma. In a study of human multistep hepatocarcinogenesis,[46] mean TRF2 mRNA levels were considerably higher in hepatocellular carcinoma (34, $n = 31$) than in normal livers (10, $n = 9$, *p*-value not stated) and furthermore, levels tended to increase throughout disease progression: TRF2 mRNA levels of chronic hepatitis (CH) and liver cirrhosis (LC) tissue were higher than that of normal liver tissue ($p = 0.019$); a significant increase was also observed in the transition from low-grade to high-grade dysplastic nodules ($p = 0.016$); and a further marked increase was observed in HCC ($p = 0.036$). The data are reproduced in Table 3. TRF2 mRNA levels were also significantly higher in HCCs with poorer differentiation ($p = 0.028$) and positively correlated with the mitotic activity ($p < 0001$).

TRF1 and TIN2 also showed raised levels in HCC compared to normal liver tissue (82 vs. 26 and 51 vs. 12, respectively) and

Table 2 – Levels of POT1 and TPP1 mRNA in human cell lines (Data from Ref. [43]).

Type	Origin	Cell line	POT1[a] mRNA	TPP1[a] mRNA
Normal	Whole embryo	173We, fetus	65	25
	Colon	CCD-18 Co, fetus	31	11
	Colon	CCD-841 CoTr, fetus	76	8
	Lung	FHs738Lu, fetus	63	31
	Endothelial	HMVEC-Ad	46	12
Transformed	Kidney	293	331	74
Cancer	Colorectal	COLO205	165	47
		DLD-1	140	62
		HCT-116	353	58
		HCT-15	210	101
		HT 29	92	151
	Cervix	C33A	313	67
		CaSki	164	47
		HeLa	74	113
		SiHa	70	102
	Brain	IMR-32	181	19
		YCC-BRN	60	22
		T98G	188	33
		U87MG	148	38
	Liver	SNU-423	36	14
		SNU-182	69	21
		SNU-398	63	13
		SNU-449	84	12
		SNU-739	46	34
		SK-Hep-1	27	32
	Breast	MCF/ADR	146	79
		MCF-7	116	20
		MDA-MB-231	58	13
		MDA-MB-435	151	72
		SK-BR-3	168	5
		T47D	181	24
		YCC-B1	66	13
		YCC-B2	99	9
	Gastric	AGS	140	14
		MKN-45	445	76
		NCI-N87	144	36
		SNU-1	163	19
		SNU-484	217	14
		YCC-1	216	13
		YCC-2	318	10
		YCC-3	186	9
		YCC-6	165	18
		YCC-7	65	4
		YCC-10	63	17
		YCC-11	52	7
		YCC-16	200	19
		KATO III	147	46
		MKN-1	196	13
		MKN-28	172	10
		MKN-74	96	10
		SNU-5	101	14
		SNU-16	358	43
		SNU-216	50	14
		SNU-638	395	48
		SNU-668	163	35
		SNU-719	235	27
	Lung	A549	233	7
		NCI-H1299	819	73
		NCI-H460	177	19
		NCI-H596	96	11
		NCI-H647	178	10

a POT1 and TPP1 mRNA levels relative to *beta-actin* determined by quantitative RT-PCR ([target gene mRNA of sample/beta-actin of sample] × 100).

Table 3 – TRF2 mRNA levels and telomere length in human multistep hepatocarcinogenesis (Data from Ref. [46]).

Tissue[a]	N[b]	TRF2[c]		Telomere length[d] (kb)	
		Range	Mean ± SD	Range	Mean ± SD
Normal	9	4–24	10 ± 7.3	8.1–9.8	8.7 ± 0.5
CH	14	7–29	17 ± 6.7	6.8–9.6	8.3 ± 0.94
LC	24	4–56	17 ± 10.8	5.6–11.0	7.8 ± 1.19
LRN	5	7–22	13 ± 5.5	5.2–9.1	7.8 ± 1.63
LGDN	14	6–29	14 ± 6.4	3.9–9.8	7.4 ± 1.77
HGDN	7	14–41	25 ± 8.4	4.5–8.1	5.3 ± 1.25
DN with HCC	10	9–58	22 ± 14.7	4.4–8.5	6.4 ± 1.31
HCC	31	9–92	34 ± 20.2	4.7–14.3	7.3 ± 2.26

a CH, chronic hepatitis; LC, liver cirrhosis; LRN, large regenerative nodule; LGDN, low-grade dysplastic nodule; HGDN, high-grade dysplastic nodule; DN, dysplastic nodule; and HCC, hepatocellular carcinoma.
b Large nodules, HCCs and the adjacent non-neoplastic liver tissues collected from 10 explanted livers and 28 resected specimens; the 9 normal controls were resected liver for benign lesion or metastatic carcinoma.
c mRNA levels relative to 18S rRNA determined by quantitative real-time PCR: ([target gene mRNA copies of sample/18S rRNA copies of sample] $\times 10^5$).
d Determined by Southern blotting and hybridisation with a 3′-end digoxigenin-labeled d(TTAGGG)$_4$ probe.

similarly tended to increase in steps throughout intermediate lesions. TRF1 (only) protein expression was evaluated by immunohistochemical staining and found to correlate well with mRNA levels ($p < 0.001$). TRF1 mRNA levels (but not TIN2) were higher in tumours with poorer differentiation ($p = 0.004$); mitotic activity correlated with TIN2 ($p < 0.001$) but not with TRF1.

Telomere lengths in the tissues analysed for TRF2 mRNA expression revealed a gradual shortening during hepatocarcinogenesis, though there was no statistically significant relationship between mRNA levels of TBP genes and telomere length in the HCC tissues. Interestingly, significant negative correlations between TRF2 mRNA levels and telomere length were observed in the subset of nodular lesions with shortened telomeres compared to their respective adjacent CH and LC tissue. These results suggest that a causal association between raised TBP levels and shortened telomere length possibly can exist, but that this relationship can either be masked by other factors or be absent where telomere length is apparently dictated by a different set of molecular drivers. Thus, it was not completely resolved by this study whether the role played by raised levels of TBPs in multistep hepatocarcinogenesis related directly to the modulation of telomere length. The authors suggest that an excess of telomere-binding proteins may balance telomerase activation to maintain telomeres in the development of HCC (though the study did not analyse telomerase activity).

In another study, analysing 31 pairs of HCC and adjacent non-cancerous liver tissues, POT1 mRNA levels (measured by real-time quantitative PCR) were significantly higher in cancer compared to normal tissue (cancer range 12–421, mean 58 ± 78; normal range 5–92, mean 23 ± 17.1; $p = 0.018$). TRF1 levels were also raised in cancer tissue (mean 84 vs. 34, $p < 0.001$). Neither protein correlated with 3′ overhang or telomere length, whereas hTERT levels did.[47]

2.1.6. *Skin cancer (non-melanoma)*

Real-time PCR analysis of TRF2 levels relative to β-actin was performed on a series of human primary tumours and compared to normal skin.[48] Levels were elevated 10–50-fold in 3 of 15 basal cell carcinoma (BCC) samples and 1 of 3 squamous cell carcinoma (SCC) samples compared to normal tissue; this was confirmed at the protein level in 1 BCC and 1 SCC tumour by immunofluorescence analysis. None of the 15 BCC and 1 of the 3 SCC samples showed a greater than 10-fold reduction in relative TRF2 level compared to the corresponding normal tissue, and the remaining showed no significant difference in relative TRF2 mRNA level from normal control tissue.

The authors suggested therefore that increased expression of TRF2 can be associated with human skin carcinogenesis and experiments with mice suggested a mechanism for the apparent role of TRF2 in the pathogenesis of skin cancer involving XPF nuclease and increased sensitivity to UV irradiation.[49]

2.1.7. *Cervical cancer*

An *in vitro* study showed raised levels of POT1 mRNA in 4 cervical cancer cell lines compared to 5 normal human cell lines ($p \leqslant 0.05$) (see Section 2.1.2 and Table 2).[43]

2.2. *Studies showing down-regulation of TBP levels in tumour tissue compared to those in normal tissue*

2.2.1. *Tumours of haematopoietic origin*

In leukaemic cells from 16 acute leukaemia patients – 3 acute lymphoblastic leukaemia (ALL) and 13 acute myelogenous leukaemia (AML), peripheral blood or bone marrow samples – mean expression of TRF2 mRNA was significantly lower than in normal leukocytes – normal granulocyte, monocyte and B- and T-lymphocyte fractions obtained from peripheral blood of 6 healthy donors ($p < 0.01$); levels were measured relative to GADPH by quantitative TaqMan RT-PCR.[50] There were parallel findings for TRF1 ($p < 0.01$) and TIN2 ($p < 0.05$). Statistical significance was higher for TRF2 and TRF1 ($p < 0.001$ for both) when comparing only the 13 AML patients with normal controls, whereas there was no significant difference for the 3 ALL patients compared to the normal lymphocyte fractions. Actual levels were not stated but changes in means were less than an order of magnitude. The opposite trend was observed for telomerase activity, being approximately 5–10-fold higher

in patient samples ($n = 12$) than in normal B- and T-lymphocytes ($n = 4$), respectively, and no activity was detected in normal monocyte and granulocyte fractions. Telomere length was shorter in patient samples ($n = 12$) than the normal leukocyte fractions ($n = 3$, $p < 0.05$). (No significant difference was observed between normal cell types). The model proposed to fit the results was one of telomerase reactivation by shortening of telomere length during haematopoietic carcinogenesis, with the down-regulation of negative regulators of telomere length (i.e. TRFs, TIN2) to maintain telomere length. *In vitro* studies on the HL-60 cell line showed a gradual increase in TRFs and TIN2 mRNA upon the induction of differentiation by TNF 471 and all-trans retinoic acid (4–4.5-fold over 4–5 days); this was accompanied by a significant decrease in telomerase activity (~98%), again suggesting an inverse relationship between telomerase activity and TBP levels.

A significant decrease in mRNA expression of TRF2, TRF1 and POT1 was detected in total blood samples from 42 B-cell chronic lymphocytic leukaemia (B-CLL) patients as compared to 20 healthy donors ($p = 0.036$, $p < 0.001$ and $p < 0.001$, respectively), measured by quantitative PCR and normalised to three reference genes.[51] Although results were not confirmed at the protein level, reduction in the expression of factors involved in telomere capping was postulated to facilitate telomere degradation and shortening and thus possibly explain the shortened telomeres reported in B-CLL cells and associated with poorer prognosis (Ref. within [51]). Levels of hRAP1 were also decreased in B-CLL ($p < 0.001$), TPP1 levels were raised ($p < 0.001$) and no significant change was observed in TIN2.

2.2.2. *Gastric tumours*

Cancerous and adjacent non-cancerous tissue samples were obtained from 32 patients undergoing surgery[52]; non-cancerous mucosa included normal mucosa, atrophic gastritis and intestinal metaplasia. Mean TRF2 mRNA levels, normalised to GADPH (determined by quantitative RT-PCR) were significantly down-regulated in gastric cancer tissue (13.7 ± 9.0, $n = 24$) compared to the non-cancerous gastric mucosa tissues (25.3 ± 20.2, $n = 53$, $p < 0.002$). Although data were not shown, the authors reported that there was no down-regulation of TBPs in the intestinal metaplasia and atrophic gastritis tissues compared to the normal mucosa, interesting because both represent high-risk conditions for the development of cancer; by contrast, changes in TRF2 levels (up-regulation) were detected in precursor lesions of liver and of lung cancer ([46,53] see above and below, respectively). Significant down-regulation of TRF1 (mean 14.5 ± 9.9 vs. 32.7 ± 25.1, $p < 0.0001$) TIN2 and tankyrase (but not hRAP1) was also detected in the gastric cancer tissue. The authors speculated that the modulation of telomere length by alteration in levels of these proteins would not seem a sufficient explanation for the observation since TRF2 and tankyrase have been reported to have opposing effects on telomere length (in separate studies: [54,55]); note though that telomere length was not actually measured in this study.

Evidence of down-regulation of TRF2 protein in a further study,[56] which obtained cancer and ~3 cm-separated non-cancerous gastric tissue specimens from surgically resected stomach tissue, was obtained using immunohistochemistry. TRF2 (and TRF1) protein staining was ubiquitous, therefore staining was scored positive when cells were judged to have greater TRF staining intensity than co-present interstitial lymphocytes; a total of 1000 cancerous and 1000 normal epithelial cells were observed and positive staining ratios calculated. The average positive staining ratio for TRF2 was $65.6 \pm 20.7\%$ in non-cancerous tissue and significantly lower, $42.9 \pm 22.1\%$, in cancerous tissue ($p < 0.01$) (range 17.1–95.3% and 6.9–85.0%, respectively). The TRF2 positive staining ratio correlated negatively with depth of invasion and histological classification, e.g. T0/1, mean 62.6 vs. T4, mean 18.1, $p < 0.01$; G1 and G2, mean 60.9 vs. G3 and G4, mean 32.1, $p < 0.001$. There were parallel findings of similar magnitude with TRF1. Expression of TRFs did not correlate with telomerase activity which was detected in a significantly higher proportion of cancer than non-cancer tissues, and activity levels were significantly higher in cancer; however, telomerase activity did not increase with depth of tumour. The authors proposed that the suppression of TRF-mediated telomerase inhibition with tumour progression, through the down-regulation of TRF levels, may help to maintain the proliferative capability of the cancer cells and efficient telomerase activity.

POT1 levels were compared in 51 pairs of gastric cancer tissues and the corresponding non-neoplastic mucosae with no significant inflammatory involvement.[57] Tissue pairs were maintained and quantitative RT-PCR used to compare POT1 (only) mRNA levels in tumours relative to the corresponding normal tissue to give a T/N ratio; a T/N ratio > 2.0 was deemed to represent up-regulation and a T/N < 0.5 to represent down-regulation. Up-regulation of POT1 was found in 23.5% of cases (12/51) and down-regulation in 35.3% of cases (18/51). Approximately half the GC patients showed no changes in POT1 expression. Down-regulation of POT1 (T/N < 0.5) was observed significantly more frequently in stage I/II GC (incidence 52.4%, 11 of 21) than in stage III/IV GC (incidence 23.3%, 7 of 30; $p = 0.033$) and conversely, up-regulation of POT1 (T/N > 2.0) was observed significantly more frequently in stage III/IV GC (33.3%, 10 of 30) than in stage I/II GC (9.5%, 2 of 21; $p = 0.048$). There was also a positive correlation in T/N ratio with tumour progression, which was significantly higher in stage III/IV tumours than in stage I/II tumours (3.84 ± 8.02 vs. 0.75 ± 0.72, respectively, $p = 0.005$). Changes in POT1 expression levels would therefore seem to be associated with stomach carcinogenesis and GC progression. Telomere length and 3′ telomeric overhang signals were analysed in 20 of the 51 GC tissues (randomly selected). POT1 mRNA expression levels decreased in accordance with telomere shortening ($r = 0.713$, $p = 0.002$) and decreases in 3′ telomeric overhang signal ($r = 0.696$, $p = 0.002$) suggesting a role for POT1 in the regulation of telomere length (or alternatively an association between POT1 levels and telomere length). Further work with POT1 antisense oligonucleotides suggested that the inhibition of POT1 may induce telomere dysfunction (telomere shortening, reduction in overhang and appearance of anaphase bridges).

A further study[58] demonstrated that POT1 expression in 4 gastric cancer cell lines was decreased compared to that in lymphocytes obtained from healthy individuals ($n = 3$) (levels in cell lines determined from the signal density of immunoblotting standardised to that in lymphocytes, which was set to 100%, ranged 25–47%). The cancer cells also showed shorter

telomere length (range across the 4 cell lines was 2.8–4.7 kb, compared to 9.9 kb in lymphocytes), moreover, POT1 levels were shown to be significantly correlated with telomere length (Spearman $r = 0.9998$, $p = 0.0167$). In surgical specimens resected from 24 gastric cancer patients, POT1 index, assessed by immunohistochemistry compared to infiltrating lymphocytes, was reduced in stage III-IV gastric cancers ($23 \pm 14\%$, $p < 0.0001$) compared to stage I-II gastric cancers ($39 \pm 14\%$, $p < 0.0001$) (stage III-IV vs. I-II, $p < 0.05$). POT1 index in gastric epithelia distant from the cancer was $84 \pm 14\%$ ($p < 0.0001$) and $72 \pm 24\%$ ($p < 0.0001$) in peritumoral epithelia. Additionally, POT1 index was compared with telomere volume determined previously by FISH and the two found to correlate ($p < 0.0001$, Spearman $r = 0.7137$). The results suggest that POT1 expression corresponds to telomere length in gastric cancer tissue. POT1-low cases showed advanced cancer invasion ($p < 0.05$) but no difference in nodal metastasis, peritoneal dissemination or histological type.

2.2.3. *Breast tumours*

A study of breast tissue using *in vivo* samples[59] revealed some interesting trends: in 127 breast cancer tissues and 33 normal background tissues collected after surgery, the levels of POT1 mRNA measured by real-time PCR were lower in malignant compared to non-malignant tissues (means 85,454 vs. 144,218, respectively); this was statistically significant only when comparing mean levels in normal tissues (144,218) with TNM3 (1,327, $p = 0.012$) and TNM4 (28,206, $p = 0.011$), although there was not a consistent decreasing trend for stages TNM1 to TNM4. Comparing clinical outcomes, POT1 levels were significantly higher ($p \leqslant 0.05$) in tumours of patients who remained disease free than in those who suffered either local recurrence, bony metastasis, or died from breast cancer, possibly suggesting a tumour suppressive effect of POT1. Mean data for POT1 and TRF2 are reproduced in Table 4.

The trend was similar for TRF2 with non-cancerous tissues expressing higher mRNA levels than malignant (mean 255 versus 178.5); however, statistically significant differences were not observed. TRF2 mRNA decreased with increasing grade of the tumour (p-values not stated). Levels were particularly low in TNM stage 3 – with statistically significant differences between TNM1 and TNM3 (means 255 vs. 0.74, $p = 0.05$), and TNM2 and TNM3 (85 vs. 0.74, $p = 0.0021$) but again without a consistent decreasing trend for stages TNM1 to TNM4. There also was not a clear trend of decreasing levels with deteriorating clinical outcomes but levels were higher in tumours from patients who remained disease free compared with those who developed local recurrence (means: 174 vs. 79, $p = 0.43$) or distant metastasis (means: 174 vs. 26, $p = 0.12$) though these differences were not statistically significant; levels in patients who died of cancer were intermediate between disease free survivors and those with local recurrence or distant metastasis (132.7).

It was suggested that POT1 (and TANK2) being significantly over-expressed in this study in benign tumours compared to advanced tumours, may possess tumour suppressor properties, perhaps through negative regulation of telomerase activity (not measured directly though component gene expression supports this). No clear trends emerged for TRF1 for which mean levels were higher in malignant tissues but not significantly.

The mRNA expression of TANK2, TIN2, hTERT, TANK1, TEP1, EST1 and hTR was also measured, but the findings are outside the scope of this review. Briefly, the levels of TANK2 and TIN2 transcription were also lower in malignant tissues, whereas hTERT, TANK1, TEP1, EST1 and hTR were higher in tumour samples compared with normal breast tissue.

In another study of breast cancer,[60] expression of TRF2 and TRF1 mRNA was significantly lower in cancerous tissue ($n = 38$) than control tissue from the same resected breasts ($n = 16$) by an approximately 2- to 3-fold difference in mean levels, measured by quantitative TaqMan RT-PCR ($p < 0.0001$). Levels were higher in breast cancer tissues without detectable telomerase activity ($n = 13$) compared to those with ($n = 25$) (TRF2: $p < 0.016$; TRF1: $p < 0.001$). There was a non-significant negative correlation between expression of the TRFs and telomere length. A requirement in breast cancer for escape from negative regulation of telomerase activity by TRFs was proposed as an explanation for the findings.

2.3. *Studies showing no change in TBP levels in tumour tissue compared to those in normal tissue*

2.3.1. *Lung*

A study of 148 non-small cell lung cancer (NSCLC) patients[c] (mainly adenocarcinoma or squamous cell carcinoma) did not detect any significant difference in mean expression levels of TRF2 or POT1 mRNA between the samples of tumour tissue (T) and paired adjacent normal tissue (N) (TRF2: N, 1.03 ± 1.22; T, 1.06 ± 1.16; $p = 0.81$; POT1: N, 0.61 ± 0.36; T, 0.54 ± 0.33; $p = 0.10$).[53] Levels were determined by real-time quantitative RT-PCR and normalised to the geometric mean of 3 internal housekeeping control genes. However, TRF2 expression in tumour tissues was found to be significantly inversely correlated with tumour grade whereby the higher the grade, the lower the gene expression, suggestive of a protective role of TRF2 against cancer progression (grade 1, well differentiated, 1.95; grade 2, moderately differentiated, 0.98; and grade 3, poorly differentiated, 0.87; $p = 0.0114$.) POT1, TRF1 and RAP did not show such a correlation. In contrast to POT1 and TRF2, the TRF1 mRNA level was significantly lower in tumour tissues compared to normal tissue (N, 65.7; T, 30.14; $p < 0.0001$) but did not correlate with tumour grade. Interestingly, higher RAP1 expression was associated with significantly better survival though no difference in RAP1 mRNA level between normal and tumour tissues was observed (N, 0.63; T, 0.97). Though data on telomere length were not presented, the authors stated that no association between telomere length and TBP gene expression was observed, nor between telomere length and overall survival, suggesting that the possible protective effect of TRF2 and indeed predictive property of RAP1 might be independent of telomere length. The study did not examine telomerase activity levels.

In another study of lung cancer,[61] equivalent incidence of positive expression of TRF2 mRNA was detected by RT-PCR

[c] Histology breakdown: adenocarcinoma, 70; squamous cell carcinoma, 53; large cell carcinoma, 4; adenosquamous carcinoma, 3; bronchioloalveolar carcinoma, 13; and incomplete, 5.

Table 4 – Mean levels of POT1 and TRF2 mRNA in patient subgroups (Data from Ref. [59]).

Parameter		POT1[a]	TRF2[a]
Tissues	Normal	144,218 ± 168,208	255 ± 776
	Malignant	85,454 ± 164,892	178.5 ± 52.7
Prognostic groups	NP-1 (<3.4)	89,015 ± 179,735	178.5 ± 112.7
	NP-2 (3.4–5.4)	98,371 ± 177,011	363 ± 1075
	NP-3 (>5.4)	54,586 ± 101,849	260 ± 566
Tumour grade	Grade-1	122,026 ± 235,211	492 ± 1585
	Grade-2	85,662 ± 146,850	122.3 ± 522.8
	Grade-3	77,066 ± 161,689	0.741 ± 1.420
Tumour staging	TNM1	82,062 ± 153,519	31.4 ± 44.4
	TNM2	79,924 ± 126,179	85.5 ± 139.8
	TNM3	1327 ± 2249	0.741 ± 1.420
	TNM4	28,206 ± 34,863	28.4 ± 44.6
Clinical outcome over 10 years	Disease free	87,094 ± 175,248	173.9 ± 50.9
	Local recurrence	43,625 ± 319,993	78.6 ± 171.8
	Distant metastasis	49,994 ± 106,784	26.2 ± 51.8
	Died of breast cancer	47,502 ± 99,785	132.7 ± 281.0

a Mean mRNA levels, determined by real-time quantitative PCR, normalised against CK19 expression already measured in the specimens, with standard deviation.

in paired tumour (83.3%) and normal (82.7%) lung tissues ($n = 78$; 42 squamous cell carcinoma (SCC), 36 adenocarcinoma). In contrast, TRF1 was expressed at a higher rate (incidence) in tumour tissues compared to normal tissues (74.4% vs. 49.3%; $p = 0.003$). There was no association between the expression of telomerase activity and TRF1 or TRF2 (or hTERT), though hTERT expression was closely related to both TRF1 ($p < 0.003$) and TRF2 ($p < 0.024$) (and c-MYC).

2.3.2. *Oesophageal SCC*

TRF2 mRNA (detected by RT-PCR) was expressed in 91.9% of surgically excised oesophageal squamous cell carcinoma tumour tissues, a rate not significantly higher than in the paired normal oesophageal mucosa tissues (90.5%) ($n = 74$ paired specimens).[62] In contrast, the TRF1 expression rate was significantly higher in tumour tissues compared to normal tissue (82.4% vs. 58.1%, $p = 0.002$). The expression rate of TRF2 mRNA (but not TRF1) was significantly higher in the tumours that were positive for telomerase activity (95% vs. 72%, $p = 0.039$) which led the authors to suggest that upon telomerase activation TRF1 expression is suppressed to prevent interference with telomerase binding and instead, TRF2 over-expression persists for more efficient T-loop formation. However, TRF1 mRNA expression was still 84% in telomerase positive tumours and 72% in telomerase negative tumours thus very limited data exist to support this. Interestingly, the study did reveal that when the T/N TRF length ratio decreased to a critical level ($\leqslant$85%) a better survival was observed, perhaps due to the failure of tumour cells to regain adequate telomere length, thus triggering apoptosis.

2.3.3. *Epithelial keratinocytes and squamous carcinoma*

In a panel of 1 human vulval epidermoid cancer cell line, 2 squamous cell carcinoma (SCC) cell lines, 1 normal oral keratinocyte (NOK) strain, 1 normal ectocervical epithelial cell strain (NCE) and 8 HPV16- or SV-40-immortalised NCE cell lines *in vitro*, constitutive expression of TRF2 mRNA, examined by RT-PCR, was observed in all normal, immortalised and cancer cells, at similar levels throughout.[63] TRF1 mRNA levels on the other hand, rose during serial passage of primary culture, and were expressed at higher levels in immortalised and cancer cells.

2.3.4. *Other tumour types*

An *in vitro* study examining POT1 mRNA level (relative to β-actin by quantitative RT-PCR) showed no significant difference from that of 5 normal human cell lines, in liver ($n = 6$), brain ($n = 3$) or colorectal ($n = 5$) cancer cell lines (see Section 2.1.2 and Table 2).[43]

2.4. *Studies comparing tumour subtypes only*

Two studies examined subtypes of leukaemias for significant differences in TRF2 levels between the cancer types and found none; both, however, did detect differences in TRF1 levels. In a subset of AML cases, characterised by chromosome aberrations specifically consistent with telomere dysfunction, no reduction in TRF2 or POT mRNA was observed compared to the other cases[64]; levels were measured by real-time quantitative PCR relative to porphobilinogen deaminase (PBGD). However, higher TRF1 mRNA expression was observed and telomere length was significantly shorter despite higher telomerase activity (not correlating with hTERT expression); telomerase-mediated telomere elongation in this group of AML therefore appeared to be repressed via TRF1. Results did not support reduced expression of TRF2 or POT1 as a cause for telomere dysfunction generating the chromosome instability in AML (although differences in expression at the protein level could not be excluded.) Ageing-related critical shortening of telomeres (crisis) was proposed as the cause of the chromosomal aberrations in this subset of AML, with up-regulation of telomerase.

A separate group of researchers showed that whereas TRF2 levels (measured relative to glyceraldehyde-3-phosphate-

dehydrogenase (G3PHD) by RT-PCR) did not discriminate between the type of leukaemia (AML versus ALL), TRF1 expression was significantly elevated in patients with ALL compared to AML ($p = 0.0232$, $n = 15$ and 29, respectively); levels also tended to be higher in patients without telomere shortening ($p = 0.077$) and with hTERT expression ($p = 0.055$).[65]

3. Discussion and conclusions

3.1. *Is there a picture emerging on the levels of TBPs in tumours?*

This review of the literature, focussing on TRF2 and POT1, has shown instances of up-regulation in cancer compared to normal tissue, (Section 2.1) balanced in possibly equal measure by instances of down-regulation (Section 2.2) and no notable difference (Section 2.3). Relative levels would seem to vary according to the particular cancer type, stage,[d] grade[e] and genetic context but with no predictable pattern yet based on the current level of knowledge and understanding. Parallel changes in TRF1 and TRF2 levels are evidenced,[39,41,46,50–52,56,60] as are divergences in trends amongst these two proteins.[40,44,45,61–65] TRF2 has been assigned the role of telomere capping,[66,67] whereas TRF1 has been implicated as a negative regulator of telomerase activity[18]; although both have been reported to negatively regulate telomere length,[55] TRF2 purportedly does so independently of telomerase[68]; these deviations in properties might rationalise the lack of a consistent relationship between TRF1 and TRF2 levels.

3.1.1. *Relationship with telomerase*

Given the mechanistic relationship between telomerase activity and TBPs, both required for chromosomal stability, some interplay in levels is expected and thus many researchers have incorporated measures of telomerase activity into their studies of TBP levels and vice versa. Whilst TBPs have demonstrated telomere-stabilising properties[66,67,69] delaying the onset of senescence,[18] paradoxically they have also been described in some reports as negative regulators of telomerase activity and thus telomere length,[24,55] perhaps by impeding telomerase access to the telomere. Thus, theoretically tumour survival may benefit on the one hand from reduced expression of TBPs (relative to normal tissue) in order to maximise telomere-maintaining activity of telomerase, but on the other hand, raised levels of telomere stabilising TBPs may help counter any shortfall in telomere-maintaining activity of telomerase by increasing the stability of short telomeres. Indeed, interpretations of the relationship between telomerase and TBPs are not consistent and appear to be highly context dependent. A common thread, however, is that of a homeostatic control mechanism for telomere stability conferred by a balance of telomerase activity and TBP levels (mutual modulation). Down-regulation of certain telomerase-inhibitory TBPs (e.g. TRF1, POT1, hPif1) may enable tumour cells to escape from negative regulation of telomerase activity and thus promote telomere stability[40,50,56,59,60]; in other cancers, high levels of TBPs may counterbalance telomerase activity to regulate telomere length.[43,44,46,64] Conversely, increased expression of TBPs may compensate for telomerase down-regulation[70] to maintain telomere stability. Also postulated[61,62] is a modulation of TRF1 levels to regulate telomerase but persistence T-loop-promoting TRF2 in carcinogenesis, and finally an additive action of telomerase activity and TBPs to maintain telomere function and chromosomal stability.[42]

3.1.2. *Relationship with telomere length*

A correlation between TBP level and telomere length might be envisaged due to the potential availability of binding sites for protein. Studies have largely failed (where investigated) to show a correlation between telomere length and TRF2 level[53,65]; a significant *negative* correlation was observed between telomere length and TRF2 levels in just a subset of hepatocellular carcinoma lesions[46] and one study[60] detected a (non-significant) negative correlation between expression of the TRFs and telomere length in breast cancer. TRF2 mRNA expression rate was demonstrated to be greater amongst NSCLC samples with relatively shorter telomere lengths (TRF length $\leqslant$ 75% of paired normal tissue, expression rate 92.3%, $n = 39$; TRF length > 75% of paired normal tissue, expression rate 75%, $n = 40$; $p = 0.03^{*}$)[71] which was also true of TRF1 (expression rate 82.5% vs. 65%, $p = 0.045$) – levels were measured by RT-PCR in surgically resected tissue.[f] Down-regulation of TRF2 mRNA has also been reported amongst tumour samples with reactivated telomerase and telomere shortening relative to normal tissue.[72] These results suggest that overall, it may be the balance of telomere length, TBP levels and telomerase activity that determine the rate of DNA damage signalling emanating from the chromosome termini and in turn, cell survival, rather than a simple correlation between two of these factors (see Fig. 2).

However, there is some evidence that POT1 mRNA expression levels may correlate with telomere (and overhang) length – a decrease in POT1 in accordance with telomere shortening was demonstrated ($r = 0.713$, $p = 0.002$)[57] and decreases in 3′ telomeric overhang signal ($r = 0.696$, $p = 0.002$) suggesting a role for POT1 in the regulation of telomere length; furthermore, a recent study demonstrated a significant correlation between telomere length and levels of POT1 in gastric cancer, both in cell lines and cancer specimens from patients.[58] One in vitro study, however, failed to detect a correlation between POT1 mRNA levels and telomere (or 3'-overhang) length across 56 cancer cell lines compared to 5 normal human cell lines.[43]

3.1.3. *Influence of genetic context*

Intuitively, the carcinogenic history and other features and contexts of the particular cancer would seem to dictate whether there is advantage and therefore selection pressure for alteration in the levels of TBPs compared to normal tissue.

[d] See Ref. [42], Section 2.1.2 (gastric, no correlation of TRF2 levels with stage) and Ref. [57], Section 2.2.2 (gastric, changes in POT1 levels according to tumour stage).

[e] Studies have shown, variously, correlation of TRF2 levels with grade (Ref. [46] – hepatocarcinogenesis, Section 2.1.5, also observed of TRF1); inverse correlation with grade (Ref. [53] – lung, Section 2.3.1 and Ref. [59] – breast, Section 2.2.3, no such correlations for TRF1); and no correlation with grade (Ref. [44] – lung, Section 2.1.3, but an increase observed for TRF1).

[f] TRF2 was expressed in 83.3% of tumour tissues overall and TRF1 in 74.4%, but the expression rates in normal tissue were not stated.

A role for an elevation of TRF2 promoting carcinogenesis is implied by results from the studies showing a significant increase in TRF2 levels in cancer compared to normal tissues (i.e. Section 2.1) but there is some evidence for a changing role for POT1 from tumour initiation to progression ([57], Section 2.2.2). Speculatively, low levels may initially be selected through promoting chromosomal instability (in the absence of telomerase activity) and thus the acquisition of the necessary genetic changes for cancer initiation; however, as the cancer progresses, continued low levels may lead to unsustainable genetic instability, inducing crisis and cell death, thus cells with raised expression would be selected. Presumably several other factors would influence the net process – e.g. telomere length initially, rate of proliferation, at what point and to what level telomerase was activated, and other modifiers of telomere stability such as DNA damage response proteins (see Fig. 2). If at early stages of carcinogenesis TBPs are critically involved, does their function remain critical to the advanced tumour?

There is also a body of studies, covered in Section 2.2, showing *lowering* of TRF2 in cancer compared to normal tissues, indicative of the potential for tumour *suppression* by TRF2, though this does not necessarily exclude the possibility that TRF2 may play a role in early carcinogenesis as just discussed: with the dynamic nature of cancers and increasing genetic instability, the set of conditions favouring initial cancer development may not favour progression, considering parallel changes in other telomere length/stability and telomerase modulating genes altering the selective pressure on individual TBP expression. It is worth noting that leukaemias may be distinct from solid tumour types in the interplay between the levels of TBPs, telomerase expression and activity and telomere length, because normal B- and T-lymphocytes have been shown to express telomerase even in the non-cancerous state, sometimes at levels comparable with leukaemia tumour samples, which would be expected to impact differentially on telomere length dynamics during carcinogenesis.[64,73,50] Whether raised or lowered levels of TBPs prevail may depend on the particular cancer and its unique genetic fingerprint.

3.2. *Possible reasons for discrepancies in findings*

The reviewed studies have improved our knowledge of how TBP levels can vary between cancers and throughout the carcinogenic process but have shown a lack of consistency in findings between tumours and/or studies. As discussed in Section 3.1, the specific tumour's biology will influence whether and if so how the levels of TBPS are altered compared to counterpart non-cancerous tissues; notwithstanding this, differences in study design are likely to be involved to some extent. For example:

(i) Source of control tissue – whether from the same individual as the cancer samples when paired tissues were collected (e.g. [47,52,53,56,61,62]) or whether from healthy volunteers (e.g. [41,50,51]) or from patients with other conditions (e.g. [39,46]); where control tissue is from that adjacent to the cancer, whether that tissue is sufficiently demarcated from the cancer in gene expression terms, as tissues close to the cancer may show gene expression changes compared to normal tissue.[58,74]

(ii) Whether the study has examined (relative) levels of gene expression (e.g. [39,41,42,46,51,53]) or rates of expression (all or nothing according to arbitrary threshold) (e.g. [44,61,62]) – if a balance of TBPs, telomere length and telomerase activity is required to maintain telomere stabil-

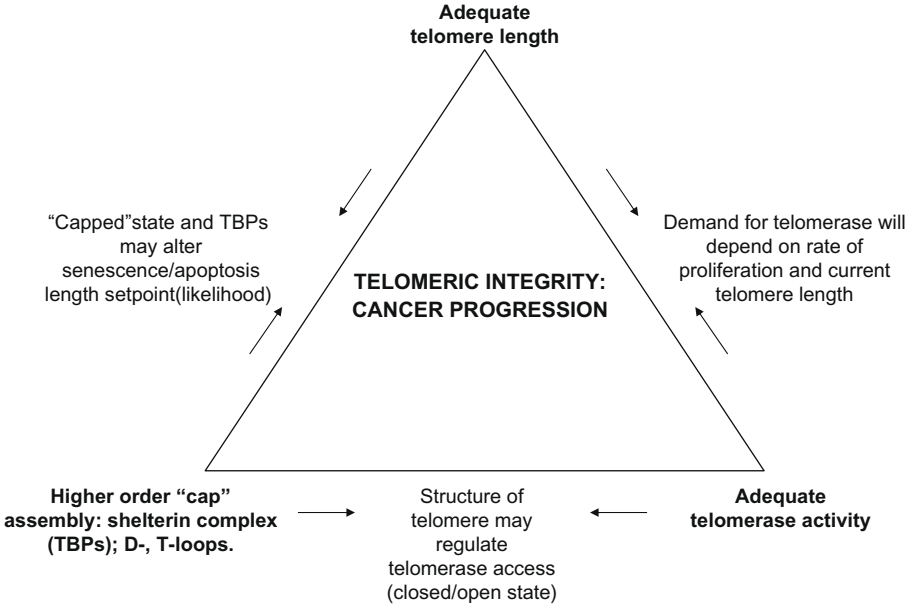

Fig. 2 – The complexity of telomeric integrity. Individual tumours may achieve telomere stability through a differing balance between the key mediators of telomeric integrity – telomere length, telomerase activity and capping status. This variability may explain the lack of consistent trends in the literature reports examining levels of individual TBPs in groups of cancers.

ity and in turn tumour viability, then an experiment detecting expression above a threshold may not tell the whole story.

(iii) When examining relative expression levels, the potential for fluctuations in 'housekeeping' genes must be considered; studies normalising to three genes are likely to give more reliable results (e.g. [51,53]).

(iv) Whether tumour and the corresponding normal tissue samples are maintained as pairs and a ratio of expression obtained for each pair (e.g. [57]) – or pooled into separate tumour and control populations for the analysis of mean levels or rates of expression in each population (all other examples cited in this section except [42]); if there is a wide variability in expression between individuals the latter approach may mask a trend to differences between cancer and normal tissue.

(v) Whether RNA or protein was measured (note that in Ref. [45] both were measured and discrepancies detected). Only a minority of the studies discussed in this review have confirmed that protein levels reflect changes observed in mRNA levels ([39]; limited confirmation in [46,48]) thus all findings must be interpreted with caution.

(vi) The technique used for detection, e.g. *in situ* hybridisation allows visual confirmation at the cellular level to avoiding the potential problem of contamination in PCR experiments by lymphocyte infiltration for example, but needs controlling for non-specific binding of probe TRF2 staining dispersed throughout the nuclei has been demonstrated suggesting not all TRF2 is telomere-associated[45]; indeed changes in the localisation of TBPs may be of critical importance in acquisition and maintenance of telomere end protection[75] and this may not be revealed through studies of levels.

(vii) Caution must be exercised in extrapolating results from cultures of cells since growth conditions may have pronounced effects on gene expression compared to the tissues from which the cells were derived; with surgically resected tissue, the speed with which the tissue is frozen would be critical in maintaining a true reflection of gene expression.

3.3. *Potential significance of findings for therapies targeting telomere maintenance/integrity*

Do raised levels of TBPs in cancers relative to normal tissue indicate greater reliance of the tumour on those proteins than normal cells, conferring target status and implying a high susceptibility of such cancers to agents interfering with the binding of those proteins? Or do raised levels simply provide a higher reservoir of target protein to pose greater resistance to therapies aiming to displace those proteins? Would tumours with lowered TBP levels compared to normal tissue be more sensitive to telomere and telomerase targeted therapies? Influence of TBP levels on the outcome of treatment with telomere or telomerase targeted therapies can certainly be envisaged, through both direct capping effects and through indirect effects on telomere length mediated through telomerase. Actually in several studies a wide range of levels of TBP gene expression has been detected in tumour samples, as much as a log difference,[46,56,65] suggesting that if levels do modulate response to therapy there is a scope for a broad distribution of responsiveness/sensitivity, possibly necessitating pre-screening. Aside from protein levels, there may also be the potential for changes in the localisation of TBPs,[75] and this may too be speculated to modulate the response to telomere targeted agents.

Important questions as to the possible toxicological sequelae associated with telomere integrity/maintenance targeted therapies which displace TBPs are beyond the scope of this review, but clinical trials are certainly needed to assess the utility and limitations of such agents. Simply considering the levels of TBPs in tumours compared to normal tissue, no one individual TBP stands out as a particularly accessible target; nonetheless given the reliance of telomeres on TBPs as fundamental factors conferring telomeric integrity and hence permitting cancer progression (Fig. 2), they should logically be pursued as therapeutic targets in their own right with investigation into whether a therapeutic window can be achieved. What is suggested by the data covered in this review, however, is that whether and how the levels of TBPs impact on attempts to disrupt telomere maintenance for therapeutic gain is likely to be very tumour specific and that we are unlikely to be able to predict sensitivity to such therapies based on TBP levels alone.

3.4. *Suggestions for future studies*

Questions addressing the levels of TBPs at telomeres may be rather too simple and perhaps we should be asking what is the state of the protein complexes at telomeres and how do they interconnect with the activity and localisation of telomerase and current telomere length and higher structure. To capture a picture of this dynamic aspect of chromosome biology, clearly critical in cancer, will demand an experimental strategy that is consistent and highly sophisticated in its approach, with the design of integrative biological studies incorporating genomics and proteomics for TBPs and associated proteins, assays of telomerase activity and localisation, and telomere length, to gain a fuller understanding of the functional telomere complex.

Conflict of interest statement

J.C. is an employee of Pharminox Ltd.

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REFERENCES

1. Moyzis RK, Buckingham JM, Cram LS, et al. A highly conserved repetitive DNA sequence $(TTAGGG)_n$, present at the telomeres of human chromosomes. *Proc Natl Acad Sci USA* 1988;**85**:6622–6.

2. de Lange T. Shelterin: the protein complex that shapes and safeguards human telomeres. *Genes Dev* 2005;**19**:2100–10.
3. Liu D, O'Connor MS, Qin J, Songyang Z. Telosome, a mammalian telomere-associated complex formed by multiple telomeric proteins. *J Biol Chem* 2004;**279**:51338–42; Wang F, Podell ER, Zaug AJ, et al. The POT1-TPP1 telomere complex is a telomerase processivity factor. *Nature* 2007;**445**:506–10.
4. Harley CB, Futcher AB, Greider CW. Telomeres shorten during ageing of human fibroblasts. *Nature* 1990;**345**:458–60.
5. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;**100**:57–70 [Review].
6. Kim NW, Piatyszek MA, Prowse KR, et al. Specific association of human telomerase activity with immortal cells and cancer. *Science* 1994;**266**:2011–5.
7. Bryan TM, Englezou A, Gupta J, Bacchetti S, Reddel RR. Telomere elongation in immortal human cells without detectable telomerase activity. *EMBO J* 1995;**14**:4240–8.
8. Blackburn EH. The end of the (DNA) line. *Nat Struct Biol* 2000;**7**:847–50.
9. Autexier C, Lue NF. The structure and function of telomerase reverse transcriptase. *Ann Rev Biochem* 2006;**75**:493–517; Gillis AJ, Schuller AP, Skordalakes E. Structure of the *Tribolium castaneum* telomerase catalytic subunit TERT. *Nature* 2008;**455**:633–7.
10. Cohen SB, Graham ME, Lovrecz GO, Bache N, Robinson PJ, Reddel RR. Protein composition of catalytically active human telomerase from immortal cells. *Science* 2007;**315**:1850–3.
11. Blackburn EH. Telomere states and cell fates. *Nature* 2000;**408**:53–6.
12. Zhou J, Hidaka K, Futcher B. The Est1 subunit of yeast telomerase binds the Tlc1 telomerase RNA. *Mol Cell Biol* 2000;**20**:1947–55.
13. Qi H, Zakian VA. The saccharomyces telomere-binding protein Cdc13p interacts with both the catalytic subunit of DNA polymerase alpha and the telomerase-associated est1 protein. *Genes Dev* 2000;**14**:1777–88.
14. Reichenbach P, Höss M, Azzalin CM, Nabholz M, Bucher P, Lingner J. A human homolog of yeast Est1 associates with telomerase and uncaps chromosome ends when overexpressed. *Curr Biol* 2003;**13**:568–74.
15. Zhou XZ, Lu KP. The Pin2/TRF1-interacting: protein PinX1 is a potent telomerase inhibitor. *Cell* 2001;**107**:347–59.
16. Banik SS, Counter CM. Characterization of interactions between PinX1 and human telomerase subunits hTERT and hTR. *J Biol Chem* 2004;**279**:51745–8.
17. Court R, Chapman L, Fairall L, Rhodes D. How the human telomeric proteins TRF1 and TRF2 recognize telomeric DNA: a view from high resolution crystal structures. *EMBO Rep* 2005;**6**:39–45.
18. van Steensel B, de Lange T. Control of telomere length by the human telomeric protein TRF1. *Nature* 1997;**385**:740–3.
19. Karlseder J, Broccoli D, Dai Y, Hardy S, de Lange T. p53- and ATM-dependent apoptosis induced by telomeres lacking TRF2. *Science* 1999;**283**:1321–5; Karlseder J, Smogorzewska A, de Lange T. Senescence induced by altered telomere state, not telomere loss. *Science* 2002;**295**:2446–9.
20. Guo X, Deng Y, Lin Y, et al. Dysfunctional telomeres activate an ATM-ATR-dependent DNA damage response to suppress tumorigenesis. *EMBO J* 2007;**26**:4709–19.
21. Takai H, Smogorzewska A, de Lange T. Damage foci at dysfunctional telomeres. *Curr Biol* 2003;**13**:1549–56.
22. Lei M, Podell ER, Cech TR. Structure of human POT1 bound to telomeric single-stranded DNA provides a model for chromosome end-protection. *Nat Struct Mol Biol* 2004;**11**:1223–9.
23. Colgin L, Reddel R. Telomere biology: a new player in the end zone. *Curr Biol* 2004;**14**:R901–2.
24. Kelleher C, Kurth I, Lingner J. Human protection of telomeres 1 (POT1) is a negative regulator of telomerase activity in vitro. *Mol Cell Biol* 2005;**25**:808–18.
25. Salvati E, Leonetti C, Rizzo A, et al. Telomere damage induced by the G-quadruplex ligand RHPS4 has an antitumor effect. *J Clin Invest* 2007;**117**:3236–47.
26. Kim SH, Kaminker P, Campisi J. TIN2, a new regulator of telomere length in human cells. *Nat Genet* 1999;**23**:405–12.
27. Li B, Oestreich S, de Lange T. Identification of human Rap1: implications for telomere evolution. *Cell* 2000;**101**:471–83.
28. Grandin N, Charbonneau M. Protection against chromosome degradation at the telomeres. *Biochimie* 2008;**90**:41–59 [Review].
29. De Cian A, Lacroix L, Douarre C, et al. Targeting telomeres and telomerase. *Biochimie* 2008;**90**:131–55 [Review].
30. Harley CB. Telomerase and cancer therapeutics. *Nat Rev Cancer* 2008;**8**:167–79 [Review].
31. Tejera AM, Alonso DF, Gomez DE, Olivero OA. Chronic in vitro exposure to 3′-azido-2',3′-dideoxythymidine induces senescence and apoptosis and reduces tumorigenicity of metastatic mouse mammary tumor cells. *Breast Cancer Res Treat* 2001;**65**:93–9.
32. Naasani I, Seimiya H, Tsuruo T. Telomerase inhibition, telomere shortening, and senescence of cancer cells by tea catechins. *Biochem Biophys Res Commun* 1998;**249**:391–6.
33. Damm K, Hemmann U, Garin-Chesa P, et al. A highly selective telomerase inhibitor limiting human cancer cell proliferation. *EMBO J* 2001;**20**:6958–68.
34. Keppler BR, Grady AT, Jarstfer MB. The biochemical role of the heat shock protein 90 chaperone complex in establishing human telomerase activity. *J Biol Chem* 2006;**281**:19840–8.
35. Kim YW, Hur SY, Kim TE, et al. Protein kinase C modulates telomerase activity in human cervical cancer cells. *Exp Mol Med* 2001;**33**:156–63.
36. Shammas M, Liu X, Gavory G, Raney K, Balasubramanian S, Shmookler-Reis R. Targeting the single-strand G-rich overhang of telomeres with PNA inhibits cell growth and induces apoptosis of human immortal cells. *Exp Cell Res* 2004;**295**:204–14.
37. Kelland L. Targeting the limitless replicative potential of cancer: the telomerase/telomere pathway. *Clin Cancer Res* 2007;**13**:4960–3 [Review].
38. Gomez D, O'Donohue MF, Wenner T, et al. The G-quadruplex ligand telomestatin inhibits POT1 binding to telomeric sequences in vitro and induces GFP-POT1 dissociation from telomeres in human cells. *Cancer Res* 2006;**66**:6908–12; Gomez D, Wenner T, Brassart B, et al. Telomestatin-induced telomere uncapping is modulated by POT1 through G-overhang extension in HT1080 human tumor cells. *J Biol Chem* 2006;**281**:38721–9.
39. Bellon M, Datta A, Brown M, Pouliquen JF, Couppie P, Kazanji M. Increase expression of telomere length regulating factors TRF1, TRF2 and TIN2 in patients with adult T-cell leukemia. *Int J Cancer* 2006;**119**:2090–7.
40. Klapper W, Krams M, Qian W, Janssen D, Parwaresch R. Telomerase activity in B-cell non-Hodgkin lymphomas is regulated by hTERT transcription and correlated with telomere-binding protein expression but uncoupled from proliferation. *Brit J Cancer* 2003;**89**:713–9.
41. Campbell LJ, Fidler C, Eagleton H, et al. hTERT, the catalytic component of telomerase, is downregulated in the haematopoietic stem cells of patients with chronic myeloid leukaemia. *Leukemia* 2006;**20**:671–9.
42. Matsutani N, Yokozaki H, Tahara E, et al. Expression of telomeric repeat binding factor 1 and 2 and TRF1-interacting

nuclear protein 2 in human gastric carcinomas. *Int J Oncol* 2001;**19**:507–12.
43. Lee ME, Rha SY, Jeung HC, Kim TS, Chung HC, Oh BK. Variation of the 3′ telomeric overhang lengths in human cells. *Cancer Lett* 2008;**264**:107–18.
44. Nakanishi K, Kawai T, Kumaki F, et al. Expression of mRNAs for telomeric repeat binding factor (TRF)-1 and TRF2 in atypical adenomatous hyperplasia and adenocarcinoma of the lung. *Clin Cancer Res* 2003;**9**:1105–11.
45. Nijjar T, Bassett E, Garbe J, et al. Accumulation and altered localization of telomere-associated protein TRF2 in immortally transformed and tumor-derived human breast cells. *Oncogene* 2005;**24**:3369–76.
46. Oh BK, Kim YJ, Park C, Park YN. Up-regulation of telomere-binding proteins, TRF1, TRF2, and TIN2 is related to telomere shortening during human multistep hepatocarcinogenesis. *Am J Pathol* 2005;**166**:73–80.
47. Lee JE, Oh BK, Choi J, Park YN. Telomeric 3′ overhangs in chronic HBV-related hepatitis and hepatocellular carcinoma. *Int J Cancer* 2008;**123**:264–72.
48. Muñoz P, Blanco R, Flores JM, Blasco MA. XPF nuclease-dependent telomere loss and increased DNA damage in mice overexpressing TRF2 result in premature aging and cancer. *Nat Genet* 2005;**37**:1063–71.
49. Muñoz P, Blanco R, Blasco MA. Role of the TRF2 telomeric protein in cancer and ageing. *Cell Cycle* 2006;**5**:718–21.
50. Yamada K, Yagihashi A, Yamada M, et al. Decreased gene expression for telomeric-repeat binding factors and TIN2 in malignant hematopoietic cells. *Anticancer Res* 2002;**22**:1315–20.
51. Poncet D, Belleville A, de Roodenbeke CT, et al. Changes in the expression of telomere maintenance genes suggest global telomere dysfunction in B-chronic lymphocytic leukemia. *Blood* 2008;**111**:2388–91.
52. Yamada M, Tsuji N, Nakamura M, et al. Down-regulation of TRF1, TRF2 and TIN2 genes is important to maintain telomeric DNA for gastric cancers. *Anticancer Res* 2002;**22**:3303–7.
53. Lin X, Lu C, Spitz MR, Wu X. Expression of telomere-asssociated genes as prognostic markers for overall survival in patients with non-small cell lung cancer. *Clin Cancer Res* 2006;**12**:5720–5.
54. Smith S, Giriat I, Schmitt A, de Lange T. Tankyrase, a poly, ADP-ribose polymerase at human telomeres. *Science* 1998;**282**:1484–7.
55. Smogorzewska A, van Steensel B, Bianchi A, et al. Control of human telomere length by TRF1 and TRF2. *Mol Cell Biol* 2000;**20**:1659–68.
56. Miyachi K, Fujita M, Tanaka N, Sasaki K, Sunagawa M. Correlation between telomerase activity and telomeric-repeat binding factors in gastric cancer. *J Exp Clin Cancer Res* 2002;**21**:269–75.
57. Kondo T, Oue N, Yoshida K, et al. Expression of POT1 is associated with tumor stage and telomere length in gastric carcinoma. *Cancer Res* 2004;**64**:523–9.
58. Fujii K, Sasahira T, Moriwaka Y, Oue N, Yasui W, Kuniyasu H. Protection of telomeres 1 protein levels are associated with telomere length in gastric cancer. *Int J Mol Med* 2008;**21**:599–604.
59. Salhab M, Jiang WG, Newbold RF, Mokbel K. The expression of gene transcripts of telomere-associated genes in human breast cancer: correlation with clinico-pathological parameters and clinical outcome. *Breast Cancer Res Treat* 2008;**109**:35–46.
60. Saito K, Yagihashi A, Nasu S, Izawa Y, et al. Gene expression for suppressors of telomerase activity (telomeric-repeat binding factors) in breast cancer. *Jpn J Cancer Res* 2002;**93**:253–8.
61. Hsu CP, Miaw J, Hsia JY, Shai SE, Chen CY. Concordant expression of the telomerase-associated genes in non-small cell lung cancer. *Eur J Surg Oncol* 2003;**29**:594–9.
62. Hsu CP, Lee LW, Shai SE, Chen CY. Clinical significance of telomerase and its associate genes expression in the maintenance of telomere length in squamous cell carcinoma of the esophagus. *World J Gastroenterol* 2005;**11**:6941–7.
63. Fujimoto R, Kamata N, Taki M, et al. Gene expression of telomerase related proteins in human normal oral and ectocervical epithelial cells. *Oral Oncol* 2003;**39**:445–52.
64. Swiggers SJ, Kuijpers MA, de Cort MJ, Beverloo HB, Zijlmans JM. Critically short telomeres in acute myeloid leukemia with loss or gain of parts of chromosomes. *Genes Chromosomes Cancer* 2006;**45**:247–56.
65. Ohyashiki JH, Hayashi S, Yahata N, et al. Impaired telomere regulation mechanism by TRF1 (telomere-binding protein), but not TRF2 expression, in acute leukemia cells. *Int J Oncol* 2001;**18**:593–8.
66. van Steensel B, Smogorzewska A, de Lange T. TRF2 protects human telomeres from end-to-end fusions. *Cell* 1998;**92**:401–13.
67. Yang Q, Zheng YL, Harris CC. POT1 and TRF2 cooperate to maintain telomeric integrity. *Mol Cell Biol* 2005;**25**:1070–80.
68. Ancelin K, Brunori M, Bauwens S, et al. Targeting assay to study the cis functions of human telomeric proteins: evidence for an inhibition of telomerase by TRF1 and for activation of telomere degradation by TRF2. *Mol Cell Biol* 2002;**22**:3474–87.
69. Baumann P, Podell E, Cech TR. Human Pot1 (protection of telomeres) protein: cytolocalization, gene structure, and alternative splicing. *Mol Cell Biol* 2002;**22**:8079–87.
70. Escoffier E, Rezza A, Roborel de Climens A, et al. A balanced transcription between telomerase and the telomeric DNA-binding proteins TRF1, TRF2 and Pot1 in resting, activated, HTLV-1-transformed and Tax-expressing human T lymphocytes. *Retrovirology* 2005;**2**:77.
71. Hsu CP, Ko JL, Shai SE, Lee LW. Modulation of telomere shelterin by TRF1 [corrected] and TRF2 interacts with telomerase to maintain the telomere length in non-small cell lung cancer. *Lung Cancer* 2007;**58**:310–6.
72. Frías C, García-Aranda C, De Juan C, et al. Telomere shortening is associated with poor prognosis and telomerase activity correlates with DNA repair impairment in non-small cell lung cancer. *Lung Cancer* 2008;**60**:416–25.
73. Broccoli D, Young JW, de Lange T. Telomerase activity in normal and malignant hematopoietic cells. *Proc Natl Acad Sci USA* 1995;**92**:9082–6.
74. Chandran UR, Dhir R, Ma C, Michalopoulos G, Becich M, Gilbertson J. Differences in gene expression in prostate cancer, normal appearing prostate tissue adjacent to cancer and prostate tissue from cancer free organ donors. *BMC Cancer* 2005;**5**:45.
75. Chen LY, Liu D, Songyang Z. Telomere maintenance through spatial control of telomeric proteins. *Mol Cell Biol* 2007;**27**:5898–909.