Research Article

Cancer Epidemiology, Biomarkers & Prevention

A Rare Variant P507L in TPP1 Interrupts TPP1–TIN2 Interaction, Influences Telomere Length, and Confers Colorectal Cancer Risk in Chinese Population

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Abstract

Background: Telomere dysfunction triggers cellular senescence and constitutes a driving force for cancer initiation. Genetic variants in genes involved in telomere maintenance may contribute to colorectal cancer susceptibility.

Methods: In this study, we firstly captured germline mutations in 192 patients with colorectal cancer by sequencing the coding regions of 13 core components implicated in telomere biology. Five potential functional variants were then genotyped and assessed in a case–control set with 3,761 colorectal cancer cases and 3,839 healthy controls. The promising association was replicated in additional 6,765 cases and 6,906 controls. Functional experiments were used to further clarify the potential function of the significant variant and uncover the underlying mechanism in colorectal cancer development.

Results: The two-stage association studies showed that a rare missense variant rs149418249 (c.C1520T and p.P507L) in the 11th exon of *TPP1* (also known as *ACD*, gene ID 65057) was significantly associated with colorectal cancer risk with the ORs being 2.90 [95% confidence interval (CI), 1.04–8.07; $P = 0.041$], 2.50 (95% CI, 1.04–6.04; $P = 0.042$), and 2.66 (95% CI, 1.36–5.18; $P = 0.004$) in discovery, replication, and the combined samples, respectively. Further functional annotation indicated that the TPP1 P507L substitution interrupted TPP1–TIN2 interaction, impaired telomerase processivity, and shortened telomere length, which subsequently facilitated cell proliferation and promoted colorectal cancer development.

Conclusions: A rare variant P507L in TPP1 confers increased risk of colorectal cancer through interrupting TPP1–TIN2 interaction, impairing telomerase processivity, and shrinking telomere length.

Impact: These findings emphasize the important role of telomere dysfunction in colorectal cancer development, and provide new insights about the prevention of this type of cancer.
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Introduction

Colorectal cancer is a common diagnosed malignancy and one of the most lethal cancers worldwide (1). Although environmental factors play the primary role in causing sporadic cancers, the effect of hereditary should not be ignored, as about one-third of colorectal cancer cases are attributable to genetic defects (2).

The *TERT-CLPTM1L* region mapped to chromosome *5p15.33* is a pleiotropic cancer risk locus identified by genome-wide association studies of multiple cancers including colorectal cancer (3). Telomerase reverse transcriptase (*TERT*), a plausible attributive gene in this region, encodes the catalytic subunit of telomerase, which is responsible for adding *TTAGGG* repeats to chromosome ends, known as telomeres (4). Telomere dysfunction triggers cellular senescence and constitutes a driving force for cancer initiation by causing genome instability (5). While telomerase is critical for telomere length regulation, telomere maintenance requires cooperation of different telomere-binding proteins (6–8). Several mutations in these telomeric factors have been found associated with a spectrum of disorders called telomeropathies. Remarkably, because of the extremely crucial role of telomere in chromosome protection and genome integrity control, pathologic mutations in these factors would be subject to strong negative selection, leading to their rarity in general population (9).

In this study, we hypothesized that genetic variants in coding regions of the core components involved in telomere biology could predispose individuals to colorectal cancer. To capture both common and rare variants in telomeric factors harbored by patients with colorectal cancer, we firstly scanned targeted telomeric regions using next-generation sequencing. Subsequently, a two-stage case–control set with a total of 21,271 subjects was

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performed to identify colorectal cancer associated variants in Chinese population. Functional exploration and characterization were further introduced to illustrate the underlying pathogenic mechanisms.

Materials and Methods

Study subjects

We enrolled 192 newly diagnosed colorectal cancer patients from Wuhan, China, to screen candidate genetic variants in targeted regions. A total of 3,761 colorectal cancer cases and 3,839 healthy controls were recruited from Wuhan for prioritizing the promising associations in the discovery stage. In the replication stage, 6,765 colorectal cancer cases and 6,906 cancer-free controls were enrolled from Beijing, China. Our study was conducted in accordance with the ethical guidelines of National Natural Science Foundation of China. Informed consent was obtained from each participant, and this study was approved by the institutional review board of Huazhong University of Science and Technology (Hubei, China). The details in sample recruitment, selection criteria and data collection are provided in Supplementary Methods. The basic characteristics of the enrolled subjects are shown in Supplementary Table S1.

Targeted sequencing

We sequenced the coding exons of 13 core genes involved in telomere maintenance using peripheral blood DNA of 192 patients with colorectal cancer. These candidate genes included the gene encoding the human telomerase (*TERT*), six components of the shelterin complex (*TERF1*, *TERF2*, *TINF2*, *TPP1*, *POT1*, and *TERF2IP*), three components of the MRN complex (*MRE11A*, *RAD50*, and *NBX*), and genes encoding the CST complex (*CTC1*, *OBFC1*, and *TEN1*). The targeted regions were amplified with a total coverage of 98.7% (Supplementary Table S2). The primary sequences were generated by using Ion Torrent Personal Genome Machine (PGM) platform (Life Technologies) as reported previously (10). Details in primer design, sequencing procedure, and data analysis are available in Supplementary Methods. Sequence data has been deposited at the European Genome-phenome Archive (EGA), under accession number EGAS00001002977.

Screening of candidate variants and genotyping

Genetic variants in noncoding regions and synonymous variants captured by targeted sequencing were initially removed. Bioinformatics resources including SIFT (11), PolyPhen2 (12), PROVEAN (13), and MutationAssessor (14) were used to prioritize functional missense variants, and only the most likely damaging variants, which were predicted to be deleterious by all the 4 algorithms, were considered for further investigation. Candidate variants were genotyped using ABI TaqMan SNP Genotyping Assays (Applied Biosystems). Specifically, both positive and negative controls were applied in each genotyping plate.

Cell lines

The human colon cancer cell lines HCT116 and LoVo were purchased from the cell bank of the Chinese Academy of Sciences (Shanghai, China) in August 2016, and the human embryonic kidney cell line HEK293T was a generous gift from Dr. Kun Huang in the School of Pharmacy, Huazhong University of Science and Technology (Wuhan, China). All the cells were tested routinely by DNA sequencing using the Applied Biosystems AmpF/STR Identifier Kit (Applied Biosystems) in September 2016.

Coimmunoprecipitation and Western blot analysis

Cells were cotransfected with wild-type (or mutant) Flag-TPP1 and HA-TIN2 plasmids and harvested 48 hours post-transfections. Anti-Flag antibody (Sigma F1804) or HA-tag antibody (Abcam ab137838) was mixed with cellular protein and incubated for 3 hours at 4°C. Resuspended Protein A/G PLUS-Agarose (Santa Cruz Biotechnology sc-2003) was then added to each tube and incubated at 4°C on a ricker platform overnight. Beads were washed with lysis buffer and followed by Western blot analyses. Detailed procedures about coimmunoprecipitation (co-IP) and immunoblot are present in Supplementary Methods.

Telomerase processivity

The quantitative determination of telomerase processivity was assayed by *Telo* TAGGG Telomerase PCR ELISA Kit (Roche), an improved method based on the telomerase repeat amplification protocol assay (15). Briefly, total protein was extracted from transfected cells and used for telomerase-mediated elongation. The PCR products derived from this assay were split into two aliquots, denatured and hybridized separately to digoxigenin (DIG) labeled detection probes and immobilized via the biotin label to a streptavidin-coated microplate. Immobilized amplicons were subsequently detected with an antibody against DIG conjugated to horseradish peroxidase (anti-DIG-HRP). At last, the absorbance at 450 nm of each sample was measured after adding the stop reagent.

Telomere length determination

Relative telomere length (RTL) in peripheral blood cells was measured using a validated method, which measures the relative average TL in genomic DNA by determining the ratio of telomere repeat copy number to a single-copy gene *36B4* (Supplementary Methods; ref. 16).

Cell proliferation

Cancer cell proliferation was measured by using the Cell Counting Kit-8 (Dojindo Laboratories). In brief, human HCT116 and LoVo cells after transfection were seeded in 96-well plates. CCK-8 solution was added to each well and incubated in a humidified CO_2 incubator at 37°C for 2 hours. Then, the absorbance at 450 nm of each well was measured. The CCK-8 solutions were added per 24 hours until 96 hours after cells seeding into the plates.

Results

Germline rare variant *TPP1* rs149418249 (*C*>*T*) was associated with colorectal cancer risk

Over 98% of the raw sequences produced by sequencing were aligned to hg19 human reference genome. All the sequencing depths of aligned reads were above 78-fold, with an average of 553-fold. The sequencing initially captured 82 exonic variants, of which 12 were common variants and the remaining 70 were low-frequency or rare variants. A list of the 82 variants is provided in

Table 1. Comparisons about the minor allele frequency of candidate variants between cases and controls in discovery case–control study

Chr	Position	Rs	Allele	Variation	Gene	MAF		OR	*P* Fisher
						Cases	Controls		
chr5	131977907	rs199579239	*G>A*	p.Leu1264Phe	*RAD50*	0.22%	0.12%	1.83	0.164
chr14	24709325	rs192423622	*T>C*	p.Ile389Thr	*TINF2*	0.13%	0.13%	1.03	1.000
chr16	67691701	rs149418249	*C>T*	p.Pro507Leu	*TPP1*	0.19%	0.07%	2.91	0.038
chr17	8132702	rs183966301	*G>A*	p.Ala1025Val	*CTC1*	0.48%	0.38%	1.28	0.384
chr17	8141754	rs200137992	*G>A*	p.Gly131Arg	*CTC1*	0.27%	0.17%	1.57	0.225

Supplementary Table S3. Thirty-six synonymous variants and 9 variants in untranslated regions were filtered and the other 37 nonsynonymous variants were retained for function prediction. Finally, 5 nonsynonymous variants with strong predicted functions were selected as our candidates. The results about function prediction for all the 37 variants are supplied in Supplementary Table S4, and the workflow for variant screening is displayed in Supplementary Fig. S1.

We then genotyped 3,761 cases and 3,839 controls using TaqMan genotyping assay and after quality control, 3,745 cases and 3,833 controls were reserved for the association analyses. Among the 5 candidates, only one rare variant, rs149418249 (c.*C*1520*T* and p.P507L) in the gene *ACD* (gene ID 65057, well known as *TPP1*, and referred as *TPP1* below for convenience), was significantly associated with colorectal cancer risk [OR = 2.90; 95% confidence interval (CI), 1.04–8.07; P = 0.041; Tables 1 and 2]. We replicated this association in an independent sample set with a total of 6,765 colorectal cancer cases and 6,906 controls. The general call rate of genotyping was 98.3% and specifically, 6,652 cases and 6,792 controls were successfully genotyped. In this stage, rs149418249 still exhibited a significant association with colorectal cancer susceptibility (OR = 2.50; 95% CI, 1.04–6.04; $P = 0.042$; Table 2). When combining all the samples, we found a more robust association (OR = 2.66; 95% CI, 1.36–5.18; $P = 0.004$; Table 2). Subgroup analyses were further conducted with stratification of age or sex. As a result, the variant significantly associated with colorectal cancer risk in both young and the elderly subjects ($P = 0.034$ and 0.045, respectively; Supplementary Table S5). When stratified by sex, we only detected a significant association in males (OR = 2.93; 95% CI, 1.24–6.95; $P = 0.014$; Supplementary Table S5). However, the effect in females showed a similar trend, although it was not significant (OR = 2.31; 95% CI, 0.80–6.67; $P = 0.120$; Supplementary Table S5).

TPP1 P507L change repressed telomerase processivity through perturbing TPP1–TIN2 interaction

The missense variant rs149418249 (c.C1520*T* and p.P507L) is a novel identified variant with a minor allele *T* but not *G* as reported previously, accordingly leading to Pro507Leu change instead of Pro507Arg in translation. The amino acid encoded by this variant resides in the TIN2-binding domain of TPP1 and is well conserved across mammal species (Fig. 1A). We thus speculated that the variation might damage telomere maintenance through interrupting TPP1–TIN2 interaction. To test this hypothesis, we assessed the interplay between TIN2 and TPP1 by performing co-IP assays. The wild-type TPP1 efficiently precipitated with TIN2, while TPP1 with P507L change showed a modest reduction in TIN2 interaction (Fig. 1B and C). Otherwise, co-IP of TIN2 with cotransfected wild-type or mutant TPP1 also showed abolished TPP1–TIN2 interaction in mutant TPP1 compared with the wild-type, suggesting deficient binding affinity of mutant TPP1 to TIN2.

The telomerase recruitment role of TPP1 depends on its interaction with TIN2, which tethered to duplex telomeric repeats through TRF1/TRF2. We next questioned whether this TPP1 mutation was defective in stimulating telomerase processivity. To figure out this issue, we quantified telomerase activity and observed increased telomerase activity in cells over expressed with the wild TPP1. However, the telomerase processivity was significantly decreased in the presence of mutant TPP1 in both cancer cells and normal cells when compared with the wild-type (Fig. 1D and E).

The *TPP1* rs149418249 affected telomere length

Because we have demonstrated that TPP1 P507L impaired telomere biology, we next sought to interrogate whether this mutation affected telomere length (TL) in general population. On the basis of our large-scale case–control study, we captured 31 colorectal cancer cases and 12 controls carrying the risk allele of rs149418249. The rare variant rs149418249 was visualized and validated (Supplementary Fig. S2). All the carriers were heterozygous, of which 25 cases and all the controls with qualified DNA samples were selected as risk allele carriers in the following analysis. Each three noncarriers were matched to one heterozygous carrier according to age, gender, and group (case or control). RTL of each subject was measured, and after removing undetectable outliers, we included 37 heterozygous carriers and 105 noncarriers in our analysis. In line with the recognized inverse correlation between age and TL, lymphocyte RTL was progressively shortened with the increasing of age ($r = -0.170$; P = 0.043; Fig. 2A). The RTL in risk allele carriers prominently

Table 2. Association of *TPP1* rs149418249 and colorectal cancer risk in the discovery, replication, and combined analyses

Stage	Genotype	Cases	Controls	OR[a]	95% CI	*P*
Discovery	*CC*	3,663	3,802	Reference		
	CT	14	5	2.90	1.04–8.07	0.041
Replication	*CC*	6,635	6,785	Reference		
	CT	17	7	2.50	1.04–6.04	0.042
Combined	*CC*	10,298	10,587	Reference		
	CT	31	12	2.66	1.36–5.18	0.004

[a]Adjusted for sex, age, smoking, and drinking status. ORs calculated with a genotypic model are presented.

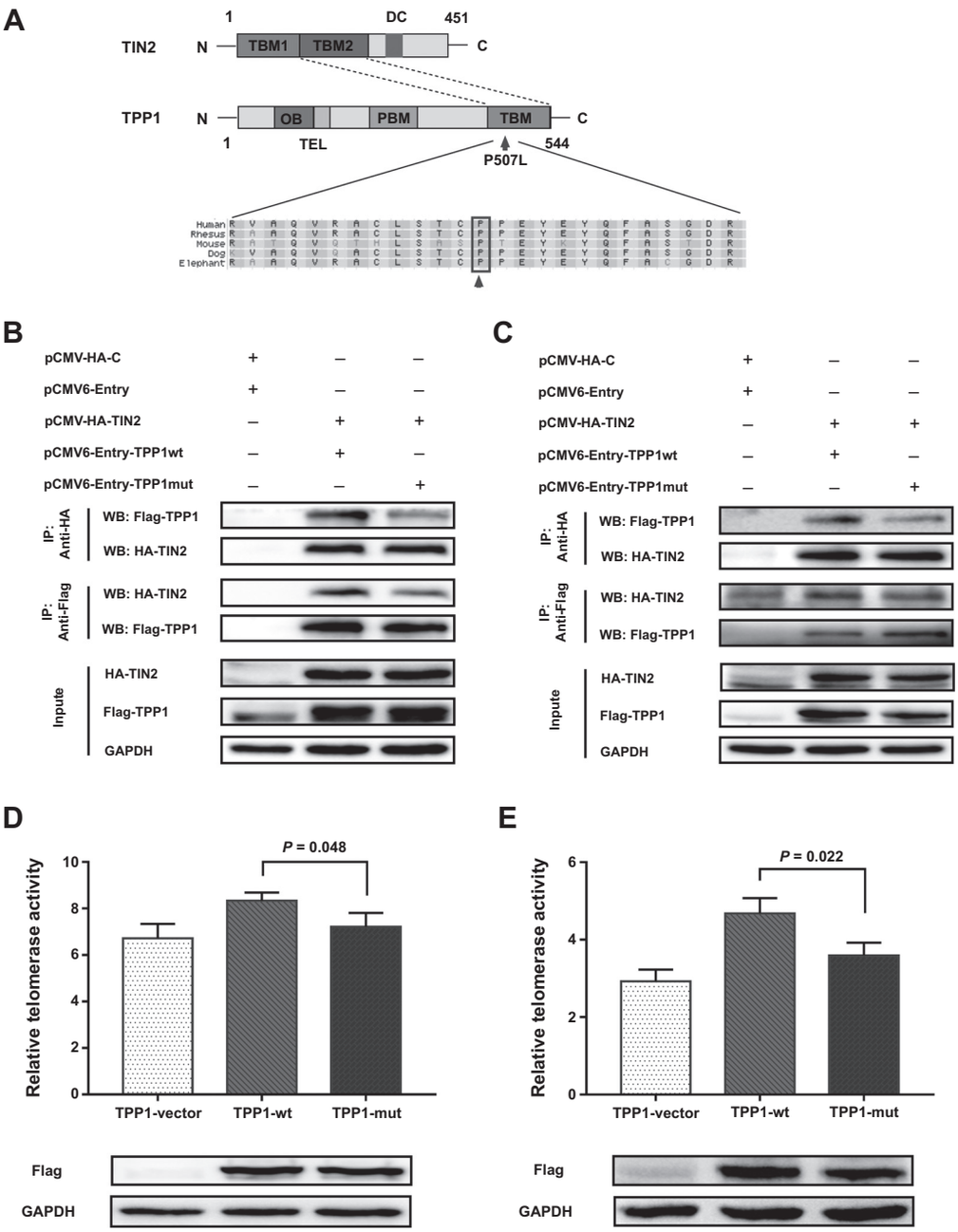

Figure 1.

The TPP1 P507L mutation caused telomerase deficiency through disturbing TPP1–TIN2 interaction. **A,** TPP1 P507 was predicted to reside in the TIN2-binding domain of TPP1 and the amino acid was well conserved across mammal species. **B** and **C,** The P507L change was marginally inhibited TPP1–TIN2 interaction in both HCT116 cells (**B**) and LoVo cells (**C**). **D** and **E,** As a result of the abolished TPP1–TIN2 interplay, the telomerase processivity was significantly decreased in both cancer cell line LoVo (**D**) and normal cell line 293T (**E**) overexpressed with mutant TPP1. The data in histograms are shown as the mean ± SD from three independent experiments. Comparisons between mutant group and wild-type group are using two-sided unpaired Student *t* test.

decreased when compared with noncarriers ($P = 2.39 \times 10^{-4}$; Fig. 2B). Moreover, the discrepancy between carriers and noncarriers were consistently significant in both colorectal cancer cases and healthy controls (Fig. 2C and D).

TPP1 P507L change facilitated cancer cell proliferation

Telomerase activity and TL are important factors in the pathobiology of cancer. We next examined whether TPP1 P507L mutation affected colorectal cancer cell proliferation. As a result, over

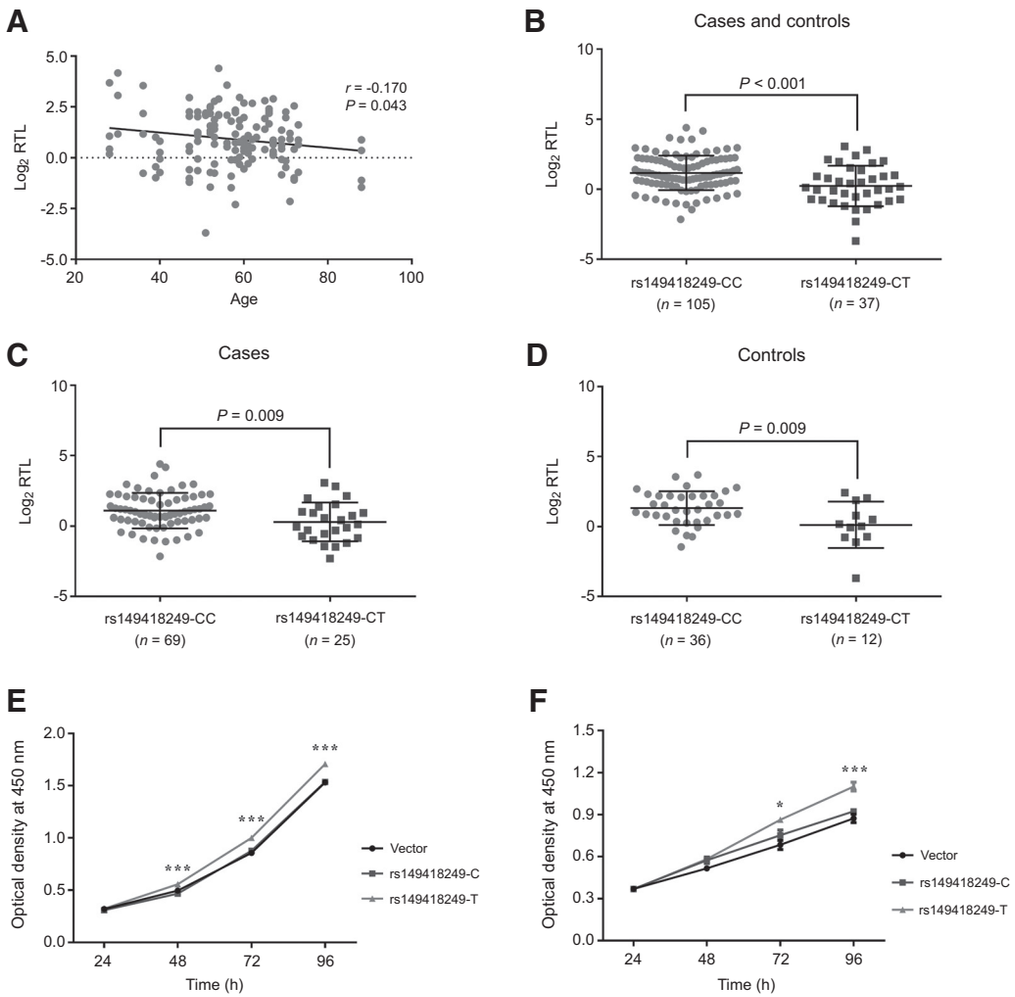

Figure 2.

TPP1 rs149418249 shortened telomere length in both colorectal cancer cases and healthy controls and accelerated cancer proliferation. **A**, The lymphocyte RTL was inversely associated with age. **B**, The RTL in people carrying the risk allele prominently decreased when compared with noncarriers. **C** and **D,** The discrepancy between carriers and noncarriers was consistently significant in both colorectal cancer cases (**C**) and healthy controls (**D**). In scatter plots, each dot represents the RTL of a sample. Comparisons between the RTL in carriers and noncarriers are using two-sided unpaired Student *t* test. **E** and **F,** Overexpression of mutant TPP1 significantly enhanced cell proliferation compared with vector control and wild-type TPP1 in HCT116 cells (**E**) and LoVo cells (**F**). Data are shown as the mean ± SD. *, $P < 0.05$; ***, $P < 0.001$, compared by two-sided unpaired Student *t* test.

expression of *TPP1* rs149418249 *T* allele significantly enhanced cancer cell proliferation compared with vector control and *TPP1* rs149418249 C allele (Fig. 2E and F).

Discussion

Telomeres are special heterochromatic structures that cap the ends of eukaryotic chromosomes, thus protecting them from degradation and preventing the aberrant and fatal activation of DNA damage repair machinery (17). Telomere attrition induces chromosomal instability and contributes to genomic rearrangements, which are permissive for tumorigenesis (18). In this study, we newly identified a rare variant rs149418249 (c.C1520*T* and p.P507L) in shelterin component *TPP1*, which confers risk of sporadic colorectal cancer in Chinese population, especially in males. It was reported that telomeres shortened more slowly in women than in men due to hormonal control of telomerase activity (19, 20). We supposed that the gender disparity might be attributed to effects of estrogen on telomerase. However, the concrete mechanism should be illustrated in future. Besides, the nonsignificant association in females may be subject to limited sample size, thus the results need to be verified in future.

The missense variant rs149418249 increased cancer susceptibility through interfering TPP1–TIN2 physical interaction, impairing telomerase processivity, and shortening telomere length (Fig. 3). TPP1 acts as a molecular hub coordinating end-replication and end-protection functions of the shelterin complex (21, 22). To date, disease-associated mutations detected in TPP1 were clustered in the POT1-binding domain or the OB-fold, which is responsible for the recruitment of telomerase (23, 24).

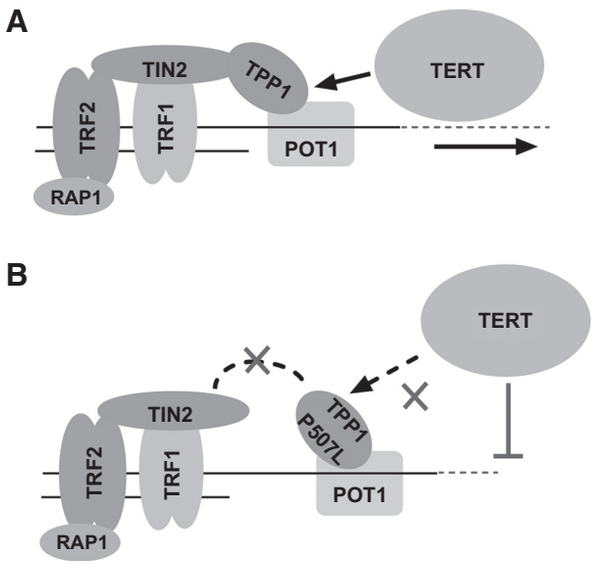

Figure 3.

A schematic representation about the cancer promoting role of TPP1 P507L mutation. **A,** Wild-type TPP1 tethered to TIN2 ensures the proper assembly of shelterin complex. **B,** While in the mutated situation, the P507L change inhibits its binding affinity to TIN2, interferes telomerase recruitment to telomeres, and finally results in impaired telomerase processivity.

Intriguingly, the missense variant identified in our study resides in the TIN2-binding domain and the P507L change in TPP1 marginally inhibited its interaction with TIN2. The physical link between TPP1 and TIN2 is important for the functional balance of the shelterin machinery. Binding of TPP1 to TIN2 is the sole mechanism by which TPP1/POT1 links to other shelterin components and it is required for POT1-mediated telomere protection (25). Moreover, TIN2-tethered TPP1 participates in recruiting telomerase to telomeres in human cells (26). We found that cells expressing mutant TPP1 indicated telomerase activity defects, suggesting the diminution in TIN2 binding indeed impinges on the telomerase recruitment. Measurements of the RTL in general population further revealed a reduction of TL in people harboring TPP1 risk allele. This observation is in accordance with the previous studies showing short telomeres were prone to telomere dysfunction and colorectal malignancy (27). Nevertheless, a study demonstrated an U-shaped association between TL and colorectal cancer initiation (28). On the basis of the combination of 8 datasets, a meta-analysis showed that TL was not contributed to colorectal cancer risk (29). Hence, the relationship between TL and colorectal cancer development needs to further investigate.

One of the strengths of this study is the identification of the convincing association between rare variant in *TPP1* and colorectal cancer susceptibility. Despite the limited contribution of single-risk variant to the public due to its rarity in population (the population attributable fraction of rs149418249 in our population is about 0.19%), our approach highlights the role of rare variants in cancer development and provides new insights into the prevention of colorectal cancer. In addition, functional annotation suggests the interruption of TPP1–TIN2 interaction as a driver mechanism for colorectal tumorigenesis. Nevertheless, certain limitations should be addressed. First, in spite of the large sample size, replication efforts would be necessarily owing to the rare frequency of rs149418249. Second, to better screen potential candidates, scanning in both colorectal cancer cases and healthy controls is expected instead of sequencing in patients alone. Finally, although we have detected TPP1–TIN2 interaction, exploration toward the functional significance on the whole complex remains challenging. Systematic interrogation is required to recover detailed biological mechanisms and further studies are warranted to elaborate the role of other telomeric factors in cancer development in addition to the core components discussed in this study.

In summary, a rare missense variant rs149418249 (c.C1520T and p.P507L) in TPP1 confers colorectal cancer susceptibility in a Chinese population. Functional evidence revealed that the P507L change in TPP1 impaired TPP1–TIN2 interplay, triggered telomere shortening and subsequently facilitated cell proliferation and promoted colorectal cancer development. This strongly supports the causative role of telomere dysfunction in the etiology of colorectal cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: J. Li, J. Chang, Y. Gong, X. Miao
Development of methodology: J. Li, X. Miao
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Li, J. Chang, J. Ke, Y. Zhu, Y. Yang, D. Zou, X. Peng, N. Yang, X. Wang, W. Hu, R. Zhong, X. Miao
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Li, J. Chang, J. Tian, J. Ke, J. Gong, X. Miao
Writing, review, and/or revision of the manuscript: J. Li, J. Chang, X. Miao
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Ke, L. Cheng, X. Miao
Study supervision: J. Ke, X. Miao

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