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The enigma of excessively long telomeres in cancer: lessons learned from rare human POT1 variants

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Abstract

The discovery that rare POT1 variants are associated with extremely long telomeres and increased cancer predisposition has provided a framework to revisit the relationship between telomere length and cancer development. Telomere shortening is linked with increased risk for cancer. However, over the past decade, there is increasing evidence to show that extremely long telomeres caused by mutations in shelterin components (POT1, TPP1, and RAP1) also display an increased risk of cancer. Here, we will review current knowledge on germline mutations of POT1 identified from cancer-prone families. In particular, we will discuss some common features presented by the mutations through structure-function studies. We will further provide an overview of how POT1 mutations affect telomere length regulation and tumorigenesis.

Keywords

telomere; POT1; cancer; germline mutation

Telomere length impacts tumorigenesis

Human telomere length is highly heterogeneous, typically ranging from 5 kb to 15 kb in the population, with considerable variability between individuals, among different tissues, and across different stages of a lifetime [1]. The importance of setting telomere length in a fine range has been well demonstrated. Critically short telomeres have been linked to degenerative diseases and increased cancer risk [2–6]. However, a growing body of epidemiological evidence has shown that individuals with extremely long telomeres have an increased risk for cancers, including gliomas, melanomas, and pulmonary and pancreatic cancers [7–12]. The molecular mechanisms of how defective telomere length maintenance contributes to tumorigenesis are not well understood. The genetic association of telomere length abnormalities with cancer has led to the identification of multiple population-linked

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SNPs and rare variants found in cancer patients, many of which are telomere-related. The first connection was revealed in 2013 with the identification of a gain-of-function mutation in the TERT promoter of tumor samples derived from familial melanoma cases across five generations [13]. This mutation resulted in an up to two-fold increase in TERT transcription. Since then, a number of germline mutations have been revealed in telomeric shelterin components, including POT1, TPP1, and RAP1 [14–25]. Among them, POT1 mutations are the most common and have been reported in a broad range of cancer types. In this review, we focus on emerging evidence from cancer-prone families as well as structure-function mechanistic studies linking germline mutations of POT1 to cancer susceptibility.

Deleterious germline POT1 variants identified in cancers

Approximately 10% of cutaneous malignant melanoma (CMM) cases occur in a familial setting, and 20–40% of familial melanomas exhibit mutations in the *CDKN2A* gene. In an attempt to identify new high-penetrance susceptibility genes for familial CMM, Shi et al. and Robles-Espinoza et al. performed whole-exome and genome sequencing on tumors derived from more than 100 cases of familial melanomas and identified POT1 as a second major susceptibility gene for CMM in several populations [14,15]. One missense POT1 variant (g.124493086C>T; p.Ser270Asn) was further traced back to approximately 10 generations ago and identified as a founder mutation in five unrelated melanoma-prone families in Italy [15]. To date, the tumor spectrum has been expanded to gliomas, cardiac angiosarcomas, chronic lymphocytic leukemia (CLL), colorectal cancer, and Hodgkin's lymphoma [14–25].

POT1 directly binds single-stranded telomeric DNA sequences through its two N-terminal oligonucleotide/oligosaccharide-binding (OB) fold domains [26]. Yet due to its low abundancy inside the cell, POT1 telomeric localization requires interaction with TPP1 through its C-terminal half, which contains a third OB fold domain and a Holliday junction resolvase-like domain (Figure 1) [27–30]. This POT1/TPP1 heterodimer further stabilizes the protein complex and maintains the overall POT1 level in the cell. Once bound to the 3' telomeric overhang, POT1 has two major functions. First, it inhibits the recruitment of ataxia telangiectasia and Rad3-related (ATR) to replication protein A (RPA) coated single-stranded DNA (ssDNA) by competitively binding RPA. Therefore, it represses the subsequent ATR-dependent DNA damage response (DDR) [31,32]. Second, it blocks access of telomerase to its substrate in late S phase by direct occupation of the G-strand overhang, but also acts as a telomerase processivity factor together with TPP1 [33,34]. As expected, any structural disruption caused by POT1 mutation may affect its telomere localization, protein stability, ssDNA binding, telomerase recruitment and/or processivity, and eventually, telomere length and function. Early somatic mutations of POT1 were found enriched in the OB folds in patients with chronic lymphocytic leukemia (CLL) [35]. However, current sequencing data has indicated that the positions of POT1 germline mutations may be located throughout the entire protein. Based on their distribution and structural consequences, these mutations can be separated into three groups:

Missense mutations in POT1 ssDNA binding OB folds

OB folds are important functional modules that mediate various protein-DNA, -RNA, and -protein interactions [36]. Structural analysis of the human POT1-ssDNA complex has revealed that the telomeric ssDNA adopts an irregular and extended conformation and binds both OB folds, where 24 protein residues of POT1 are used to make protein-ssDNA contact. A total of 12 missense mutations have been identified, whereas Tyr36Cys/Tyr36His, Ile78Thr, and Asp224Asn were found twice in two separate studies (Figure 1). Seven of them are predicted to be deleterious, either by directly affecting one of the 24 residues found at the POT1-telomeric polynucleotide interface (Tyr36Cys/Tyr36His, Tyr89Cys, Gln94Glu, Asp224Asn, Ser270Asn, Arg273Leu), or by affecting an α-helix structure involved in OB folding (Arg137His). Reduction or loss of POT1-DNA complex formation was confirmed in most of these mutations by *in vitro* telomeric ssDNA binding assay. So far, only Lys90Glu was reported to have the same DNA binding affinity as wild-type(wt) POT1 [37]. Functional characterization of the mutants was conducted in cells and mice exogenously expressing individual substitutions [37,38]. These studies demonstrated that all mutant alleles were capable of localizing to telomeres as expected, confirming that the mutations did not fully abolish binding to TPP1. As the POT1 variants identified from the patients were heterozygous, with carrier cells still retaining one wt allele, limited characterization of peripheral blood mononuclear cells (PBMCs) harboring Asp224Asn and Ser270Asn showed no presence of telomere dysfunction-induced foci (TIF), suggesting that the wt allele of POT1 is sufficient for ATR repression. Only when Tyr89Cys, Lys90Glu, Gln94Glu, and Ser270Asn are overexpressed exogenously, are TIF levels increased, possibly due to titration of wt POT1 present at single-stranded ends. All carriers of these variants, including Lys90Glu, have increased telomere lengths and numbers of fragile telomeres, a feature indicative of telomere replication-dependent defects.

One outlier of this group is the Arg117Cys mutation. Residue 117 is located within the OB1 fold, yet the single amino acid change greatly disrupts the overall protein conformation. As a result, this mutation was predicted to affect the interaction between OB1 and OB2 as well as the binding of POT1 to TPP1. Carriers of this variant demonstrated a severe phenotype, exemplified by reduced telomere bound POT1, increased TIF levels, and abnormally long/fragile telomeres [17].

Missense mutations in the POT1-TPP1 binding region

POT1 and TPP1 form a stable heterodimer that caps the telomere ends and regulates the access and processivity of telomerase [33,34]. As stated earlier, POT1 sequesters the 3'-overhang, thereby preventing telomerase access. In contrast to the negative regulation of telomerase by POT1, TPP1 positively mediates telomere lengthening by recruiting TERT through the seven amino acids, known as the TEL patch [39]. Recent structural work has revealed POT1 C- terminal binding to TPP1 spans a large region and makes extensive interactions [29,30]. POT1 mutations occur in this region, which involve residues 376, 497, 532, 598, and 623 (Figure 1). POT1 Ala532Pro and Gln623His mutants have been characterized in detail at the cellular level. Residue 623 is located in the binding pocket of POT1, which directly engages TPP1. The Gln623His mutant protein displayed a 4–5-fold decreased binding affinity to TPP1 and was less stable compared to the wt protein. When the

Gln623His mutant was overexpressed in U2OS cells, it was able to localize to telomeres, though with decreased efficiency. However, this mutant was unable to completely repress DDR activation at telomeres. The Gln623His mutant displayed low to medium levels of TIF formation. This was a surprising result, since the Gln623His mutant did not significantly impact the ability of POT1 to interact with telomeric ssDNA, based on *in vitro* ssDNA binding results. Thus, either the overall expression of POT1 was too low to protect the telomere ends, or the TIFs arose from a capping-independent mechanism, such as defective telomere replication. Finally, cells carrying the Gln623His mutation showed a significant increase in telomere length and fragility after long term culture [29,30].

Unlike the Gln623His mutant, the phenotype of the Ala532Pro mutant is less severe. Structurally, residue 532 is located away from the TPP1 binding site and does not appear to have a significant impact on POT1/TPP1 complex formation. As expected, the mutant protein displayed almost the same binding affinity to TPP1 as wt POT1. However, *in vitro* protein purification demonstrated decreased expression of the Ala532Pro protein, possibly due to unfolded protein response-mediated degradation. When overexpressed in cells, the mutant still localized to telomeres and did not significantly impact telomere length during prolonged culture. The only significant phenotype presented by the Ala532Pro mutant was an increased incidence of fragile telomeres [30].

Nonsense and splicing mutations

Human POT1 has 19 exons and is predicted to produce 5 splice variants; transcripts 2, 3 and 5 are nonsense mediated mRNA decay candidates. Variants 1, 2, 3, and 5 share the same N-terminal sequence (encoding OB1 and OB2 domains) and terminate through different splicing to generate truncated versions distinct from the widely studied POT1 variant 1 protein. Variant 4 encodes a 55 kDa protein with an in-frame start codon ATG in exon 9, thereby missing most of the N-terminal OB1 domain [40]. Similar to the naturally occurring POT1 splice variants, nonsense and splicing mutations are likely to result in truncated products. Indeed, one such mutant (g.124510965C>T, c.255G>A) is predicted to cause skipping of exon 7 to generate an isoform that corresponds to the natural POT1 variant 4 [21]. Disruptive mutations represent the second most common type of mutations identified besides OB folds mutants. 11 disruptive mutations have been identified, most of which are located in the C-terminal half of POT1 (Figure 1). The expression of the majority splice mutants has been confirmed by the presence of an aberrant transcript shown by RT-PCR. While little functional characterization has been conducted in this group, current structural information indicates that the mutants will be impacted in two major ways at the protein level. First, there is predicted to be decreased POT1 expression due to nonsense mediated decay (E450*), misfolded protein degradation, or complete/partial loss of TPP1 binding and stabilization. This would elicit a phenotype similar to that exhibited by genetic knockdown of POT1 [27,41]. Second, if the mutants are stably expressed, then the phenotype will be due to gaining extratelomeric function or will be reminiscent of natural POT1 variants. Earlier characterization demonstrated that POT1 variants are expressed in multiple tissues. Intriguingly, the variants displayed different DNA binding affinities and telomere elongation abilities [42,43].

Mechanistic basis of telomere overlengthening caused by POT1 mutation

The most consistent phenotypes from the structure-function analysis of the POT1 variants are longer telomere length and elevated levels of fragile telomeres, which can be explained by several possibilities:

Loss of negative suppression by POT1

I. ***Loss of functional OB folds:*** POT1 blocks telomerase access through direct occupation of the 3' single-stranded G overhang. When the binding of OB folds to the 3' terminus is weakened by mutation, exposure of ssDNA renders constitutive telomerase access and elongation. Earlier work demonstrated that deletion of POT1 OB1 fold resulted in a dramatic increase in telomere length [27,44]. The germline OB fold mutants described here reinforce the importance of this cis-inhibitory effect of POT1 on telomerase.

II. ***Loss of TPP1 interaction:*** As mentioned earlier, TPP1 is required for the POT1 localization to telomeres. In addition, *in vitro* studies have shown that TPP1 enhances the binding of POT1 to ssDNA up to 10 fold [33,34]. Finally, TPP1 binding to POT1 stabilizes the POT1 protein, as demonstrated in recent studies of POT1 mutants. Thus, any partial or complete disruption of the POT1/TPP1 complex would result in decreased levels of functional POT1 at telomere ends, which in turn, would lead to extensive telomere elongation.

III. ***Decreased expression of POT1 protein:*** POT1 is the least abundant shelterin component, estimated to be present at around 15,000 molecules per cell. Each telomere may contain only 50–100 molecules of POT1 and TPP1 [45]. With such limited expression of POT1, any further decrease in its protein level would have a profound impact on its function at telomeres. Accordingly, a previous report demonstrated that POT1 suppression with shRNA led to an increase in telomere length [27,44]. Decreased levels of POT1 protein in POT1 mutants could occur due to a destabilized POT1/TPP1 complex, destabilized POT1 transcript, or a misfolded protein response, all of which would cause POT1 haploinsufficiency.

Loss of telomerase inhibition from CST complex

In addition to persistent telomerase access, loss of inhibition of telomerase following telomeric repeat extension can also lead to telomere elongation. In mammalian cells, the CST (CTC1-STN1-TEN1) heterotrimer appears to play a key role in this step. CST, an RPA like OB containing complex, is found transiently associated with telomeres [46,47]. In late S/G2 phase, after telomerase extends the telomeric DNA, the CST complex is brought to telomeres by TPP1 and POT1, where it limits accumulation of telomerase and terminates telomerase action [48,49]. Recent studies have confirmed significant lengthening of telomeres in the context of CST mutation or inhibition [47,50,51]. Another function of CST at telomeres is to facilitate proper DNA replication. Due to the repetitive nature of telomeres and their tendency to form secondary structures, DNA replication experiences frequent fork stalling and/or collapse in telomeres, which is manifested as fragile telomeres in metaphase spreads [52–55]. It is unclear whether POT1 regulates CST during telomere replication.

Since most POT1 mutants have elevated fragile telomere signals, it is tempting to speculate that CST function is compromised in this setting. Indeed, Pinzaru et al. observed that reduced interaction between STN1 and TEN1 was associated with the Lys90Glu mutation, suggesting that destabilized CST complex assembly might contribute to the phenotype [37].

Gain-of-function variants generated by POT1 mutation

In addition to the loss-of-function or haploinsufficiency mutants as discussed above, there are also possibilities of gain-of-function mutants. The truncation mutants generated by nonsense or splicing mutation are such candidates. Previous studies have shown that naturally occurring POT1 splice variants display different DNA binding and telomere elongation capacities [42,43]. Recent CRISPR knockout of individual POT1 isoforms further demonstrated differences in their ability to block ATR activation and to regulate telomere overhang length [56]. Notably, most of the truncation mutants are predicted to lose their TPP1 interaction domain partially or completely, and thus are not expected to be recruited to telomeres. If such proteins are stable, would they gain extratelomeric functions, find new binding partners, or be reminiscent of natural POT1 variants? Work on the POT1a paralog in *Arabidopsis thaliana* demonstrated that POT1a is part of the telomerase complex and acts as a positive regulator of telomere length by stimulating repeat addition processivity. Alternatively, could human POT1 splice variants gain more access to existing partners, for example, CST complex? Again, recent work from *Arabidopsis thaliana* indicates that POT1a is able to displace TEN1 (T) by competing for the same binding site on STN1 (S) and forming a complex with the remaining CST components-STN1 (S) and CTC1 (C). The interaction between POT1a-CS is proposed to promote a telomere extendable state [57,58]. Thus, it is possible that by switching or competing the subunit within the CST heterotrimer, the dynamic POT1-CST interaction can either destabilize/titrate away functional CST to mimic CST deficiency phenotype as described above, or lead to increased assess and/or utilization of its telomeric DNA substrate by telomerase.

Do POT1 mutations confer a selective advantage to promote tumorigenesis and replication stress?

Cancers with POT1 germline mutations are often described as early onset. Pedigree analysis from published cancer-prone families is consistent with a profile of genetic anticipation. That is, POT1 mutations are inherited across generations, and cancers are diagnosed at an earlier age in later generations, with early mortality observed in some cases. This pattern supports a general role for POT1 variants in cancer predisposition. However, the overall penetrance of POT1 alleles associated with cancer is modest or incomplete. Not all mutation carriers develop cancers in their lifetime. Other than CMM, in which POT1 mutations have been detected in 2–4% families, the overall frequency of POT1 mutation is low. In parallel, work from mouse models indicated that POT1a (mouse paralog) inactivation alone is insufficient to initiate tumorigenesis. Only when coupled with p53 deficiency, does loss of POT1 function result in formation of various cancers [37,38,59]. Collectively, it is likely that these POT1 mutations provide a selective advantage to cancer cells and facilitate cancer development.

Recent large-scale studies on the elderly population indicate that telomere lengthening may offer modest benefits for health-span, but could concomitantly increase the risk of cancer [60]. We consider that the increased telomere length evoked by POT1 mutations could provide a selective advantage to cancer cells through a variety of mechanisms. ***(I) delayed replicative senescence checkpoint:*** Telomere shortening and the associated replicative apoptosis/senescence is the first barrier to limit cell growth and block tumorigenesis. With excessively long telomeres, cells are capable of undergoing more replications and are likely to accumulate more mutations. If some of them are driving mutations, or each of them is associated with some fitness advantage, then ultimately the chance of having a clonal expansion and tumor formation is increased. From the cancer spectrum, POT1 mutations seem to have a preference to CMM. It is known that melanoma genomes have the highest mutation load of any cancer [61,62]. ***(II) increased genome instability:*** Telomeres are prone to replication stress due to their repetitive nature and tendency to form G-quadruplex-like structures. The excessively long telomere length associated with POT1 mutations would exacerbate the replication burden. In accord with this notion, characterization of different POT1 mutations have consistently shown elevated levels of fragile telomeres, which indicates telomere replication defects. Moreover, other chromosomal aberrations, including chromosomal breaks, sister telomere fusions, and chromosomal fusions are also prominent. ***(III) bypass the requirement for TERT in tumorigenesis:*** Enabling unlimited replicative potential is one of the hallmarks of cancer. This is achieved most commonly by telomerase reactivation, or less frequently, via an alternative homologous recombination-based telomere maintenance mechanism. It is estimated that more than 85% of cancer cells need to acquire telomerase reactivation to gain a growth advantage. However, Taboski et al. demonstrated that excessively long telomeres bypass the requirement for TERT in tumorigenesis [63]. Thus, bypassing telomerase activation may account for the accumulation of tumor promoting mutations and increase the risk of neoplasms.

Conclusion

Emerging evidence has shown that individuals with excessively long telomeres have an increased risk for cancers, which has led to the identification of a number of genomic rare variants in shelterin components, including POT1. In addition to the loss-of-function or haploinsufficiency variants, there are also possibilities of existence of gain-of-function variants. POT1 variants may confer a selective advantage to promote tumorigenesis through a variety of mechanisms. The study of rare POT1 variants offers a unique opportunity to dissect POT1 function *in vivo*. It is noteworthy that evolution naturally produces POT1 variants in other organisms, such as mice and *Arabidopsis thaliana*. For example, the Brassicaceae lineage of POT1a is under positive selection and the amino acids that were changed serve to enhance the interaction of POT1a with the STN1 component of CST [58]. Studies from these organisms may help our understanding of how rare POT1 variants in humans contribute to tumorigenesis. Finally, rapid advances in whole-exome and -genome sequencing technologies are transforming knowledge from genetic sequence to biological consequence. It is likely that more rare variants associated with shelterin and other telomere length maintenance genes will be identified in the future, and can render new insight into the role of extremely long telomeres in the susceptibility to cancer development.

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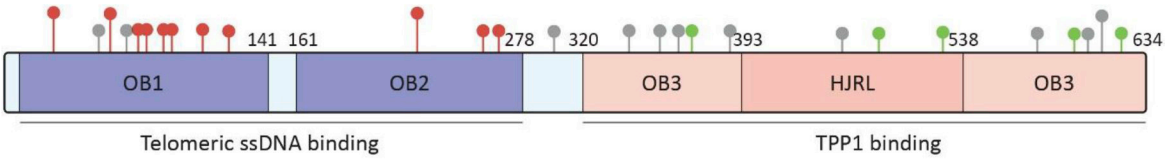
A.



B.

variant type	location
missense mutations in ssDNA binding domain	Y36C, Y36H, I78T, Y89C, K90E, Q94E, G95C R137H, D224N, S270N, R273L, R117C
missense mutations in TPP1 binding domain	Q376R, T497K, A532P, D598S, Q623H
disruptive variants (nonsense, splice acceptor or donor, and frameshift)	nonsense: Q301*, E344*, R363*, E450* splice site: g.124510965C>T, C.255G>A g.124481233C>T, C.1164G>A g.124465412C>T, C.1687G>A g.124465306C>T, C1792G>A frameshift: N75Kfs, Q358Sfs, D617Efs

Figure 1.

Rare germline variants in POT1 identified in familial cancers. (A) Schematic structure of human POT1 protein and conserved domains. OB1 and OB2 folds of POT1 mediating telomeric ss-DNA binding are colored in blue. The C-terminal OB3 and embedded HJRL domain mediating TPP1 binding are colored in red. The positions of variants identified in familial cancers are shown as pins on top of the protein. The taller pins represent the mutations that have been identified more than once. (B) Table of deleterious germline mutations identified in the POT1 gene.

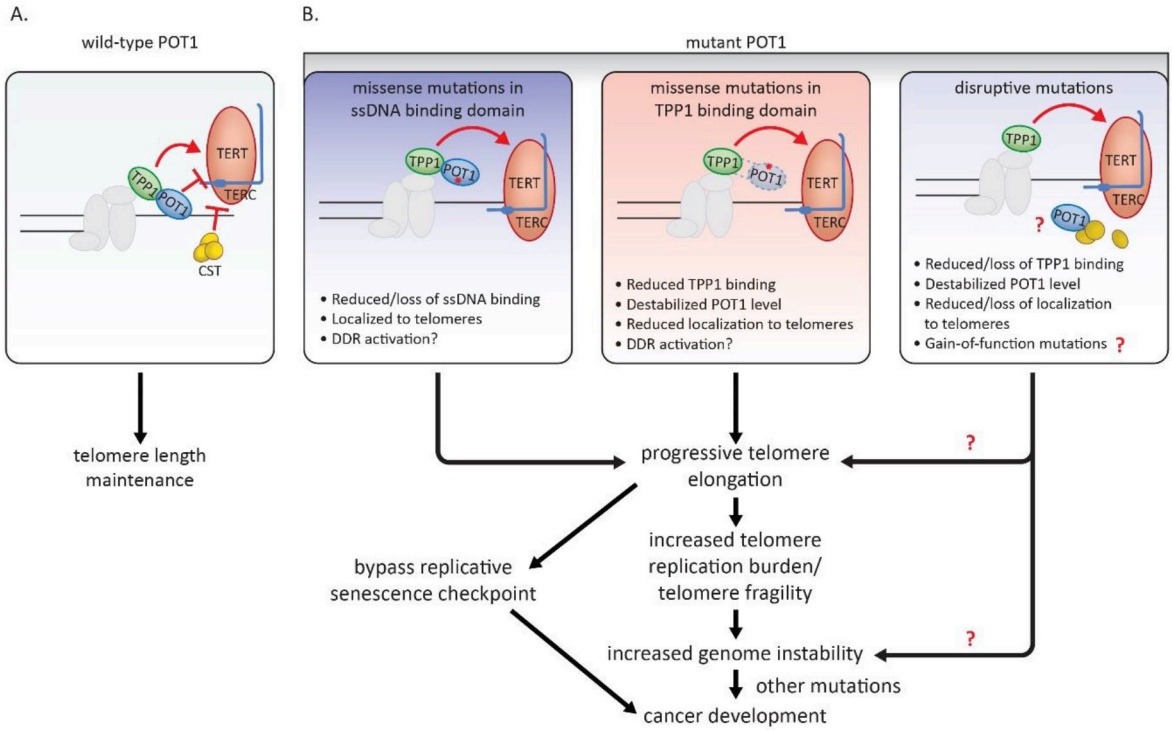


Figure 2.

Impact of rare POT1 variants on telomere length, telomere DNA replication, genome integrity, and cancer. (A) Regulation of telomere length homeostasis by POT1. Wild-type POT1 together with TPP1 functions to protect telomere ends and to modulate telomerase access to 3’ ssDNA. The POT1-TPP1 complex both negatively and positively regulates telomerase recruitment and processivity. The CST complex facilitates telomere DNA replication and terminates telomerase action. (B) Rare POT1 variants lose the ability to protect telomeres through different mechanisms, shown in the boxes. Ultimately, cells undergo progressive telomere elongation, which further exacerbates replication burden and telomere fragility, consequently contributing to genome instability and cancer. Also, excessively long telomeres may allow bypass of the replicative senescence checkpoint and promote tumorigenesis.