POT1 mutations cause telomere dysfunction in chronic lymphocytic leukemia

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Chronic lymphocytic leukemia (CLL) is the most frequent leukemia in adults1–3. We have analyzed exome sequencing data from 127 individuals with CLL and Sanger sequencing data from 214 additional affected individuals, identifying recurrent somatic mutations in POT1 (encoding protection of telomeres 1) in 3.5% of the cases, with the frequency reaching 9% when only individuals without IGHV® mutations were considered. POT1 encodes a component of the shelterin complex and is the first member of this telomeric structure found to be mutated in human cancer. Somatic mutation of POT1 primarily occurs in gene regions encoding the two oligonucleotide-oligosaccharide-binding (OB) folds and affects key residues required to bind telomeric DNA. POT1-mutated CLL cells have numerous telomeric and chromosomal abnormalities that suggest that POT1 mutations favor the acquisition of the malignant features of CLL cells. The identification of POT1 as a new frequently mutated gene in CLL may facilitate novel approaches for the clinical management of this disease.

A long-standing barrier to understanding leukemogenesis has been an inability to accurately identify the genetic alterations that underlie this disease4. To gain further insight into the genetic basis of CLL, we have analyzed the exomes of matched tumor and normal samples from the peripheral blood of 127 untreated individuals with CLL, identifying telomeric somatic mutations using the previously described Sidrón algorithm5,6. Several of the driver genes, such as SF3B1, NOTCH1 and MYD88, have previously been assessed for their functional and clinical roles in CLL5–8. We also observed that POT1 is a frequent genetic target for somatic mutation in CLL, and, given its prominent role in telomere capping and telomerase recruitment9,10, we hypothesized that mutations in this gene might be a source of genomic instability and represent a new tumor-driving factor. In fact, when the number of mutations, gene size and codon composition were taken into account, POT1 was the gene that was second most frequently affected by point mutations (P = 4.2 × 10−6), with only SF3B1 having a higher frequency (P = 8.6 × 10−11). POT1 was found to be mutated in approximately 5% of CLL cases (Supplementary Table 1) and, conversely, was exclusively altered in samples with wild-type IGHV®, suggesting that inactivation of POT1 could represent a distinct molecular alteration for the development of this aggressive CLL subtype. We next extended our sequence analysis of POT1 by performing Sanger sequencing in a validation set consisting of 214 matched tumor and normal samples from individuals with CLL (Supplementary Table 2), with the finding of 5 additional somatic mutations. RNA sequencing (RNA-seq) analysis of available cases confirmed the expression of mutant POT1 alleles in CLL tumor cells (data not shown). Therefore, we have identified 12 somatic point mutations in 341 CLL cases (3.5%; Table 1). This finding also implies that 9% of individuals with wild-type IGHV® have a POT1 mutation, which is a comparable mutational frequency to that of SF3B1 (11%) in individuals of this CLL subtype9. Our findings make POT1 one of the most frequently mutated genes reported so far in CLL along with NOTCH1, SF3B1, TP53 and ATM6–8. Notably, 3 of the 12 mutations found in POT1 are predicted to result in a truncated protein. The remaining somatic mutations were all nonsynonymous and were predominantly predicted to be deleterious by a consensus of five bioinformatics tools11, supporting the idea that POT1 mutations constitute a driver event in CLL (Table 1).

Shelterin proteins (namely TRF1, TRF2, TIN2, TPP1, RAP1 and POT1) are critical regulators of telomere integrity by modulating telomere capping, replication and telomere extension by telomerase12–14. Structurally, POT1 is the only shelterin that contains two N-terminal OB domains, which confer to this protein affinity for the single-stranded DNA (ssDNA) sequence TTAGGG, in addition to a C terminus that binds TPP1 and anchors POT1 to the shelterin complex. Notably, 9 of the 12 POT1 somatic mutations we have detected in CLL samples reside within gene regions encoding the OB folds (Fig. 1a). Comparative sequence analysis of the OB fold somatic alterations showed that most mutated residues are phylogenetically conserved and suggests that they have an essential role in...
### Table 1. Somatic mutations affecting POT1 in individuals with CLL

<table>
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<tr>
<th>Case</th>
<th>Mutation</th>
<th>Exon</th>
<th>Amino-acid change</th>
<th>Mutation type</th>
<th>Prediction</th>
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### Figure 1

#### Structural impact of POT1 somatic mutations.

(a) Impact of mutations on the primary structure of the POT1 protein. The alterations identified in this study (red) are depicted on a representation of the protein, with the positions of the OB domains indicated (green boxes). Below, additional somatic alterations in other tumors and CLL studies are shown in black. (b) Phylogenetic features of the mutated residues. Protein sequence alignments around the non-truncating mutated residues (arrows) in evolutionarily diverse species are shown. Completely and partially conserved residues are highlighted in dark and light purple, respectively. (c) Ribbon representation of the POT1 OB domains (green) bound to a telomere-like oligonucleotide (orange). Detailed views of several mutated amino acids identified in individuals with CLL are shown to the right.
capacity was seen when the p.Phe62Ala substitution was introduced into the OB domain of the POT1 mouse homolog, Pot1a\(^\text{18}\). These experiments show that mutant POT1 proteins are able to localize to telomeres with other shelterin components but are unable to bind telomeric G-strand overhangs.

We then sought to determine whether mutant POT1 localized at telomeres causes telomere elongation in a dominant-negative fashion, as previously described for the ΔOB POT1 mutant\(^\text{10,17}\). Expression of wild-type and mutant POT1 proteins was maintained over the 45 population doublings examined (Supplementary Fig. 1b), and, in agreement with previous reports, exogenous expression of wild-type POT1 did not significantly increase telomere length in HT1080 cells, whereas the mutant POT1 proteins significantly increased telomere length in each of the population doubling points examined (Fig. 2e and Supplementary Fig. 1c,d) without altering telomerase activity (Supplementary Fig. 1e,f). Consistent with the mutations behaving in a dominant-negative manner to facilitate partial and/or full loss of POT1 function, ablation of human POT1 (ref. 19) and of mouse POT1 orthologs\(^\text{18,20}\) resulted in a dysfunctional telomeric phenotype characterized by elongated telomeres and unprotected telomere ends that lead to chromosomal alterations. Accordingly, we examined the frequency of chromosomal aberrations in HT1080 cells expressing wild-type and mutant POT1 proteins after 5, 15 and 45 population doublings by telomeric FISH analysis of metaphase spreads. At population doubling 45, HT1080 cells expressing mutant POT1 proteins showed a marked propensity for the fusion of sister chromatids (>5 fusions per metaphase; Fig. 2f,g), with the frequency of incidence increasing significantly with progressive population doublings (Supplementary Fig. 2a). As shelterin components are gatekeepers guarding against telomere fragility\(^\text{12,13}\), we also measured the presence of multitelomere signals (MTSs), a telomere aberration that has been associated with increased replication fork stalling at telomeres. Notably, HT1080 cells expressing mutant POT1 proteins had a tendency to have higher numbers of MTSs, suggesting that there was elevated telomere fragility due to the actions of the mutant POT1 proteins (Supplementary Fig. 2b).

We also observed significantly more chromosomal breaks and fusions in cells expressing the mutant POT1 proteins in comparison to cells expressing wild-type POT1 or an empty vector (Fig. 2h and Supplementary Fig. 2c,d). In contrast, overexpression of wild-type POT1 led to no change in the frequency of each of the chromosomal events examined (Fig. 2f,g and Supplementary Fig. 2a–d), showing that the chromosomal alterations were due to the specific activities of mutant POT1 in the heterologous cell lines rather than to a generalized increase in POT1 protein amounts.

Unprotected telomeres can lead to chromosomal instability and the acquisition of genomic aberrations, a key event in tumorogenesis\(^\text{21}\). In individuals with CLL, increased genomic complexity has been correlated with decreased survival\(^\text{22,23}\). We first assessed telomere lengths in purified tumor cells from CLL cases harboring POT1 mutations in comparison to those in age-matched control CLL.
cases with wild-type IGHV containing no mutation in POT1, TP53 or ATM (Supplementary Table 3), observing no significant differences in telomere lengths or in telomerase activities (Supplementary Fig. 3a,b). The absence of changes in telomere length due to POT1 mutations potentially derives from the variance in telomere lengths within human populations, a situation that is distinct from those present in the analysis of isogenic cell lines and inbred mouse strains. Next, we examined the integrity of telomeres in the POT1-mutated CLL samples through telomeric FISH analysis of metaphase spreads (Fig. 3a). Notably, similar to observations made in HT1080 cells, we found significantly more sister chromatid–type end-to-end fusions (SCFs) in POT1-mutated samples than in control samples, with up to five fusions occurring per metaphase (P = 0.01) (Fig. 3a,b). We also detected significantly more chromosome-type fusions containing TTAGGG signals in POT1-mutated samples than in control samples (P = 0.042) (Fig. 3c), but the number of chromosome-type fusions with no detectable TTAGGG signal was not significantly different between the two populations (P = 0.072) (Supplementary Fig. 3c), in agreement with the normal telomere lengths observed in POT1-mutated samples. As in HT1080 cells, we detected a modest but significant increase in the presence of MTSs in POT1-mutated CLL cells compared to the controls (2.2-fold increase, P = 0.03; Fig. 3d), reflecting the presence of greater telomere fragility in these individuals.

Collectively, the observation of a high incidence of telomere-containing chromosomal fusions coincident with no change in the frequency of telomere-deficient fusions is consistent with these fusions being produced by POT1 mutation–mediated telomere uncapping rather than by overt telomere shortening, resembling the chromosomal phenotype of mouse cells with complete ablation of Pot1 activity18,20. This notion is further supported by the fact that we did not find significantly greater numbers of signal-free ends per metaphase in POT1-mutated CLL cells or in HT1080 cells expressing mutant POT1 proteins (Supplementary Figs. 3d and 2e, respectively). We also observed significant increases in the frequency of nuclear aberrations and the presence of unresolved mitotic structures in POT1-mutated samples relative to control samples (Supplementary Fig. 3e–g). Notably, the higher incidence of micronuclei in POT1-mutated CLL cells suggests a potential link between POT1 function and the recently identified phenomenon of chromothripsis24,25. Further, POT1-mutated CLL cells and HT1080 cells expressing mutant POT1 proteins showed no difference in telomeric DNA damage relative to control cells (Supplementary Fig. 4a–c), as has been observed with complete ablation of POT1 proteins from mammalian cells18,19,21. This discrepancy potentially derives from the presence of wild-type POT1 in both cell systems, which has been implicated in suppressing the telomeric DNA damage response in cells expressing the ΔOB POT1 isoform26. Finally, the correlation between genomic instability and poor clinical outcomes, previously observed for individuals with CLL, prompted us to assess the clinical features of individuals with POT1 mutations. Individuals with somatic mutations in POT1 presented with more advanced disease at diagnosis (Binet B: 13% in cases with wild-type POT1 versus 42% in cases with mutated POT1; P = 0.02) and with more adverse biological features, such as the more frequent presence of wild-type IGHV (100% versus 46%; P = 0.001) and the expression of ZAP-70 (75% versus 31%; P = 0.001), than individuals without POT1 somatic mutations (Supplementary Table 4).

The International Cancer Genome Consortium was formed on the idea that next-generation sequencing techniques would reshape and extend understanding of cancer biology to generate novel clinical approaches, including for CLL27. The identification of POT1 as a new frequently mutated gene in CLL and the first shelterin found to be mutated in human cancer will hopefully be useful for improved patient prognosis and will provide additional knowledge for the development of novel therapeutic approaches to treat this disease.


METHODS
Methods and any associated references are available in the online version of the paper.

Accession code. Sequencing, expression and genotyping array data have been deposited at the European Genome-phenome Archive (EGA), which is hosted at the European Bioinformatics Institute (EBI), under accession EGAS000000000092.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Subjects. All individuals gave informed consent for their participation in the study following the guidelines of the International Cancer Genome Consortium (ICGC). Clinical characteristics of the 341 subjects are shown in Supplementary Table 1.

Collection and preparation of samples. The tumor samples used for exome sequencing were obtained from fresh or cryopreserved nonneoplastic cells. To purify the CLL fraction, samples were incubated with a cocktail of magnetically labeled antibodies directed against T cells (CD2 and CD3), natural killer (NK) cells (CD1b and CD56), monocytes (CD11b and CD13) and granulocytes (CD11b, CD14 and CD15), with adjustment for the percentage of each contaminating population (AutoMACS, Miltenyi Biotec). The degree of contamination by non-CLL cells in the CLL fraction was assessed by immunophenotyping and flow cytometry. Whole blood was sedimented with 2% dextran, and the leukocyte fraction was obtained. DNA was extracted from purified samples using a FlexiGene DNA kit (Qiagen). The quality of purified DNA was assessed by SYBR Green staining on agarose gels, and DNA was quantified using a Nanodrop ND-100 spectrophotometer. The tumor samples for exome sequencing contained 29% neoplastic cells, and the contamination from neoplastic cells in normal DNA was <2%. The samples for the CLL validation cohort contained >30% neoplastic cells.

Exome enrichment. We sheared 3 µg of genomic DNA from each sample and used it for the construction of a paired-end sequencing library as described in the paired-end sequencing sample preparation protocol provided by Illumina. Enrichment of exon sequences was then performed for each library using the SureSelect Human All Exon 50Mb kit (Agilent Technologies) following the manufacturer’s instructions. Exon-enriched DNA was pulled down by magnetic beads coated with streptavidin (Invitrogen), washed and eluted, and 18 additional cycles of amplification of the captured library were performed. Exon enrichment was validated by RT-PCR on a 7300 Real-Time PCR System (Applied Biosystems) using a set of two pairs of primers to amplify exons and one pair of primers to amplify an intron. Enriched libraries were sequenced in one lane of an Illumina Genome Analyzer IIx sequencer using the standard protocol.

Read mapping and processing. For exome sequencing, reads from each library were mapped to the human reference genome (GRCh37) using Burrows-Wheeler Aligner (BWA) with the sample option, and a BAM file was generated using SAMtools. Optical or PCR duplicates were removed using Picard. Bases were initially called using the Mapping and Assembly with Qualities (MAQ) consensus model implemented in SAMtools. For the identification of somatic substitutions, we used the Sídúrón algorithm, which has previously been described.5,6 This algorithm was adapted to account for sites with very high coverage resulting from exome enrichment. Thus, we added new cutoff values for the S parameter (30 for coverage higher than 50 and 50 for coverage higher than 100).

Sanger sequencing. PCR products were cleaned using ExoSap IT (USB Corporation) and sequenced using ABI Prism BigDye Terminator v3.1 technology (Applied Biosystems) with 5 µmol of each primer (Supplementary Table S). Sequencing reactions were run on an ABI-3730 automated sequencer (Applied Biosystems). All sequences were manually examined.

Characterization of mutations. The amino-acid sequences of POT1 from evolutionarily diverse species were gathered from NCBI and aligned with CLUSTALX2 (ref. 28). Alignment was depicted with Jalview v2.7 (ref. 29). The structure of the OB domains of POT1 was obtained from the accession 3KJO in the Protein Data Bank (PDB) and was rendered with PyMol v0.99. To statistically assess mutational selection of residues that cluster within the OB folds, we compiled a list of all residues closer than 3.5 Å to the telomeric DNA in the crystal structure of POT1 (PDB 3KJP). This defined an interface between both molecules comprising 24 residues (residues 31, 33, 36, 39–42, 48, 60, 62, 87, 89, 94, 159, 161, 223, 224, 243, 245, 266, 267, 270, 271 and 273). We then performed a Monte Carlo simulation with 10 million sets of 8 random nonsynonymous mutations. All had less than 6 residues in this interface (only 1 set had 5).

Retroviral expression. The pLPC myc human POT1 plasmid was a gift of T. de Lange (Addgene plasmid 12387)56. The POT1 variants encoding p.Tyr36Asn and p.Tyr223Cys changes were generated by site-directed mutagenesis of the wild-type cDNA using the QuikChange XL site-directed mutagenesis kit (Stratagene) and the corresponding oligonucleotides (Supplementary Table 6). Retroviruses were packaged in HEK 293T cells using a vesi-cular stomatitis virus G (VSVG)-based package. HT1080 cells were seeded in 6-well plates at 20–30% confluency 24 h before infection. The following day, 1 ml of viral supernatant was added to the growth medium supplemented with 5 µg/ml polybrene (Millipore), and plates were centrifuged at 200g for 1 h at room temperature and incubated for 8 h. This protocol was repeated twice, and cells were allowed to recover for 24 h in growth medium before undergoing selection with puromycin for 3 d. Cells then underwent serial passaging and were collected at the indicated population doubling points.

Confocal microscopy. HT1080 cells expressing Myc-tagged wild-type and mutant (Tyr36Asn and Tyr223Cys) POT1 were plated on sterile glass coverslips and allowed to adhere overnight. After 24 h, cells were fixed with 2% formaldehyde and were permeabilized by incubation with 1% Triton X-100 in PBS. Cells were washed, blocked with 0.2% cold-water-fish gelatin (Sigma) and 0.5% BSA in PBS and incubated for 4 h at room temperature with 9E10 antibody to Myc (Abcam; 1:1,000 dilution) in combination with an antibody to TRF1 (Abcam, ab10579; 1:1,000 dilution) in blocking buffer. For immuno-fluorescence detection of TIF proteins, HT1080 cells and peripheral blood mononuclear cells (PBMCs) were similarly fixed and permeabilized before incubation with antibodies to 53BP1 (Novus Biologicals, NB100-304; 1:500 dilution) and TRF1. After multiple washes, cells were incubated with Alexa Fluor 488– and Alexa Fluor 568–conjugated goat secondary antibodies to rabbit IgG (1:250 dilutions) for 1 h at room temperature. Nuclei were stained by incubating cells with 4.6-diamidino-2-phenylindole (DAPI), after which coverslips were mounted on slides, and cells were imaged with a Leica SP2 confocal microscope (Leica Microsystems). Images were processed using ImageJ and displayed using CorelDraw.

Protein blot analysis. Whole-cell lysates were collected in a buffer containing 1% Triton X-100, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl and protease inhibitor cocktail (Roche Applied Science), and protein concentrations were determined by bicinchoninic acid assay (BCA; Pierce). Equal amounts of lysates (20 µg) were separated by SDS-PAGE and transferred to a nitrocellulose membrane that was blocked with 5% nonfat dry milk. Membranes were incubated overnight at 4 °C with 9E10 antibody to Myc (Abcam, ab32; 1:1,000 dilution), antibody to phosphorylated CHK1 (Cell Signaling Technology, 2341; 1:1,000 dilution) or antibody to β-actin (Sigma, A5441; 1:10,000 dilution) and then washed before incubation with species-appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. Membranes were analyzed using an LAS-3000 mini imaging system (Fujifilm).

ChIP assays. For ChIP analysis, 3 × 10⁶ cells were used per condition. Formaldehyde was added directly to the tissue culture medium to a final concentration of 1%, and cells were incubated for 15 min at room temperature (25 °C) on a shaking platform. Cross-linking was then stopped by the addition of glycine to a final concentration of 0.125 M. Cross-linked cells were washed twice with cold PBS, collected and lysed at a density of 20 × 10⁶ cells/ml for 10 min at 4 °C in a buffer of 1% SDS, 50 mM Tris-HCl (pH 8.0) and 10 mM EDTA containing protease inhibitors. Lysates were sonicated to obtain chromatin fragments of <1 kb in size and centrifuged for 15 min in a microcentrifuge at room temperature. Chromatin was diluted 1:10 with a buffer composed of 1.1% Triton X-100, 2 mM EDTA, 150 mM NaCl and 20 mM Tris-HCl (pH 8.0) containing protease inhibitors and precleared with a salmon sperm DNA–protein A agarose slurry (Upstate). Chromatin fragments were incubated with 10 µg of one of the following antibodies at 4 °C overnight on a rotating platform: 9E10 antibody to Myc, antibody to TRF1 (Abcam, ab1423), antibody to TRF2 (Millipore, 05-521), antibody to POT1 (Abcam, ab21382) and antibody to RAP1 (Bethyl Laboratories, A300-306A). Salmon sperm DNA–protein A agarose beads (60 µl) were then added, and incubation was continued for 1 h. Immunoprecipitation pellets were washed with 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl.
Telomere length assays. Using the TeloTAGGG Telomere Length Assay kit (Roche), 2 µg of DNA for each sample were digested and subjected to Southern blot analysis, and determination of mean terminal restriction fragment (TRF) length was performed according to the manufacturer’s instructions. Densitometry arrays of 30–40 quadrants were generated across the entire lane for each sample using Multi-Gauge V3.0 software (Fujifilm). After background subtraction, absorbance (A) was obtained in each of these quadrants corresponding to different DNA lengths (L). Mean TRF was defined as \( \Sigma(A_i)/\Sigma(A_i/L_i) \) for each sample.

TRAP assays. Telomerase activity was measured with a modified telomeric repeat amplification protocol (TRAP) assay as previously described\(^{31}\).

Telomeric FISH on metaphase spreads and HTQFISH. For telomeric FISH on metaphase spreads, 3 × 10\(^4\) PBMCs or HT1080 cells expressing Myc-tagged wild-type and mutant (Tyr36Asn and Tyr223Cys) POT1 were stimulated with 7.5 ng/ml 12-O-tetradecanoylphorbol-13-acetate (TPA) in RPMI medium supplemented with FBS, penicillin-streptomycin, β-mercaptoethanol, sodium pyruvate, nonessential amino acids and L-glutamine. After 72 h, metaphase spreads were obtained, and telomeric FISH was performed as described\(^{32}\). At least 5–10 metaphase spreads per subject or HT1080 cell line were analyzed, and chromosomal aberrations were quantified and represented as frequency per metaphase. High-throughput quantitative telomeric FISH (HTQFISH) was performed as described\(^{33}\), seeding at least 150,000 cells in duplicate wells and analyzing >5,000 nuclei per HT1080 cell line or subject sample.