RESEARCH ARTICLE

Acquired *TERT* promoter mutations stimulate *TERT* transcription in mantle cell lymphoma

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Mantle cell lymphoma (MCL) is an aggressive lymphoid neoplasm with poor prognosis. Acquired telomerase reverse transcriptase gene promoter (*TERT*p) mutations are among the most frequent somatic non-coding mutations in cancers. In this study, the prevalence of *TERT*p mutations in 24 MCL and 21 other lymphoid neoplasias (oLN) was investigated. Eight MCL samples (33%) carried *TERT*p mutations, two homozygous and six heterozygous (seven C228T and one C250T), which directly correlated with higher *TERT* transcription, mitochondrial DNA copy number, and *IGHV* mutational status in MCL neoplastic cells. *TERT*p mutations were not found in oLN. *TERT*p mutations correlated with more lymphoma proliferation and tumor burden, as suggested by the higher number of lymphoma cells circulating in peripheral blood, and tended to associate with longer MCL telomeres, especially in homozygous mutants, although not statistically significant. Telomere-biology genes were overexpressed in MCL cells in comparison to healthy lymphocytes, but were not influenced by mutation status. The findings described for the first time that acquired *TERT*p mutations are common in MCL but not in other lymphoid neoplasms. It was also demonstrated that *TERT*p mutations are associated with higher *TERT* mRNA expression in MCL cells in vivo and higher tumor burden, suggesting these mutations as a driver event in MCL development and progression.
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Introduction

Lymphoid malignancies constitute a heterogeneous group of lymphoproliferative disorders with specific clinical, morphological, immunophenotypic, and genetic features. Mantle cell lymphoma (MCL) is a distinct entity genetically characterized by the t(11;14) translocation and *cyclin D1* overexpression [1]. MCL displays an aggressive clinical behavior with poor response to current therapeutic strategies and a median survival between 3 and 5 years. Such an aggressive feature relates to a high number of recurrent secondary chromosomal aberrations and different molecular mechanisms causing gene deregulation. The transcription factor *SOX11* (SRY [sex determining region Y]-box 11) has been found aberrantly expressed in most aggressive MCL patients, being considered a reliable biomarker in this pathology [2]. This gene is commonly associated with embryonic neurogenesis and tissue remodeling, and is not expressed under normal conditions in any adult normal tissue [3].

Telomeres are ribonucleoprotein structures at the end of linear chromosomes that protect them from end-to-end fusion and recombination. Telomeres shorten with mitotic cell division and telomere excessive attrition predisposes cells to chromosomal aberrations and malignant transformation, including hematologic malignancies [4,5]. The shelterin complex (TRF1, TRF2, TIN2, RAP1, TPP1, and POT1) comprises a group of proteins coating telomeres and regulates telomere length (TL) by modulating telomerase activity [6]. Telomerase is a reverse transcriptase composed of the catalytic subunit TERT, the RNA component TERC, and stabilizing proteins, such as dyskerin (encoded by *DKC1*). By adding telomeric DNA repeat to chromosome ends, telomerase maintains TL and compensates for the continued replicative loss of telomeres. Normal mature cells express low to undetectable levels of *TERT*, whereas more than 90% of human tumor cells overexpress *TERT* [7].

Recently, somatic mutations in the *TERT* promoter (*TERT*p) region were detected in many solid tumors [8–10]. However, the presence of acquired *TERT*p mutations in lymphoid malignancies has not been determined. In this study, we investigated the prevalence of *TERT*p mutations in MCL and other lymphoid neoplasias (oLN). Mutational status was correlated with TL, mtDNA copy number, and the expression of telomere-biology genes in tumor cells.

Additional Supporting Information may be found in the online version of this article.

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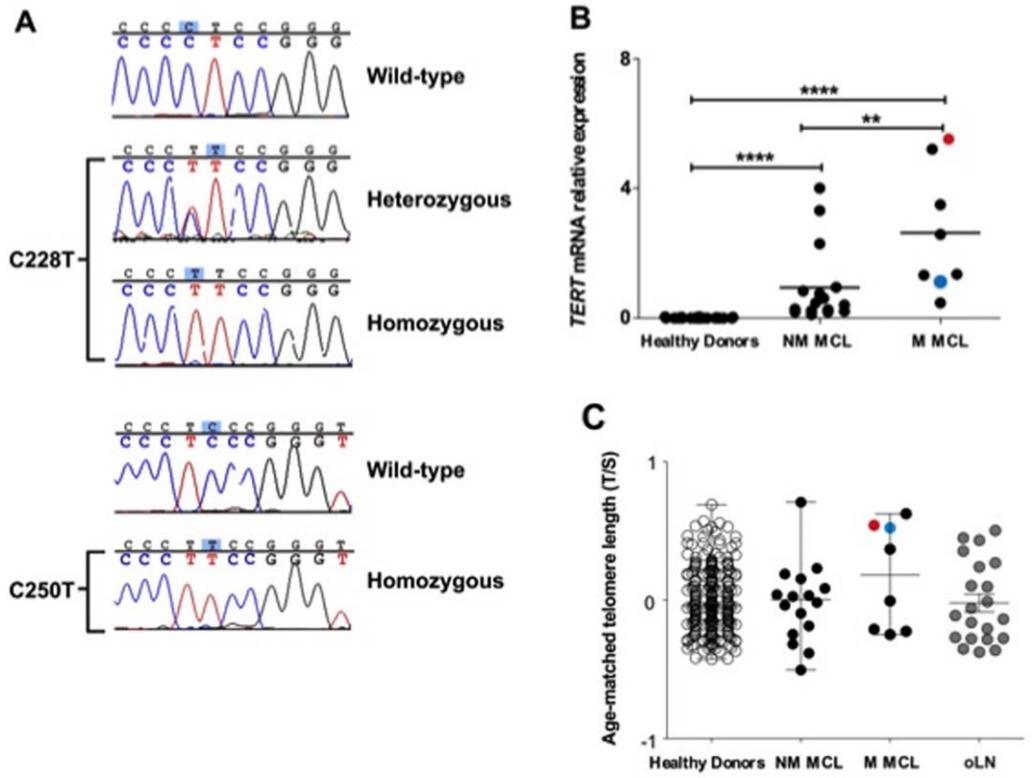

Figure 1. Influence of *TERT* promoter (*TERT*p) mutations on gene expression in MCL. **(A)** Chromatogram of the *TERT* promoter region including the C228T and C250T mutations. Eight MCL samples carried the mutations: six heterozygous and two homozygous. **(B)** *TERT* mRNA expression level in healthy control peripheral blood leukocytes and MCL cells, according to the *TERT*p mutation status. Transcripts levels were measured using quantitative RT-PCR of peripheral blood cells of 25 healthy donors and 24 mantle cell lymphoma (MCL): 16 non-mutated *TERT*p MCL (NM MCL) and 8 mutated *TERT*p MCL (M MCL), (**$P = 0.0047$; ****$P < 0.0001$). **(C)** Telomere length analysis was performed by qPCR in MCL and other lymphoid neoplasias (oLNs) cases. MCL samples tended to be longer in some mutant cases, especially in those bearing homozygous mutations. TL in the oLNs was comparable to MCL. The homozygous C228T and C250T *TERT*p mutant MCLs are depicted in *red* and *blue*, respectively. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Materials and Methods

Patients. We collected peripheral blood samples of 24 patients with leukemic MCL (7 female; mean age: 67 years; range: 49–88 years) and 21 patients with oLN (8 female; mean age: 63 years; range: 45–79 years) including hairy-cell leukemia, follicular lymphoma, Waldenström macroglobulinemia, B-prolymphocytic leukemia, plasmacytic leukemia, and T-cell lymphoma. Patients were seen at the University Hospital, University of São Paulo, Ribeirão Preto, SP, Brazil, and were diagnosed according to the World Health Organization criteria [11]. The study was approved by the local Ethics Committee and all individuals gave their written informed consent.

Mutational analysis. Total genomic DNA was extracted and purified from peripheral blood samples of MCL patients and controls using PureGene Gentra kit (Qiagen). The promoter region of *TERT* (−424 to +65) was amplified from genomic DNA according to Killela et al. [9]. PCR products were purified with a QIAquick PCR purification kit (Qiagen), and direct sequencing was performed with BigDye Terminator version 3.1 (Applied Biosystems) in an automated genetic-sequence analyzer (ABI Prism 3500 XL, Applied Biosystems).

Gene expression by real-time quantitative PCR (qPCR). Total RNA was extracted, purified and reverse transcribed from peripheral blood samples of MCL patients and controls using standard methods [12]. Gene expression was quantified by qPCR using a LightCycler system (Roche Diagnostics, Mannheim, Germany) with TaqMan methodology. The PCR conditions as well as the sequences of primers and probes were previously described [13,14]. For all targets, the PCR amplification protocol was initiated at 95°C for 10 minutes followed by 45 PCR cycles consisting of at 15 sec at 95°C followed by 60°C for 1 min. All measurements included a determination of the standards and the use of a no-template as a negative control, in which water was substituted for the cDNA. For data normalization to correct for variations in RNA quality and quantity, *SOX11* expression was performed with the reference gene *GUSB* (*β-glucoronidase*) (Hs00939627_m1, Applied Biosystems, Foster City, CA) and the analysis of shelterin components was conducted with the reference gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). All samples were assayed in duplicate, and values were expressed as mean ± standard deviation.

Telomere length measurement. Mean telomere length was measured by qPCR based on a modification of the method described by Cawthon [15], as we previously described [16,17]. Briefly, qPCR was conducted in triplicate and all qPCR reactions were prepared on a QIAgility automated pipettor (Qiagen, California). Amplification was conducted in the Rotor-Gene Q (Qiagen) real-time PCR cycler. The telomere length for each sample was determined using the telomere to single copy gene ratio (*T/S* ratio) by calculating the ΔCt [*Ct*(telomere)/*Ct*(single gene)]. The *T/S* ratio for each sample (x) was normalized to the mean *T/S* ratio of reference sample [$2^{-(\Delta Ctx - \Delta Ctr)} = 2^{-\Delta\Delta Ct}$], which was also used for the standard curve, both as a reference sample and as a validation sample. In every run, two reference samples were included to validate each reaction. The experiment was considered acceptable if control sample *T/S* ratio ranged within the 95% variation interval (0.95–1.05).

Immunoglobulin heavy chain variable region (IGHV) mutational analysis. The *IGHV* gene sequences were determined by PCR of *IGHV* regions on cDNA obtained from total RNA from MCL patients. VH framework region one consensus family specific primers (VH1–VH6) and one antisense JH primers were used. When amplifications of these primers were unsuccessful, an alternative set of primers that anneal to sequences in the leader region (LH1–LH6) and one antisense Cμ primer were used. PCR products were purified in 2% agarose gels, sequenced bidirectionally, and analyzed on an automated DNA sequence analyzer (377 ABI Prism, PE biosystem, Foster City, CA) [18]. Sequence data were analyzed by using IgBLAST (immunoglobulin BLAST) (http://www.ncbi.nlm.nih.gov/igblast) and the ImMunoGeneTics database (IMGT) (http://imgt.cines.fr). Sequences were subdivided according to Hadzidimitriou et al. [19] criteria into "truly unmutated" (TU), with 100% germinal identity (GI), "minimally/borderline mutated" (MBM) with 99.9%–97% GI, or "highly mutated" (HM) with less than 97% GI.

Analysis of mtDNA copy number by qPCR. Total genomic DNA was extracted from MCL patients using PureGene Gentra kit (Qiagen), and the concentration of DNA was measured using DenoVix. mtDNA copy number was performed according to Gu et al. [20]. Briefly, *ND4* gene was used to represent the mtDNA, and *pyruvate kinase* (*PK*) gene was used to represent the nuclear DNA. Relative mtDNA copy numbers were assessed after *ND4* normalization by the single-copy nuclear gene *PK*. All reactions were performed in 96-well plates on an ABI 7500 instrument. The corresponding real-time PCR efficiencies for each mitochondrial and nuclear gene amplification were calculated according to the equation: $E = 10^{(-1/\text{slope})} - 1$. Relative mtDNA copy number (mtDNA amount/nDNA amount) was calculated by a comparative *Ct* method using the following equation: $\text{mtDNA/nDNA} = 2^{-\Delta Ct}$, where $\Delta Ct = Ct_{\text{mitochondrial}} - Ct_{\text{nuclear}}$.

Statistical analysis. All statistical tests were performed using GraphPad Prism version 6.0 (2008). Mann–Whitney test was used to compare data from patients, controls

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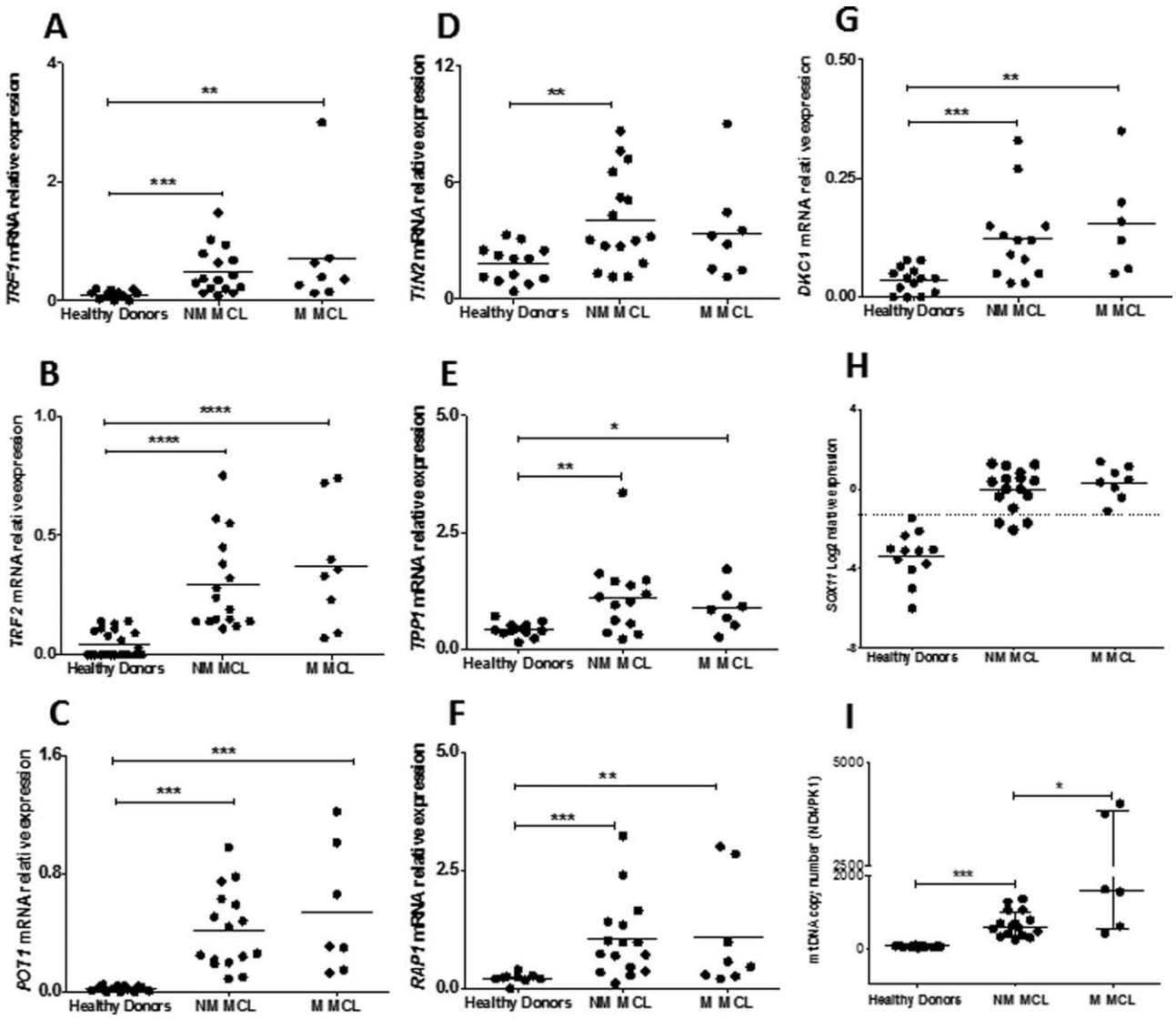

Figure 2. Expression of telomerase and telomere biology genes and molecular characterization of MCL patients. **(A)** mRNA expression levels of *TRF1* (**P = 0.0013; ***P = 0.0001), **(B)** *TRF2* (****P = 0.0001), **(C)** *POT1* (***P < 0.0001), **(D)** *TIN2* (**P = 0.0035), **(E)** *TPP1* (**P = 0.0063; *P = 0.01), **(F)** *RAP1* (**P = 0.0002; ***P = 0.0075), and **(G)** *DKC1* (**P = 0.0024; ***P = 0.0009) in healthy control peripheral blood leukocytes and MCL cells, according to the *TERT*p mutation status. Results were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene. **(H)** *SOX11* mRNA expression level in healthy control peripheral blood leukocytes and MCL cells, according to the *TERT*p mutation status. **(I)** mtDNA copy number in MCL patients. MCL cells had increased mtDNA copy number compared with normal lymphocytes ($P < 0.0001$), and *TERT*p-mutant MCL cells displayed significantly higher mtDNA copy numbers as compared with non-mutated MCL patients ($P = 0.03$).

and subgroups group-wise comparison of the distribution of clinical and laboratory variables were performed with the Student t test (for quantitative variables) and the χ^2 or Fisher's exact test (for categorical variables). Receiver operating characteristic (ROC) curves analysis was applied to calculate gene expression cut-off values, with the highest sensitivity and specificity. A $P < 0.05$ was regarded as statistically significant.

Results

*TERT*p mutations, *TERT* expression and TL in MCL and oLN patients

MCL and oLNs samples were screened for *TERT*p mutations. The probed promoter region contained the two commonly mutations to date described (C228T and C250T), located 124 and 146 bp upstream of the ATG start site, respectively. We identified *TERT*p mutations in eight MCL cases (33%) (six heterozygous and two homozygous) (Fig. 1A). The C228T transition was the most frequently mutated site in our series (7/8 mutated samples), whereas the C250T mutation was found in homozygosity in only one MCL sample. No mutations were detected in 21 oLN samples.

Next, we explored whether *TERT*p mutations modulated *TERT* expression *in vivo*. *TERT*p-mutant MCL samples showed significantly higher *TERT* mRNA levels in comparison to non-mutated MCL cases ($P = 0.0047$; Fig. 1B) and the homozygous C228T *TERT*p-mutant case displayed the highest *TERT* transcript levels, providing evidence that *TERT*p mutation enhances *TERT* expression in cancer cells in vivo. To our knowledge, this is the first report showing that *TERT*p mutations modulate *TERT* transcription in hematologic malignancies.

Clinical features were available for 12 MCL cases (Supporting Information Table S1). Patients with mutated MCL presented higher white blood cell count (WBC) compared with non-mutated MCL ($P = 0.0081$). Although lactate dehydrogenase levels were high in the MCL cohort, no significant difference between non-mutated or mutated MCL patients was detected.

Given the higher *TERT* mRNA expression levels in *TERT*p mutant lymphoma cells and their higher proliferative capacity suggested by increased WBC counts, we explored whether *TERT*p mutations modulated telomere lengths. Although telomere length tended to be

longer in some mutant cases, especially in those bearing homozygous mutations, the difference was not significant (Fig. 1C). TL in the oLNs was comparable to MCL (Fig. 1C).

*TERT*p mutation and shelterin and *DKC1* expression

We then determined whether *TERT*p mutations also influenced the expression of other telomere-biology genes. We found that shelterin genes and *DKC1* were all up-regulated in MCL cells in comparison to healthy controls (Supporting Information Fig. S1), *TERT*p-mutant MCL samples exhibited a trend to overexpress *TRF1*, *TRF2, POT1*, and *DKC1*, but it was not statistically significant (Fig. 2A-G).

Molecular characterization of MCL patients

IGHV mutational status, gene usage, and *SOX11* expression were evaluated in all MCL patients. In *IGHV* analysis, we found that 29.2% (7/24) of our cases were TU, 54.2% (13/24) were MBM and 16.7% (4/24) were HM. The most frequently used IGHV families were IGHV3 ($n = 15/24$; 62.5%), IGHV4 ($n = 7/24$; 29.2%), and IGHV1 ($n = 2/24$; 8.3%). The other families were not found in our cohort. Analysis of the *IGHV* gene usage was biased, since only five genes accounted for 70.7% of the series. *IGHV3-21* and *IGHV3-23* (20.8%, each) were the most commonly used, followed by *IGHV4-34* (12.5%), *IGHV1-8* and *IGHV4-39* (8.3%, each) (Fig. 3). Among them, cases expressing *IGHV3-21* gene had the highest proportion of TU status (60%), while *IGHV3-9, IGHV3-11, IGHV4-34, IGHV4-39,* and *IGHV4-4* were all MBM. As for *SOX11* expression, 83% of patients overexpressed this gene, meanwhile the remaining 17% showed *SOX11* downregulation.

As *IGHV* hypermutation is an early event during lymphomagenesis, HM cases are generally related to a subtype of MCL with a more indolent behavior. Most *TERT*p-mutant MCL samples were TU or MBM (Table I) with a distribution similar to the whole cohort. *SOX11* mRNA expression was positive in all mutated cases (Fig. 2H), suggesting that *TERT*p mutations may correlate with molecular biomarkers of a more aggressive disease.

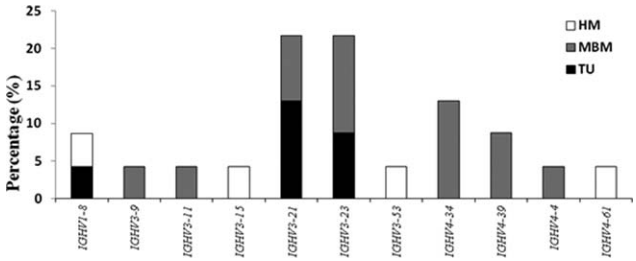

Figure 3. Somatic hypermutation and *IGHV* gene usage in our series of MCL. Most *TERT*p-mutant MCL cases were TU or MBM, with a distribution similar to the one observed in the whole cohort. TU, truly unmutated; MBM, minimally/borderline mutated; HM, highly mutated.

mtDNA copy number and *TERT*p mutations

As we found that *TERT*p stimulated *TERT* expression and correlated with higher cell proliferation, we investigated whether these mutations also modulated mitochondrial function. MCL cells had increased mtDNA copy number compared with normal lymphocytes ($P < 0.0001$; Fig. 2I); additionally, *TERT*p-mutant MCL cells displayed significantly higher mtDNA copy numbers as compared with non-mutated MCL patients ($P = 0.03$).

Discussion

The present study describes for the first time the frequency of acquired *TERT* gene promoter mutations in lymphoid neoplasms. We found that among a variety of leukemic lymphoid neoplasms, somatic hotspot *TERT*p mutations were exclusively found in mantle cell lymphoma cells and correlated with higher *TERT* mRNA expression levels and higher number of circulating lymphoma cells. Six heterozygous and two homozygous *TERT*p mutations were found in a total of 24 MCL samples. Seven cases carried the C228T mutation, which also is the most frequently mutated site in other cancers [9]; C250T was found only in homozygosity in one MCL sample. Those two out of eight mutant cases were homozygous that is relevant, given that homozygosity for *TERT*p mutations is very rare and was only previously described in basal cell and squamous cell carcinomas [21]. In a series of 1,230 samples from 60 different tumor types, all identified *TERT*p mutations were in heterozygosity and were mutually exclusive [9].

In the present study, *TERT*p mutations directly correlated with higher *TERT* transcription in MCL neoplastic cells. To the best of our knowledge, this is the first direct rigorous evidence that these mutations stimulate *TERT* transcription levels in cancer in vivo. Huang et al. [8] had previously demonstrated that C228T and C250T mutations conferred approximately two to fourfold increase in *TERT* using an in vitro reporter assay, possibly related to a potential binding site for E-twenty-six (ETS) transcription factor that stimulate the promoter activity. More recently, Huang et al. [22] also found that *TERT* expression may be increased in tumor cells, but the sample group was a heterogeneous set of neurological cancers and other cancer intrinsic molecular modulators could not be ruled out. Here, a very homogeneous set of lymphoma patients was studied, diminishing the potential confounding effects of sample heterogeneity. That some non-mutated MCL cases in our series also showed high *TERT* mRNA levels suggests that additional genetic or epigenetic factors also modulate *TERT* expression in MCL.

Higher *TERT* expression levels caused by *TERT*p mutations appeared to influence molecular, cellular, and clinical behavior of MCL. First, *TERT*p-mutant MCL telomeres tended to be longer, especially in homozygous mutants, although not statistically significant. Lack of difference in telomere length may be due to the small sample size. Second, *TERT*p mutations correlated with more lymphoma proliferation and tumor burden, as suggested by the significantly higher number of lymphoma cells circulating in peripheral blood. Peripheral

TABLE I. Percentage of Homology, *IGHV* Gene Usage and *SOX11* Expression in *TERT*p-Mutant MCL Patients

Patients	Mutation type	*IGHV* % homology	Gene involved	*SOX11* expression (log_2)
UPN 7	C228T, heterozygous	100 (TU)	VH3-23-01	1.143
UPN 11	C228T, heterozygous	100 (TU)	VH3-21-01	0.083
UPN 19	C228T, heterozygous	98.2 (MBM)	VH3-21-01	0.342
UPN 20	C228T, heterozygous	98.6 (MBM)	VH3-23-01	0.815
UPN 21	C228T, heterozygous	98.5 (MBM)	VH4-34-01	0.464
UPN 24	C228T, heterozygous	97.5 (MBM)	VH4-34-01	−1.097
UPN 32	C228T, homozygous	95.2 (HM)	VH1-08-01	−0.420
UPN 5	C250T, homozygous	100 (TU)	VH1-08-01	1.376

UPN, unique patient number; TU, truly unmutated; MBM, minimally/borderline mutated; HM, highly mutated.

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blood involvement is common in MCL patients [23] and associates with a poorer outcome [24–27]. Third, *TERTp*-mutant samples had TU or MBM *IGHV* status and all of them overexpressed *SOX11*. Finally, *TERT*p mutants were not found in other lymphoid neoplasms. Taken together, these findings suggest that somatic *TERT*p mutations may contribute to MCL more aggressive clinical behavior among mature B cell neoplasm and might be associated with worse prognosis within MCL cases. Larger studies are warranted to determine the implications of *TERT*p mutations in the clinical outcome of MCL.

Dysfunctional telomeres may arise either from critically short telomeres or by disruption of the shelterin complex [28]. In some hematologic malignancies, altered expression of shelterin proteins might disrupt the telomere structure, contributing to malignant transformation [13,28–31]. Particularly in MCL, to our knowledge, there is only one report on the expression levels of *TRF1*, *TRF2*, *TNKS*, and *PIF1* in a limited number of patients (7–8 cases) [30] and no differences in gene expression were found in comparison to lymph nodes. This is the first report determining the expression profile of a broad range of telomere-biology genes (*TRF1*, *TRF2*, *POT1*, *TPP1*, *TIN2*, *RAP1*, and *DKC1*) in MCL. An upregulation of all genes in MCL cells compared with healthy individuals was observed, suggesting that telomere structure is affected. However, we failed to detect a correlation between *TERT*p mutations and telomere-biology gene expression, possibly due to the small number of mutated cases.

Telomerase also appear to play a role in cell metabolism. Telomerase deficiency influences cause mitochondrial dysfunction [32] and mitochondrial telomerase appears to protect neoplastic cells from DNA damage [33]. Telomerase travels from the nucleus to the mitochondria triggered by oxidative stress. In our study, we found that mitochondrial DNA (mtDNA) copy number was higher in lymphoma cells carrying an acquired *TERT*p mutation, suggesting that *TERT* overexpression modulates mtDNA regulation. The exact mechanism through which altered mtDNA may contribute to lymphomagenesis still remains unclear; however, mitochondrial biogenesis is a biologic pathway to be considered [34].

In summary, we describe for the first time that acquired *TERT*p mutations are a common event in MCL but not in other lymphoid neoplasms. We also demonstrate that *TERT*p mutations were associated with higher *TERT* mRNA expression in MCL cells, suggesting an important role of these mutations as a driver event in mantle cell lymphoma development and maintenance.

Author Contributions

JP performed experiments, interpreted the data and wrote the manuscript; RMA-P, AR, BS-L and CS performed experiments, interpreted data and edited the manuscript; RPF provided patients' samples; IS and RTC designed the study, interpreted data and wrote the manuscript.

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