ORIGINAL ARTICLE

Analysis of telomere length in mantle cell lymphoma

Alejandra S. H. Cottliar[1]*, Julieta Panero[1]*, Estela Pedrazzini[1], Maria Fernanda Noriega[1], Marina Narbaitz[2], Andrea Rodríguez[3], Irma Slavutsky[1]

[1]Departamento de Genética, Instituto de Investigaciones Hematológicas ''Mariano R. Castex'', Academia Nacional de Medicina, Buenos Aires, Argentina; [2]División Patologia, Instituto de Investigaciones Hematológicas ''Mariano R. Castex'', Academia Nacional de Medicina, Buenos Aires, Argentina; [3]Departamento de Oncohematología, Instituto de Investigaciones Hematológicas ''Mariano R. Castex'', Academia Nacional de Medicina, Buenos Aires, Argentina

Abstract

Mantle cell lymphoma (MCL) is a well defined lymphoid neoplasm genetically characterized by the t(11;14)(q13;q32). Telomeres play an essential role in preserving chromosomal integrity and genomic stability; their shortening can lead to telomere dysfunction and chromosomal instability, a critical factor in cancer development. In this study, telomere length (TL) measured by terminal restriction fragments (TRF) assay in DNA samples of tumor cells from 20 patients with MCL was evaluated. Results were correlated with clinical, morphologic and cytogenetic characteristics. In all cases, the presence of the *CCND1/IGH@* rearrangement was confirmed by fluorescence *in situ* hybridization and/or PCR analysis. TL in total MCL patients revealed a mean TRF value (4.51 ± 0.79 kb) significantly shorter than those observed in controls (7.49 ± 1.94 kb) ($P < 0.001$); 30% of patients had TL shorter than 4.0 kb. TRF length was not associated with patients age ($P = 0.07$; $r = 0.17$) nor with sex (females: 4.33 ± 0.51 kb and males: 4.57 ± 0.85 kb; $P = 0.63$). No significant differences were found between patients studied at diagnosis (13) (4.44 ± 0.81 kb) respect to those analyzed at relapse (7) (4.63 ± 0.82 kb) ($P = 0.53$). In addition, we compared patients with (4.84 ± 1.09 kb) and without (4.40 ± 0.68 kb) complex karyotypes ($P = 0.45$) and cases with typical morphology (4.48 ± 0.79 kb) vs. blastoid variant (4.63 ± 1.04 kb) ($P = 0.83$), and no significant differences between them were found. Although the number of cases of our series is not large, our results showed that TL reduction in MCL is independent of the clinical characteristics, morphology and karyotype.

Key words mantle cell lymphoma; telomere length; terminal restriction fragments

Correspondence Irma Slavutsky, MD, PhD, Departamento de Genética, Instituto de Investigaciones Hematológicas ''Mariano R. Castex'', Academia Nacional de Medicina, Pacheco de Melo 3081, 1425 Buenos Aires, Argentina. Tel: 5411 4805 8803 ext. 241; Fax: 5411 4803 9475; e-mail: islavutsky@hematologia.anm.edu.ar

*These authors contributed equally to this work.

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Mantle cell lymphoma (MCL) is a well defined lymphoid neoplasm that accounts for approximately 5–10% of all non-Hodgkin lymphomas (NHL). It is characterized by a male predominance, frequently advanced clinical stage at diagnosis, and a poor prognosis with a median survival range of 3–4 yr (1). The genetic hallmark is the translocation t(11;14)(q13;q32) that juxtaposes the proto-oncogen *CCND1* at chromosome 11q13, to the immunoglobulin heavy chain (*IGH@*) gene at chromosome 14q32, leading to the constitutive overexpression of cyclin D1, a cell cycle regulator that is normally not expressed in B-cells. Most of MCL showed no mutations in variable sequences of immunoglobulin (*IGVH*) gene according to a pregerminal-center cell origin. However, up to one-third of cases display mutated *IGVH* genes suggesting that some tumors may originate in specific subsets of B cells that have entered the follicular germinal center (2). No association between *IGVH* mutational status and outcome was observed (3).

Telomeres are linear DNA structures located at the ends of chromosomes, composed by (TTAGGG)n tandem repeats and associated proteins. They play an essential role in preserving chromosomal integrity and genomic stability, constituting a critical factor in cell

survival (4, 5). A core of six telomere binding proteins, termed the shelterin complex, serves to protect telomeric ends and to prevent recognition of chromosome ends as damaged DNA (6). Both critically short telomeres and direct disruption of the shelterin structure can initiate telomere dysfunction. As normal cells undergo repeated rounds of DNA replication, their telomeres shorten because of the inability of DNA polymerases to completely replicate the ends of the chromosomal DNA. This shortening continuous until the cells reach a proliferative block named crisis, which is characterized by chromosomal instability, end-to-end fusions, and cell death (7, 8). This process can be compensated by the *de novo* synthesis of telomerase, a RNA-dependent DNA polymerase that functions as a reverse transcriptase, which provides the template for the synthesis of new telomeric DNA repeats at the chromosome termini (9). Telomerase expression is usually low or absent in most normal somatic cells, with few exceptions, whereas its activity is detected in germline, stem cells, and in the majority of tumor cells (10, 11). Particularly, the analysis of telomere length (TL) and telomerase activity in normal B cells has shown specific characteristics (12, 13). A significant telomere lengthening was observed as naïve cells matured into centroblasts and when centroblasts matured into centrocytes. Simultaneously, high level of telomerase activity was observed in centroblasts, which was increased in centrocytes, whereas resting naïve, activated naïve and memory B cells were telomerase negative. Several studies suggest that tumor cells have dysfunctional telomeres, often characterized by a marked shortening of their sequences and high telomerase activity (11, 14). In solid tumors, telomere dysfunction was associated with genomic instability that causes bridge-breakage events leading to a reorganization of the malignant cell genome (15).

Different reports showed a wide heterogeneity among TL in B cell malignancies (16–20). In this study, we evaluated at molecular level, the modifications in the TL, measured by terminal restriction fragments (TRF) assay in tumor cells from patients with MCL. Results were correlated with clinical, morphologic and cytogenetic characteristics.

Materials and methods

Patients

Twenty patients with MCL (15 males and 5 females; median age: 60 yr, range: 31–82 yr) were studied. For comparative analysis, peripheral blood mononuclear cells from 17 controls (12 males and 5 females; median age 61 yr, range: 33–70 yr) were also evaluated. All tumors were histological reviewed and they fulfilled WHO diagnostic criteria for MCL (1) including the demonstration of Cyclin D1 overexpression, the presence of t(11;14)(q13;q32) cytogenetically, and/or *CCND1-IGH@* gene rearrangement by fluorescence *in situ* hybridization (FISH) or PCR. Immunophenotypical analysis including the following monoclonal antibodies: CD5, CD19, CD22, CD23 and CD10, was also performed. B-cell phenotype (CD20 +) was confirmed in all patients. The blastoid variant of MCL was diagnosed in three cases. Clinical characteristics at the time of diagnosis are summarized in Table 1. Thirteen patients were studied at diagnosis and seven after treatment at true relapse (occurred after a previous complete or a very good partial remission). Treatment was heterogeneous and not within a single clinical trial. All patients gave their informed consent. The study was approved by the ethics committee of our institution.

Table 1 Clinical and biological parameters at diagnosis

Variable	
No. of patients (*n*)	20
Median age (yr; range)	60 (31–82)
Sex: male/female	15/5
Advanced stage, III–IV (%)	90
Median WBC count, $\times 10^9$/L (range)	11.7 (6.3–260)
Median hemoglobin level, g/dL (range)	12 (6–13)
Median platelet count, $\times 10^9$/L (range)	154 (48–685)
Median lactate dehydrogenase level, IU/L (range)	435 (210–780)

Telomeric length evaluation

Telomeric length (TL) was evaluated as previously described (17). Briefly, for TRF analysis, high-molecular weight DNA was extracted from total sample or isolated mononuclear cells (according to the percentage of tumor cells by immunophenotyping; ≥50%). Fresh tissue of bone marrow (BM), lymph node (LN) and spleen biopsies were processed. Aliquots of 10 μg DNA were digested with *Hin*fI and *Rsa*I restriction enzymes at 37°C, overnight. The digested products were separated by electrophoresis in 0.8% agarose gels during 20 h at 35 V. Gels were first depurinated, then denatured and neutralized and finally transferred to nylon membranes by Southern blotting. The telomeric fragments were detected through hybridization with a 5′ end-labeled telomeric probe $(TTAGGG)_7$. The hybridized membranes were exposed to films during 10–15 days at −70°C before developing. Hybridization signals were evaluated in the autoradiograph by densitometric scanning in each lane respect to a λ/*Hin*dIII molecular weight standard and were analyzed using an Image Master 1D Prime software (Pharmacia-Biotech, Uppsala, Sweden). The mean TL of a given sample was calculated considering the peak

observed, matching it to the molecular weight standard and evaluating over the range of 2–23 kb. TRFs were measured by estimating the band size corresponding to the point with the highest intensity. In addition, as internal controls for telomere shortening we used K-562 cell line (positive control), which has short telomeres because of its immortalized status, and cord blood cells (negative control) as a very young tissue without telomere reduction.

Cytogenetic and FISH studies

Cytogenetic study was performed on non-stimulated BM, LN and spleen biopsies. Cells were cultured for short time (24–48 h) in RPMI 1640 medium supplemented with 15% fetal calf serum and L-glutamine. G-banding technique was used. FISH analysis was carried out with a sequence specific LSI IGH/CCND1 XT Dual Color, Dual Fusion Translocation Probe (Vysis-Abbott, Downers Grove, IL, USA), according to standard protocols. The cut-off for this rearrangement (normal control mean + 3SD) was 1%.

Detection of *CCND1/IGH@* molecular rearrangement

Genomic DNA samples were subjected to a semi-nested PCR for *CCND1/IGH@* rearrangement using primers: BCL-1G (5′-GCATAATTGCTGCACTGC-3′) (first round), BCL-1B (5′-GGTTAGACTGTGATTAGC-3′) (second round) and JH consensus sequence (5′-AACT GCAGAGGAGACGGTGACC-3′) (21). PCR reactions were performed in 50 μL final volume using 0.5–1 μg of DNA, 0.25 μM of each oligonucleotide primer, 0.04 mM dNTPs, 2.7 mM $MgCl_2$, and 2 U Taq DNA polymerase. The first and second rounds had the same cycle conditions: first denaturation 94°C for 4 min, and 30 cycles that included denaturation 94°C for 1 min, annealing 60°C for 1 min and holding the products at 4°C. PCR products were electrophoresed in 2% agarose gels containing ethidium bromide and visualized under ultraviolet light transiluminator. Specific PCR products show bands at 180–250 bp. Each experiment included the clinical samples and positive and negative controls for the specific PCR.

Statistical evaluation

The comparison of data from patients and controls and between subgroups was performed using Mann-Whitney test. For correlations, the Kendall's coefficient was used. The cut-off point for TL was selected according to receiver operating characteristic (ROC) analysis using death as state variable. Overall survival (OS) measured from diagnosis to last follow-up or death was estimated by the Kaplan–Meier method and compared by the log-rank test. For all tests, $P < 0.05$ was regarded as statistically significant.

Results

Twenty patients with diagnosis of MCL were analyzed. In all cases, the presence of the *CCND1/IGH@* rearrangement was confirmed by FISH and/or PCR analysis. FISH was performed in 19 cases (no sample was available from one patient); all of them showed t(11;14)-positive cells. By PCR analysis, 55% of patients showed the *CCND1/IGH@* gene rearrangement. Cytogenetic studies were performed in 14 samples: 3 LN, 10 BM and one spleen. Complex karyotypes were found in 3 LN (cases 10, 12 and 19), and in 1 BM sample (case 2). Cases 2 and 19 were previously published (22); case 12 showed a near tetraploid clone. Patients 5 and 16 had a cryptic t(11;14)(q13;q32) translocation detected by FISH.

Telomere length (TL) measured by TRF assay was evaluated on tumor cells from BM (14), LN (5) and spleen (1) samples. Detailed data about sex, age, type of sample, moment of the disease as well as TRF values for each patient are shown in Table 2. No differences were found between TRF values in BM (4.34 ± 0.72 kb; range 3.57–5.90kb) and LN (4.88 ± 1.01 kb; range 3.63–

Table 2 Detailed data about sex, age, type of sample and TRF values in 20 MCL patients according to the moment of the disease

Case	Age/sex	Sample	TRF (kb)
Diagnosis			
1	57/M	BM	5.30
2	72/F	BM	3.79
3	61/M	BM	3.57
4	67/M	BM	5.90
5	58/M	BM	4.06
6	70/M	BM	3.61
7	82/M	BM	4.55
8	64/F	BM	4.00
9*	61/F	BM	4.52
10	31/M	LN	4.00
11	65/M	LN	3.63
12*	34/M	LN	5.73
13	69/F	Spleen	5.10
Relapse			
14*	56/M	BM	3.65
15	33/M	BM	5.36
16	59/M	BM	4.20
17	60/F	BM	4.22
18	82/M	BM	3.99
19	35/M	LN	5.85
20	57/M	LN	5.19

M: male; F: female.
*Blastoid variant.

5.85 kb) tumor samples ($P = 0.379$), so we analyzed both tissues as a whole. TL in total MCL patients revealed a mean TRF value (4.51 ± 0.79 kb) significantly shorter than those observed in controls (7.49 ± 1.94 kb) ($P < 0.001$) (Fig. 1); 30% of patients had TL shorter than 4.0 kb, close to the critical value reported as threshold of senescence (23). TL was not associated with patients age ($P = 0.07$; $r = 0.17$) nor with sex (females: 4.33 ± 0.51 kb and males: 4.57 ± 0.85 kb; $P = 0.63$).

Although the number of cases of our series is not large, we also performed the analysis of TRF according to the moment of the disease, morphology and cytogenetic characteristics. No significant differences were found between patients studied at diagnosis (4.44 ± 0.81 kb) respect to those analyzed at relapse (4.63 ± 0.82 kb) ($P = 0.53$), and both groups showed a significant telomere shortening compared to controls ($P < 0.001$). In addition, we compared patients with (4.84 ± 1.09 kb) and without (4.36 ± 0.69 kb) complex karyotypes ($P = 0.45$) and cases with typical morphology (4.48 ± 0.79 kb) vs. blastoid variant (4.63 ± 1.04 kb) ($P = 0.83$), and no significant differences between them were found.

The median OS was 32 months (range: 2–93 months). The patient comparison taking into account TRF values lower or higher than the best TL cut-off point generated by ROC analysis (4.13 kb) did not showed statistical differences (log rank: $P = 0.55$).

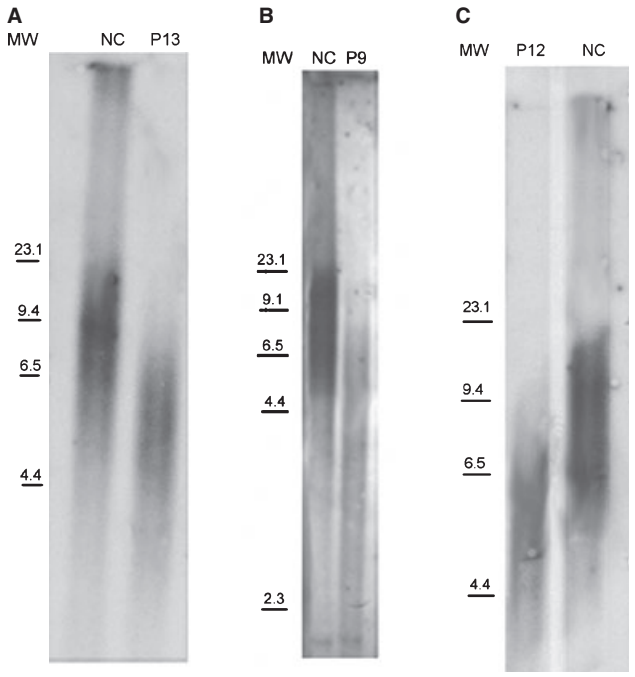

Figure 1 Examples of Southern blot analysis in MCL patients: (A) spleen sample of patient 13; (B) BM sample of patient 9; and (C) LN sample of patient 12; and their age matched controls. MW: molecular weight; NC: normal control; P: patient.

Discussion

As known, telomere erosion induces chromosomal destabilization and compromises chromosomal integrity, a critical factor in the initiation and progression of human cancers. In the present work, we studied TL measured by TRF in MCL patients. Our data showed a significant telomere reduction in MCL compared to controls and a similar mean TRF value to that reported in other MCL series (18, 20). Dysfunctional telomeres are associated to genomic alterations, including loss of heterozygosity, gene truncation and aneuploidy, and also altered gene expression, a primary source of phenotypic variability (24). The latter may drive the development of cell clones displaying progressively malignant traits, such as invasion and metastasis. Tumors with excessive telomere alterations are therefore likely to possess the most extensive phenotypic variability and have the greatest probability of containing cells with an aggressive tumor phenotype. This scenario would permit to think that altered TL may be a predictor of clinical outcome, and patients with shortest TRF would have worse prognostic. However, our data and a study by Walsh *et al.* (20) did not showed correlation between OS and TL. It is important to note that none of our patients showed an indolent MCL (25, 26). Thus, it would be interesting to analyze TL in this type of patients that had a long survival even without the need for treatment.

We have also evaluated TL in MCL patients taking into account the moment of the disease. Although the mean TRF value of samples at relapse was slightly larger than those observed at diagnosis, no significant difference between both groups was found. Similar results were found by our group in patients with multiple myeloma (MM) (17) and by Ladetto *et al.* (18) in follicular lymphoma (FL). On the contrary, Remes *et al.* (16) found a wide variation in TL (shortened, unchanged, and elongated) in relapsing lymphomas with different histological subtypes compared to TRF values obtained at diagnosis. In hematological malignancies, TL and telomerase activity has been studied at different stages of the disease. In complete remission, TL is restored and the levels of telomerase activity significantly decreases compared to that at the time of diagnosis, reflecting the elimination of the malignant clone by chemotherapy. During relapse, elevated telomerase activity and shorten telomeres could reflect the fraction of malignant cells with high proliferative capability (17, 27, 28), supporting the similar TRF length observed at diagnosis and relapse in patients with analogous tumors.

In addition, although the number of cases from our series is rather limited, we compared TRF in patients with and without complex karyotypes, and no significant difference between them was found. Analogous results were also obtained when TL from patients with typical histology and blastoid variant were compared. Studies performed in MM (16, 29) and chronic lymphocytic leukemia (CLL) (30, 31) have shown an association between TL and cytogenetics. However, our results are in line with the comparable TRF values detected by Walsh *et al*. (20) for MCL cases with and without mutations in *IGVH* genes. These findings would reflect disease homogeneity in MCL unlike CLL in which TL permit distinct different biological subsets (30, 31). More studies must be necessary to confirm these data.

A number of studies have observed telomere shortening in different histological subtypes of NHL (18–20), reflecting the heterogeneity of TL in B-cell malignancies. Ladetto *et al*. (17) found a correlation between TL and tumor histopathogenesis in relation to the germinal center (GC) origin. In this study, pregerminal tumors, like MCL and CLL, showed the shortest TRFs. GC-derived neoplasms have long telomeres, and postGC-derived malignancies showed intermediate TL. Other authors (19) found that CLL and MCL had the shortest TRFs, diffuse large B-cell lymphoma (DLBCL) and FL showed somewhat longer telomeres, whereas hairy cell leukemia and lymphoplasmacytic lymphoma/Waldenström's macroglobulinemia displayed the longest telomeres. They suggested that TL is a more dynamic factor that do not correlate simply with GC-related origin but also would reflect the telomere shortening and lengthening process. In our experience, MM had the longest telomeres (16), DLBCL (unpublished data) intermediate TL, and FL (18) and MCL the shortest TRFs, supporting the hypothesis of Walsh *et al*. (19), and probably reflecting the different proliferative activity in tumors during malignant transformation.

In conclusion, in spite of our series is not large, our data in MCL showed that TL reduction is independent of the clinical characteristics, morphology and karyotype, and did not reveal any association with prognosis. Telomerase or proteins involved in the telomere/telomerase complex are promising targets for cancer therapy (32). A group of our patients showed very short TL, close to the critical value reported as threshold of senescence. Thus, TRF analysis would permit to identify patients that were worthy to enter in protocols based in these innovative therapeutic strategies.

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