Prognostic value of telomere function in gastric cancers with and without microsatellite instability

Irene Pascua[a], Tamara Fernández-Marcelo[a], Andrés Sánchez-Pernaute[b], Carmen de Juan[a], Jacqueline Head[a], Antonio-José Torres-García[b] and Pilar Iniesta[a]

Objective To identify molecular markers that may be useful in the selection of gastric cancer patients with different prognoses, we investigated telomere function in gastric cancers with and without microsatellite instability (MSI).

Materials and methods We analyzed 83 gastric cancers and its paired-normal tissues to investigate MSI and telomere function. MSI was established using five polymorphic human repeat DNA markers. Telomere function was evaluated by determining telomerase activity, telomere length, and telomere-repeat factors 1 and 2 (*TRF1* and *TRF2*) expression.

Results Patients with high microsatellite instability (MSI-H) gastric cancers showed a significantly better prognosis than those affected by microsatellite stable or low microsatellite instability (MSS/MSI-L) tumors ($P = 0.03$). The lowest expression levels of *TRF1* and *TRF2* were associated with MSI-H gastric cancers ($P = 0.008$ and 0.006, respectively). Moreover, a clear trend toward a worse prognosis was found in the group of patients who had tumors with the shortest telomeres ($P = 0.01$). Cox multivariate analysis showed that MSI emerged as a protective prognostic factor; MSS/MSI-L tumors conferred a significantly poor prognosis in patients (relative risk = 4.862-fold greater than the MSI-H group) ($P = 0.033$). Telomere length of gastric tumors less than 2.86 kbp was a factor that led to a poor prognosis (relative risk = 4.420, with respect to tumors showing telomere length ≥ 2.86 kbp) ($P = 0.002$).

Conclusion We propose telomere status as a potential molecular marker with usefulness in the establishment of the prognosis of gastric cancers both for the mutator phenotype and for the suppressor pathway. *Eur J Gastroenterol Hepatol* 27:162–169 © 2015 Wolters Kluwer Health | Lippincott Williams & Wilkins.

European Journal of Gastroenterology & Hepatology 2015, 27:162–169

Keywords: gastric cancer, microsatellite instability, telomere, telomere-binding proteins

[a]Department of Biochemistry and Molecular Biology II, Faculty of Pharmacy, Complutense University and [b]Surgery Service, San Carlos Hospital, Institute for Health Research from San Carlos Hospital (IdISSC), Madrid, Spain

Correspondence to Pilar Iniesta, PhD, Department of Biochemistry and Molecular Biology II, Faculty of Pharmacy, Complutense University, Pza. Ramón y Cajal s/n (Ciudad Universitaria), 28040 Madrid, Spain
Tel: +34 91 3942089; fax: +34 91 3941779; e-mail: insepi@ucm.es

Received 4 September 2014 Accepted 31 October 2014

Introduction

Gastric cancer (GC) remains one of the most common cancers and although its incidence and mortality rates have been decreasing steadily, the absolute number of new cases is actually increasing because of the aging population [1]. Approximately 95% of GC are adenocarcinomas, which have been classified by anatomic site as proximal cancers or distal cancers in epidemiological studies [2] and by histological phenotype as intestinal type, diffuse type, and mixed/unclassifiable according to Lauren's classification [3]. Patients with proximal GC have a poorer survival independent of the Tumor, Node, Metastasis staging system (TNM stage) [4].

The development of gastric adenocarcinoma is a complex multistep process involving multiple genetic alterations. Most GC show genetic instability, either microsatellite instability (MSI) or chromosomal instability (CIN). Molecular studies of alterations of single genes have provided evidence that intestinal-type and diffuse-type GC evolve by different genetic pathways. Moreover, alterations in multiple single genes and complex copy number and gene expression profiles have been identified in GC over the last two decades. However, their significance in gastric carcinogenesis, tumor progression, and patient survival remains to be determined [1].

Two major carcinogenic pathways were initially described in colorectal cancer (CRC): the suppressor phenotype pathway, which constitutes 80% of sporadic CRCs; this group of tumors shows the CIN phenotype. The mutator phenotype pathway represents most cases of hereditary nonpolyposis colorectal cancer and nearly 13% of all cases of sporadic CRC. In this case, genes of the mismatch repair (MMR) system undergo alterations that affect their role in the repair of DNA base mismatches. Primary alterations in *MMR* genes lead to a huge accumulation of mutations in microsatellite sequences and short-tandem repeat sequences widespread throughout the genome. Therefore, MSI is a form of genetic instability characterized by new alleles not

0954-691X © 2015 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/MEG.0000000000000250

Copyright © 2015 Wolters Kluwer Health, Inc. Unauthorized reproduction of this article is prohibited.

present in the normal genotype. High microsatellite instability (MSI-H) tumors are considered to develop through the mutator phenotype carcinogenic pathway, whereas microsatellite stable or low microsatellite instability (MSS/MSI-L) tumors develop through the suppressor pathway [5–7]. Besides CRC, the mutator phenotype pathway has also been described in other tumor types. Thus, the incidence of MSI in GC varies from 15 to 39% [8]. The relationship between survival in gastric cancer patients and the status of MSI has not yet been well established. In a recent meta-analysis, MSI gastric cancer was associated with a good prognosis, but there was heterogeneity in other previous studies. The number of loci investigated, altered epidemiology, and effects of chemotherapy are potential causes of heterogeneity [9]. Therefore, a consensus for defining the interest of MSI evaluation in gastric cancer should be established in future investigations.

However, the role of telomeres and telomerase in the initiation and progression of human cancers has been evaluated extensively. The majority of human cancers maintain or actively lengthen telomeres through upregulation of the reverse transcriptase telomerase, and many investigations have assessed the potential to utilize these molecular markers for the diagnosis of cancer [10]. It has been suggested that telomere dysfunction is an important mechanism in genetic instability [11]. Many oncogenes, tumor-suppressor genes, and apoptosis-related genes are involved in the multistage carcinogenesis of GC; however, the structure and function of telomeres may also play an important role in the process [12]. In this context, the role of the shelterin complex is of interest in telomere function regulation. Among the shelterin components, the telomeric repeat-binding factor 1 (TRF1) and mainly the TRF2 play a crucial role in regulating the molecular events that maintain telomere integrity [13]. TRF1 and TRF2 act as negative regulators of telomere length and play an important role in telomere-induced genetic instability [14,15]. The expression of these proteins is altered in many types of tumors, indicating a fundamental role in neoplastic progression [16]. In GC, it has been suggested that the overexpression of TRF1 and TRF2 may reduce the telomere length and could contribute toward the multistage carcinogenesis of gastric cancer [12]. However, the prognostic impact of telomere function has not been established in gastric cancer.

The aim of this study was to investigate telomere function in gastric cancers with and without MSI. Also, we evaluated the prognostic implications associated with these molecular markers. With this objective, we analyzed a tumor population of 83 gastric cancers and its 83 paired-normal tissues to investigate MSI, telomere length, telomerase activity, and TRF1 and TRF2.

Materials and methods

Patients and tissue samples

Eighty-three primary sporadic gastric adenocarcinomas and their corresponding control tissue samples were obtained from patients who underwent surgery at San Carlos Hospital in Madrid (Spain). Out of 83 patients, 46 were men and 37 were women, average age 70.7 ± 1.38 years (mean ± typical error). Before surgery, none of these patients had received adjuvant treatment. Informed consent was obtained from patients before investigation and this study was approved by the Ethical Committee of the Hospital. After surgical resection, all tissue samples were snap-frozen in liquid nitrogen and stored at $-80°C$ until processed. Cryostat sectioned, H&E-stained samples from each tumor block were examined microscopically by two independent pathologists to confirm the presence of more than 80% tumor cells. Paired normal tissues from the same patient, used as controls, were obtained at least 10 cm away from the margin of the tumor and also confirmed microscopically. Most of the clinicopathological variables of cancers were available (Table 1). The median follow-up period of patients was 5 years.

Analysis of microsatellite instability

Microsatellite analysis was carried out to classify tumors into groups of high and low or null instability. Genomic DNA samples were amplified using PCR and investigated for MSI using five polymorphic human repeat DNA markers that map on different chromosome loci: BAT25, BAT26, NR-24, NR-21, and MONO-27, recommended for the National Cancer Institute [17]. PCR products were obtained after a multiplex PCR analysis using the MSI Analysis System Version 1.2 (Promega, Madison, Wisconsin, USA) according to the manufacturer's recommendations. The generated

Table 1 Clinical characteristics of the gastric tumor population

Variables	Total number of cases (%)	Number of cases without available data
Tumor location	75 (100)	8
Proximal	12 (16)	
Distal	63 (84)	
Tumor histology (Lauren's classification)	72 (100)	11
Diffuse	26 (36.1)	
Intestinal	46 (63.9)	
Primary tumor (T)	78 (100)	5
T1	7 (9)	
T2	20 (25.6)	
T3	33 (42.3)	
T4	18 (23.1)	
Regional lymph node invasion (N)	78 (100)	5
N0	29 (37.2)	
N1	15 (19.2)	
N2	13 (16.7)	
N3	21 (26.9)	
Distant metastasis (M)	78 (100)	5
M0	70 (89.7)	
M1	8 (10.3)	
TNM stage	78 (100)	5
IA, IB	17 (21.8)	
IIA, IIB	22 (28.2)	
IIIA, IIIB, IIIC	31 (39.7)	
IV	8 (10.3)	

TNM stage, tumor, node, metastasis staging system.

Copyright © 2015 Wolters Kluwer Health, Inc. Unauthorized reproduction of this article is prohibited.

fluorescence-labeled PCR products, corresponding to tumor and control tissues, were then investigated on an automated ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, California, USA) to evaluate the MSI status of gastric tumors. Thus, MSI was detected by comparing the length of microsatellite alleles amplified from tumor DNA and DNA from normal tissue in the same individual. To confirm the reproducibility of the experiments, all cases showing MSI were examined at least twice by an independently performed PCR and electrophoresis. Adenocarcinomas showing MSI on two or more of the markers considered were classified as high instability tumors (MSI-H), whereas cases with only one altered marker were classified as low instability tumors (MSI-L) and cases without MSI were considered as stable tumors (MSS). In all of the analyses for clinicopathological associations with MSI, MSI-L and MSS tumors were grouped together and indicated as MSI-L/MSS.

Telomere function evaluation

Telomere function was evaluated by determining telomerase activity using a method that is based on the Telomeric Repeat Amplification Protocol and telomere length was assessed using the terminal restriction fragment (TRF) length measurement assay.

Telomerase activity in tumor and paired normal tissues was evaluated as described previously [18] using the *Telo TAGGG* PCR ELISA (Roche Applied Science, Penzberg, Germany). Considering that the cut-off for telomeric repeat amplification protocol-ELISA negativity corresponds to an optical density at 450 nm less than 0.2, all samples with optical density at 450 nm of at least 0.2 were considered telomerase positive [19].

TRF length measurement was performed using the *Telo TTAGGG* Telomere Length Assay kit (Roche Applied Science) as described previously [19]. TRF lengths for tumor and control tissues were determined by comparing the signals relative to a standard molecular weight using Image Gauge software (version 3.46; Fujifilm, Tokyo, Japan). The TRF length ratio was determined as the ratio of the length of tumor tissue TRF and their paired normal tissue TRF (ratio T/N). All cases in which the T/N ratio was less than 1 were included in the group of telomere shortening. When T/N was at least 1, tumors were included in the group of telomere maintenance.

TRF1 and TRF2 expression analysis by qRT-PCR

TRF1 and *TRF2* expression analysis was carried out in 49 gastric cancers and its corresponding normal tissues, all of them included in the initial tissue population. RNA reverse transcription was performed using the High Capacity cDNA Reverse Transcription kit (Life Technologies, Grand Island, New York, USA). Expression data were confirmed by investigating gene expression by real-time quantitative PCR using TaqMan Gene Expression Master Mix and FAM dye-labeled TaqMan MGB probes (Life Technologies) following the manufacturer's instructions. The comparative threshold cycle (C_t) method was used to calculate the relative expression. For the quantification of gene expression, the target genes values were normalized to the expression of the endogenous gene (*GAPDH*) and each tumor sample was compared with its own control tissue. This quantification is represented by a relative quantification value (RQ), being $RQ = 2^{-\Delta\Delta C_t}$ ($\Delta\Delta C_t = \Delta C_{t\ \text{tumor sample}} - \Delta C_{t\ \text{control sample}}$); ($\Delta C_t = C_{t\ \text{gene}} - C_{t\ \text{endogenous gene}}$). Values of RQ between 0.5 and 2 were considered to indicate normal expression, RQ less than 0.5 to indicate downregulation, and RQ more than 2 to indicate overexpression.

Statistical analysis

Analyses were carried out using the IBM SPSS Statistics 19 for Windows. Statistical associations were assessed using the χ^2-test for categorical variables and Student's *t*-test, ANOVA, or the corresponding nonparametrical test for continuous variables. Distributions of disease-free survival (DFS) were estimated using the Kaplan–Meier method, and comparisons were made with long-rank statistics. For survival analysis, only patients who had undergone potentially curative surgery (stage I–III tumors) and patients who did not die in the postoperative period were included. Thus, the number of patients included in the survival study was 63. A P value of less than 0.05 was considered significant.

Results

DNA samples from patients affected by sporadic gastric cancer were classified on the basis of their MSI status. Thus, after comparing results in tumor and normal paired tissues, 15 out of 83 tumors (18.1%) were classified as MSI-H cancers, whereas 68 out of 83 (81.9%) of cases showed null or low instability at the level of the markers considered (MSS/MSI-L). The relationships between microsatellite status and clinicopathologic variables of tumors are shown in Table 2. Thus, according to the statistical analysis, regional lymph node invasion (*N*) was associated significantly with gastric cancers from the suppressor pathway ($P = 0.042$). Moreover, proximal tumor location and diffuse cancers (Lauren's classification) emerged as clinical variables that tended to associate with stable or MSI-L tumors ($P = 0.058$ and 0.070, respectively).

To evaluate telomere function in gastric cancers from both mutator phenotype and suppressor pathways, we investigated telomere length and telomerase activity in the 83 gastric cancers considered in this work, as well as in its paired normal tissues. Our data showed significantly shorter telomeres in tumors than in the adjacent normal tissues ($P = 0.002$); the mean telomere lengths were 5.24 ± 0.28 and 5.95 ± 0.24 kbp in tumors and control samples, respectively. Values of telomere length ratios

Copyright © 2015 Wolters Kluwer Health, Inc. Unauthorized reproduction of this article is prohibited.

Table 2 Microsatellite status and clinical variables in gastric tumors

Variables	Number of cases	Microsatellite status: Number of MSI-H Tumors (%)	Microsatellite status: Number of MSS/MSI-L Tumors (%)	*P* value
Tumor location				0.070
Proximal	12	0 (0)	12 (100)	
Distal	63	14 (22.2)	49 (77.8)	
Tumor histology (Lauren's classification)				0.058
Diffuse	26	2 (7.7)	24 (92.3)	
Intestinal	46	12 (26.1)	34 (73.9)	
Primary tumor (T)				0.223
T1, T2	27	3 (11.5)	23 (88.5)	
T3, T4	51	12 (23.1)	40 (76.9)	
Regional lymph node invasion (N)				0.042
N0	29	9 (31)	20 (69)	
N1, N2, N3	49	6 (12.2)	43 (87.8)	
Distant metastasis (M)				0.145
M0	70	15 (21.4)	55 (78.6)	
M1	8	0 (0)	8 (100)	
TNM stage				0.360
IA, IB	17	4 (23.5)	13 (76.5)	
IIA, IIB	32	6 (27.3)	16 (72.7)	
IIIA, IIIB, IIIC	31	5 (16.1)	26 (83.9)	
IV	8	0 (0)	8 (100)	

TNM stage, tumor, node, metastasis staging system.

indicated a slight shortening in tumor samples: 0.92 ± 0.04 kbp (mean ± typical error).

Telomerase activity investigation showed that 77.1% (64/83) of gastric tumors were positive and the remaining 22.9% (19/83) were negative. Correlation analyses between telomerase activity and telomere length of tumors did not show a positive association ($P = 0.551$); telomere length in telomerase-positive cases was 5.05 ± 0.27 kbp (mean ± typical error), whereas a mean value of 5.86 ± 0.82 kbp was detected in telomerase-negative tumors. The enzyme activity was not associated with clinical variables of cancers (data not shown).

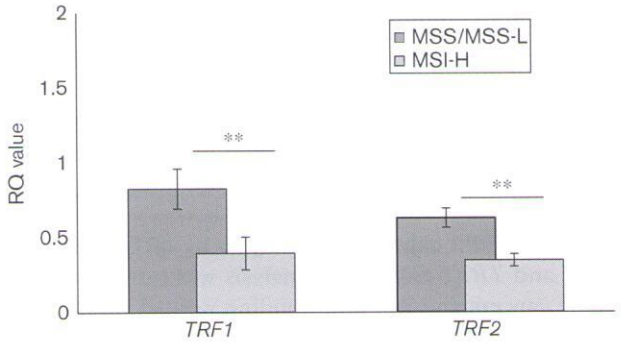
In relation to MSI status, the mean telomere length was smaller in the group of MSI-H tumors than in MSS/MSI-L cancers, without statistically significant differences (the mean ± typical error was 4.65 ± 0.54 and 5.37 ± 0.32 kbp, respectively, $P = 0.266$). However, significant differences were found for telomerase activity in relation to MSI status. Thus, our data showed that 100% of tumors from the mutator phenotype pathway were telomerase positive and 72% of cancers from the suppressor pathway showed enzyme activity ($P = 0.020$).

Also, to complete telomere function analysis in gastric cancers with and without MSI, we investigated *TRF1* and *TRF2* expression. This study was established in a group of 49 tumors and their corresponding normal tissues, 11 of which had been classified as MSI-H cancers and 38 as MSS/MSI-L. Considering all cancers as a whole, expression values by qRT-PCR were 0.73 ± 0.11 for *TRF1* and 0.56 ± 0.05 for *TRF2* (RQ values, mean ± typical error), with significant differences detected for both factors after comparing with the expression values of normal tissues ($P < 0.001$ both for *TRF1* and *TRF2*). Moreover, *TRF1* and *TRF2* expression levels indicated significant differences in relation to the MSI status of tumors: MSI-H gastric cancers showed the lowest expression values (RQ values, mean ± typical error, were 0.39 ± 0.11 for TRF1 and 0.34 ± 0.04 for TRF2). Expression values for MSS/MSI-L gastric cancers were 0.83 ± 0.13 for TRF1 and 0.62 ± 0.06 for TRF2. Differences between MSS/MSI-L and MSI-H gastric tumors were significant both for *TRF1* and for *TRF2* expression ($P = 0.008$ and 0.006, respectively) (Fig. 1).

In relation to telomerase activity, we detected higher expression levels in telomerase-negative tumors: 0.95 ± 0.21 for *TRF1* and 0.76 ± 0.12 for *TRF2*, versus 0.68 ± 0.13 and 0.52 ± 0.06 for *TRF1* and *TRF2* in telomerase-positive tumors, with significant associations ($P = 0.047$ for both *TRF1* and *TRF2*). Moreover, tumors without telomere shortening, after comparison with the corresponding normal paired tissue (T/N ratio ≥ 1), showed significantly higher *TRF1* and *TRF2* expression levels than those tumors showing telomere shortening (T/N ratio < 1): 1.15 ± 0.30 and 0.75 ± 0.13 for *TRF1* and *TRF2*, respectively, for the former versus 0.52 ± 0.05 and 0.47 ± 0.04 for *TRF1* and *TRF2*, respectively, for the latter (expression data expressed as RQ values, mean ± typical error), with significant differences ($P = 0.021$ for *TRF1* and $P = 0.05$ for *TRF2*).

Next, prognosis studies in patients with resected GC were carried out to assess the impact on the clinical course of molecular factors considered in the current work. In all cases, for survival studies, we considered DSF time as detailed in the Materials and methods

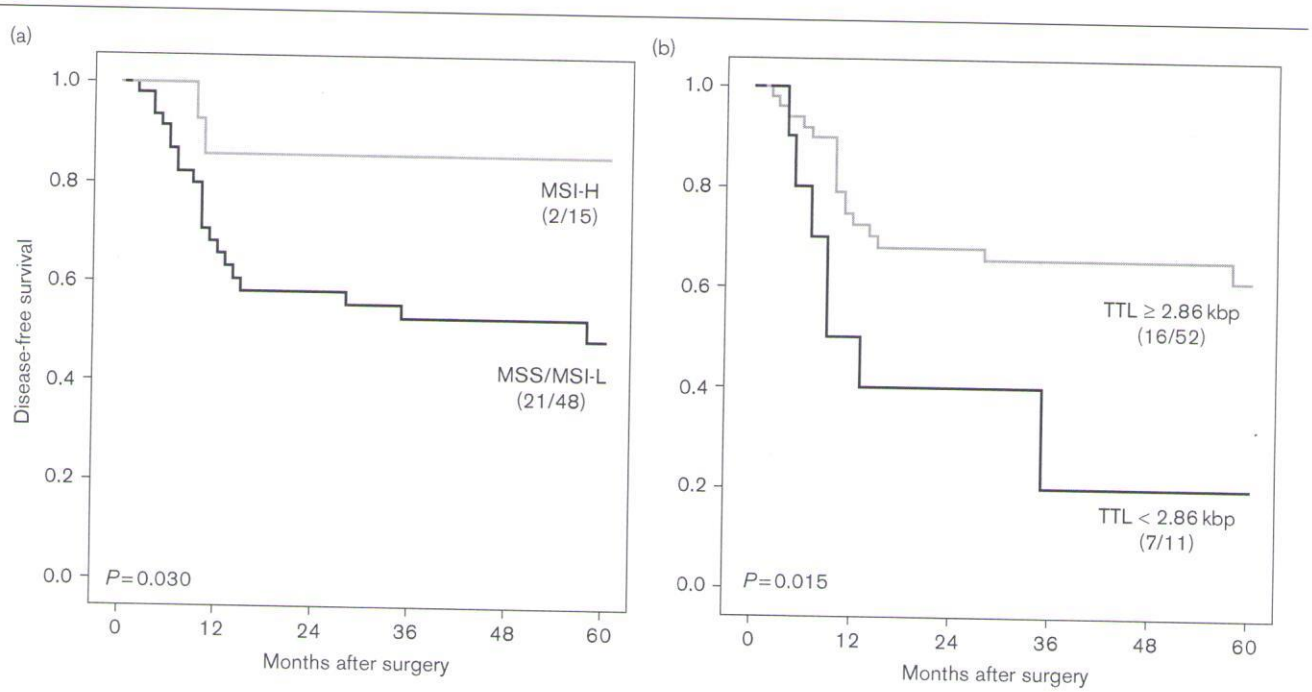
Fig. 1



TRF1 and *TRF2* expression values in MSI-H and MSS/MSI-L gastric tumors [relative quantification (RQ)]. Significant differences (**) were found for both *TRF1* and *TRF2* expression values between MSI-H and MSS/MSI-L gastric cancers ($P = 0.008$ and 0.006, respectively). MSI-H, high microsatellite instability; MSS/MSI-L, microsatellite stable or low microsatellite instability; TRF1, TRF2, telomere repeat factors 1, 2.

Copyright © 2015 Wolters Kluwer Health, Inc. Unauthorized reproduction of this article is prohibited.

Fig. 2



Kaplan–Meier survival curves for patients with gastric cancers according to (a) microsatellite status of tumors and (b) tumor telomere length. Numbers in brackets represent cases with tumor recurrence in relation to the total number of patients included in each one of the survival analyses. MSI-H, high microsatellite instability; MSS/MSI-L, microsatellite stable or low microsatellite instability.

section of the manuscript. Considering microsatellite status, during the follow-up period, tumor recurrence was detected in two of the 15 patients with MSI-H tumors; however, in the group of 48 patients classified as MSS/MSI-L who were included in the survival study, 21 patients showed tumor recurrence. Interval-free disease was 52.78 ± 4.72 months in MSI-H cancers versus 36.35 ± 3.86 months in MSS/MSI-L cases, with significant differences between both groups of patients ($P = 0.030$), (Fig. 2a).

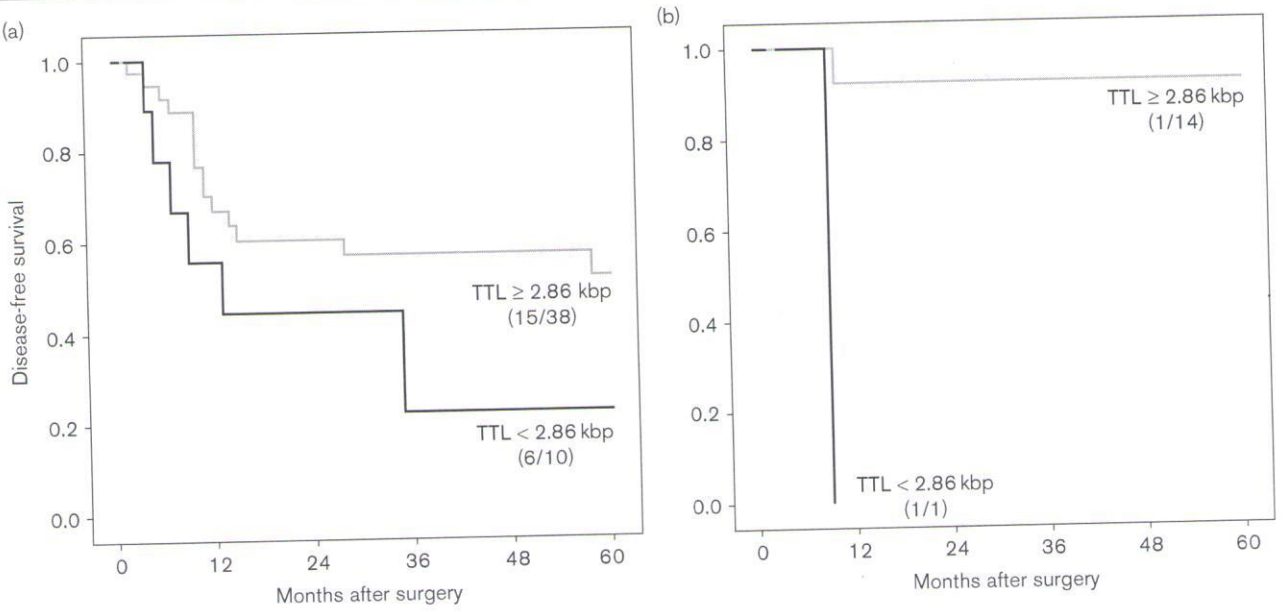
Survival analyses in relation to telomere function were carried out considering telomerase activity and telomere length of gastric tumors. In the first case, no significant differences were obtained. In fact, interval-free disease was 37.47 ± 6.69 months in patients with negative telomerase activity tumors and 41.45 ± 3.72 months in the group of positive telomerase activity cancers ($P = 0.404$).

Considering the impact on patient prognosis of telomere length of tumors, we established the cut-off value, defined as the point with the most significant (log-rank test) split, to stratify patients into distinct groups according to tumor telomere length (TTL) by the classification and regression tree technique [20]. The optimal cut-off point was set in 2.86 kbp. Thus, we detected two groups of patients with a differential prognosis: the first one included 12 patients with gastric tumors with a telomere length less than 2.86 kbp, whereas the second group of patients included 71 cases with a TTL at least 2.86 kbp. The interval-free disease was 23.70 ± 7.13 and 43.73 ± 3.45 months, respectively. Therefore, the group of tumors with shorter telomeres was associated with a worse clinical outcome ($P = 0.01$) (Fig. 2b). Moreover, shorter telomeres were associated with poor prognosis of patients considering both the MSS/MSI-L group of tumors (Fig. 3a) and MSI-H cases (Fig. 3b) ($P = 0.047$). The DFS time was 38.68 ± 4.32 (TTL ≥ 2.86 kbp) and 25.33 ± 7.73 months (TTL < 2.86 kbp) for MSS/MSI-L tumors and 56.15 ± 3.69 (TTL ≥ 2.86 kbp) and 9.00 ± 0.00 months (TTL < 2.86 kbp) for the MSI-H group of cancers. Therefore, independent of MSI status, these data indicated a poor prognosis associated specifically with telomere attrition.

Cox multivariate analysis for MSI status and TTL was carried out to show that the results obtained were independent of TNM tumor stage. As shown in Table 3, MSI of gastric cancers emerged as a protective prognostic factor. In fact, MSS/MSI-L tumors led to a significant poor prognosis in patients, with a relative risk of recurrence 4.862 fold greater than the MSI-H group, these results being independent of TNM tumor stage ($P = 0.033$). Also, our data indicated that telomere length of gastric tumors less than 2.86 kbp may be considered a factor of poor prognosis, with a relative risk equal to 4.420, with respect to tumors showing telomere length of at least 2.86 kbp; differences were significant and independent of TNM tumor stage ($P = 0.002$).

Copyright © 2015 Wolters Kluwer Health, Inc. Unauthorized reproduction of this article is prohibited.

Fig. 3



Kaplan–Meier survival curves for patients showing gastric cancers classified by function of their microsatellite status in relation to the telomere length of tumors. (a) Survival curves for patients with MSS/MSI-L tumors. (b) Survival curves for patients with MSI-H tumors. Numbers in brackets represent cases with tumor recurrence in relation to the total number of patients included in each of the survival analyses. Tumors with shorter telomeres (< 2.86 kbp) conferred the worst prognosis ($P = 0.047$). MSI-H, high microsatellite instability; MSS/MSI-L, microsatellite stable or low microsatellite instability; TTL, tumor telomere length.

Table 3 Cox multivariate analysis for TNM stage and microsatellite status (a); and TNM stage and telomere length (b) of tumors in gastric cancer

Variables	RR	95% confidence interval	*P* value
(a)			
TNM stage (I vs. II and III)	0.072	0.010–0.539	0.010
Microsatellite instability (MSS/MSI-L vs. MSI-H)	4.862	1.134–20.845	0.033
(b)			
TNM stage (I vs. II and III)	0.062	0.008–0.466	0.007
Tumor telomere length (< 2.86 vs. ≥ 2.86 kbp)	4.420	1.750–11.167	0.002

MSI-H, high microsatellite instability; MSS/MSI-L, microsatellite stable or low microsatellite instability; TNM stage, tumor, node, metastasis staging system; TTL, tumor telomere length; RR, relative risk.

Discussion

The underlying molecular mechanisms of gastric cancer have been considered previously in different studies. Results from these studies, although promising, still have limited clinical utility in predicting survival and stratifying gastric cancer patients for appropriate treatment [1]. Patients with gastric tumors with MSI are usually diagnosed at an earlier disease stage and some but not all studies showed a relationship of MSI and less aggressive tumors and improved survival of patients [21,22].

However, telomere dysfunction has been described as a potential marker in cancer. Although telomere shortening can eventually lead to telomere dysfunction, recent investigations have shown that there are multiple mechanisms that may lead to this. For example, alterations in TRF2, a major component of the shelterin complex, lead to uncapping of the telomere even in the presence of the adequate telomeric repeat sequences [10].

In the current study, we found an association between MSI-H gastric cancers and telomere dysfunction, both considering telomere shortening and a reduced *TRF1* and *TRF2* expression. Previous data concluded that the overexpression of telomeric proteins (TRF1, TRF2, and others) may be related to telomere length reduction in precancerous lesions and gastric cancer, and could contribute toward multistage carcinogenesis [12].

It has been reported that suppression of TRF2 activates an ATM-dependent DNA damage response pathway that induces apoptosis or senescence depending on the cell type. The shelterin complex is ubiquitously expressed to protect telomeres in all tissues in which the telomeres are not too short to preclude DNA binding. Recent studies challenge this prevailing paradigm by showing that the expression of shelterin subunits, notably TRF2, is increased during malignant transformation, suggesting that shelterin expression might be the target of oncogenic signaling pathways [13]. Results from the present work seem to agree with the latter study. In fact, gastric tumors with increased expression of both *TRF1* and *TRF2* conferred a poor prognosis in patients. However, MSI-H

Copyright © 2015 Wolters Kluwer Health, Inc. Unauthorized reproduction of this article is prohibited.

tumors with better clinical outcome showed significantly lower expression levels *of TRF1* and *TRF2*.

The MSI-H phenotype had been associated previously with telomere shortening in CRCs [23,24]. Also, in CRC, it has been reported that telomere attrition, low expression levels *of TRF1* and *TRF2*, and DNA damage response activation occur early in carcinogenic progression. Telomere length returns to normal levels when the full invasive potential of the tumor has been reached [25]. In gastric cancer, telomere erosion has been related to loss of heterozygosity in *DCC*, *APC*, and *MCC* genes. According to these results, it has been reported previously that telomere attrition is independent of the MSI-H phenotype, but related to the loss of heterozygosity pathway in gastric cancer [26]. In any case, studies examining the relationship between MSI and telomere lengths have been sparse. Our results seem to indicate that, independent of MSI status, critical telomere shortening constitutes a molecular factor that may be useful in the clinical evaluation of patients with gastric cancer.

Telomeres may be considered a form of super microsatellite, given their tandem repeat nature, and it may be reasonable to assume that defects in MMR genes impact telomere length [27]. In a previous work, it has been suggested that alternative lengthening of telomere-mediated telomere maintenance plays an important role in MSI-mediated stomach carcinogenesis. Defects of the MMR system may lead to homologous recombination of telomeric ends for the telomerase-independent telomere maintenance in gastric carcinomas [28].

With respect to the prognostic value of telomere length in gastric cancer, our data indicated significant differences, independent of TNM stage of tumors. Thus, gastric cancers with a critical telomere length were related to the worst prognosis of patients. We consider these results of interest as they may be useful in predicting the severity of the disease. Recently, in CRC, a classification including telomere status has been proposed. Telomere status constitutes a molecular marker with a significant value in predicting disease severity, progression, and overall prognosis, as well as in deciding on the treatment [27]. In rectal cancer, the telomere phenotype has recently been considered as a molecular parameter that may be included in analyses of tumor molecular profiles. In fact, MSS rectal cancer appears to represent a heterogeneous group of tumors that may be categorized both on the basis of CIN status and telomere maintenance mechanism [29].

Conclusion

In this work, we propose telomere status evaluation as a potential molecular marker that may be used in the establishment of the prognosis of patients with gastric cancer, both from the mutator phenotype and the suppressor pathway. Investigation of telomere length enables us to select groups of patients with gastric cancers with potentially unfavorable outcomes to establish adjuvant therapy protocols.

Acknowledgements

This work was supported by grants from Fundación de Investigación Médica Mutua Madrileña, Santander-UCM, and RTICC.

Conflicts of interest

There are no conflicts of interest.

References

1 Grabsch HI, Tan P. Gastric cancer pathology and underlying molecular mechanisms. *Dig Surg* 2013; **30**:150–158.
2 Crew KD, Neugut AI. Epidemiology of gastric cancer. *World J Gastroenterol* 2006; **12**:354–362.
3 Lauren P. The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. An attempt at a histo-clinical classification. *Acta Pathol Microbiol Scand* 1965; **64**:31–49.
4 Pacelli F, Papa V, Caprino P, Sgadari A, Bossola M, Doglietto GB. Proximal compared with distal gastric cancer: multivariate analysis of prognostic factors. *Am Surg* 2001; **67**:697–703.
5 Thibodeau SN, French AJ, Roche PC, Cunningham JM, Tester DJ, Lindor NM, *et al.* Altered expression of hMSH2 and hMLH1 in tumors with microsatellite instability and genetic alterations in mismatch repair genes. *Cancer Res* 1996; **56**:4836–4840.
6 Morán A, Ortega P, de Juan C, Fernández-Marcelo T, Frías C, Sánchez-Pernaute A, *et al.* Differential colorectal carcinogenesis: molecular basis and clinical relevance. *World J Gastrointest Oncol* 2010; **2**:151–158.
7 Huang YQ, Yuan Y, Ge WT, Hu HG, Zhang SZ, Zheng S. Comparative features of colorectal and gastric cancers with microsatellite instability in Chinese patients. *J Zhejiang Univ Sci B* 2010; **11**:647–653.
8 Kim SH, Ahn BK, Nam YS, Pyo JY, Oh YH, Lee KH. Microsatellite instability is associated with the clinicopathologic features of gastric cancer in sporadic gastric cancer patients. *J Gastric Cancer* 2010; **10**:149–154.
9 Choi YY, Bae JM, An JY, Kwon IG, Cho I, Shin HB, *et al.* Is microsatellite instability a prognostic marker in gastric cancer? A systematic review with meta-analysis. *J Surg Oncol* 2014; **110**:129–135.
10 Heaphy CM, Meeker AK. The potential utility of telomere-related markers for cancer diagnosis. *J Cell Mol Med* 2011; **15**:1227–1238.
11 Hahn WC. Role of telomeres and telomerase in the pathogenesis of human cancer. *J Clin Oncol* 2003; **21**:2034–2043.
12 Hu H, Zhang Y, Zou M, Yang S, Liang XQ. Expression of TRF1, TRF2, TIN2, TERT, KU70, and BRCA1 proteins is associated with telomere shortening and may contribute to multistage carcinogenesis of gastric cancer. *J Cancer Res Clin Oncol* 2010; **136**:1407–1414.
13 Diala I, Wagner N, Magdinier F, Shkreli M, Sirakov M, Bauwens S, *et al.* Telomere protection and TRF2 expression are enhanced by the canonical Wnt signalling pathway. *EMBO Rep* 2013; **14**:356–363.
14 Martínez P, Blasco MA. Telomeric and extra-telomeric roles for telomerase and the telomere-binding proteins. *Nat Rev Cancer* 2011; **11**:161–176.
15 Martínez P, Blasco MA. Role of shelterin in cancer and aging. *Aging Cell* 2010; **9**:653–666.
16 Cookson JC, Laughton CA. The levels of telomere-binding proteins in human tumours and therapeutic implications. *Eur J Cancer* 2009; **45**:536–550.
17 Suraweera N, Duval A, Reperant M, Vaury C, Furlan D, Leroy K, *et al.* Evaluation of tumor microsatellite instability using five quasimonomorphic mononucleotide repeats and pentaplex PCR. *Gastroenterology* 2002; **123**:1804–1811.
18 González-Quevedo R, Iniesta P, Morán A, de Juan C, Sánchez-Pernaute A, Fernández C, *et al.* Cooperative role of telomerase activity and p16 expression in the prognosis of non-small-cell lung cancer. *J Clin Oncol* 2002; **20**:254–262.
19 Garcia-Aranda C, de Juan C, Diaz-Lopez A, Sanchez-Pernaute A, Torres AJ, Diaz-Rubio E, *et al.* Correlations of telomere length, telomerase activity, and telomeric-repeat binding factor 1 expression in colorectal carcinoma. *Cancer* 2006; **106**:541–551.
20 Budczies J, Klauschen F, Sinn B, Győrffy B, Schmitt W, Darb-Esfahani S, Denkert C. Cutoff finder: a comprehensive and straightforward Web application enabling rapid biomarker cutoff optimization. *Plos One* 2012; **7**: e51862.

Copyright © 2015 Wolters Kluwer Health, Inc. Unauthorized reproduction of this article is prohibited.

21 Oliveira C, Seruca R, Seixas M, Sobrinho-Simões M. The clinicopathological features of gastric carcinomas with microsatellite instability may be mediated by mutations of different 'target genes': a study of the TGFbeta RII, IGFII R, and BAX genes. *Am J Pathol* 1998; **153**:1211–1219.
22 Kim H, An JY, Noh SH, Shin SK, Lee YC, Kim H. High microsatellite instability predicts good prognosis in intestinal-type gastric cancers. *J Gastroenterol Hepatol* 2011; **26**:585–592.
23 Rampazzo E, Bertorelle R, Serra L, Terrin L, Candiotto C, Pucciarelli S, *et al.* Relationship between telomere shortening, genetic instability, and site of tumour origin in colorectal cancers. *Br J Cancer* 2010; **102**:1300–1305.
24 Takagi S, Kinouchi Y, Hiwatashi N, Nagashima F, Chida M, Takahashi S, *et al.* Relationship between microsatellite instability and telomere shortening in colorectal cancer. *Dis Colon Rectum* 2000; **43 (Suppl)**:S12–S17.
25 Raynaud CM, Jang SJ, Nuciforo P, Lantuejoul S, Brambilla E, Mounier N, *et al.* Telomere shortening is correlated with the DNA damage response and telomeric protein down-regulation in colorectal preneoplastic lesions. *Ann Oncol* 2008; **19**:1875–1881.
26 Fang DC, Yang SM, Zhou XD, Wang DX, Luo YH. Telomere erosion is independent of microsatellite instability but related to loss of heterozygosity in gastric cancer. *World J Gastroenterol* 2001; **7**:522–526.
27 Baichoo E, Boardman LA. Toward a molecular classification of colorectal cancer: the role of telomere length. *Front Oncol* 2014; **4**:158.
28 Omori Y, Nakayama F, Li D, Kanemitsu K, Semba S, Ito A, Yokozaki H. Alternative lengthening of telomeres frequently occurs in mismatch repair system-deficient gastric carcinoma. *Cancer Sci* 2009; **100**:413–418.
29 Boardman LA, Jonhnson RA, Viker KB, Hafner KA, Jenkins RB, Riegert-Johnson DL, *et al.* Correlation of chromosomal instability, telomere length and telomere maintenance in microsatellite stable rectal cancer: a molecular subclass of rectal cancer. *Plos One* 2013; **8**:e80015.

Copyright © 2015 Wolters Kluwer Health, Inc. Unauthorized reproduction of this article is prohibited.