Relationship between telomere shortening, genetic instability, and site of tumour origin in colorectal cancers

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Colorectal cancers (CRCs) encompass a heterogeneous complex of diseases differing in molecular pathways and biological characteristics, arising through a multistep carcinogenic process, of which several genetic and epigenetic events have been characterised. There are at least two major pathways by which molecular events can lead to CRC: loss of heterozygosity with chromosomal instability, and a deficient DNA mismatch repair pathway with microsatellite instability (MSI) (Grady and Carethers, 2008; Ogino and Goel, 2008; Koopman et al, 2009). Although p53 and K-ras genes are known to be involved in chromosomal instability-associated carcinogenesis, MSI is frequently associated with the CpG island methylator phenotype (Barault et al, 2008). These two pathways are known to have a different origin and clinical outcome; right-sided proximal colon tumours are often MSI-associated lesions and have a better prognosis than left-sided distal tumours (Iacopetta, 2002; Gervaz et al, 2004; Walther et al, 2008). Rectal tumours are thought to arise through mechanisms similar to left-colon cancers; recently, it has been proposed that they constitute a third entity displaying specific characteristics (Li and Lai, 2009).

Telomere dysfunction has emerged as having a causative role in carcinogenesis by promoting genetic instability. Indeed, telomeres are specialised DNA structures located at the end of chromosomes; they are composed of (TTAGGG)n tandem repeats and are essential for stabilising chromosomes by protecting them from end-to-end fusion and DNA degradation. Telomeres are progressively shortened during each cell-replication cycle because of end-replication problems of DNA polymerase, and telomere shortening induces somatic cells to undergo senescence and apoptosis (reviewed in Blackburn et al, 2006). Further erosion of telomeres may impair their function in protecting chromosome ends, resulting in genetic instability. Although maintenance of telomere length by reverse transcriptase telomerase (hTERT) is critical to preserving the replicative potential of cancer cells (Hiyama and Hiyama, 2007; Artandi and DePinho, 2010), telomerase erosion has been proposed to have two conflicting roles: tumour suppression and genetic instability, a key event in the initiation of carcinogenesis (Hackett and Greider, 2002; Meeker et al, 2004; Perera et al, 2008).

The role of telomere dysfunction in colorectal carcinogenesis is still largely undefined. Several studies demonstrated that telomeres were shorter in CRCs than in adjacent normal mucosa (Hastie et al, 1990; Takagi et al, 1999; O'Sullivan et al, 2008), but this finding was not confirmed by other studies (Katayama et al, 1999; O’Sullivan et al, 2006). Telomeres were also found to be significantly shorter in high-grade dysplastic lesions (Raynaud et al, 2008) and large adenomas (O’Sullivan et al, 2006) than in normal adjacent mucosa; subsequent activation of telomerase may explain the increase of telomere length from preneoplastic lesions to invasive carcinoma (Raynaud et al, 2008). However, the relationship between telomere length and tumour...
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PATIENTS AND METHODS

Patients
For this study, 118 cases were selected from patients with a histopathological confirmation of CRC who underwent surgical resection at a single institution. The criteria of selection were the availability of adjacent non-cancerous mucosa and data on tumour stage, location, and markers of genetic instability.

Samples
Tissues specimens were obtained at the time of surgery, immediately shock frozen in liquid nitrogen, and stored at –80°C until use. Cryostat sections of 6 μm thickness from each tissue sample were prepared using a 1720 Digital cryostat (Leitz, Germany). One section of each sample was stained with haematoxylin–eosin for histopathology. All tumour samples analysed for telomere length contained >80% tumour cells. DNA was extracted by the standard phenol/chloroform method from frozen tissues.

Telomere length measurement by quantitative real-time PCR

Telomere length was determined using real-time PCR (Cawthon, 2002; O’Callaghan et al, 2008) with minor modifications. Two PCRs were performed for each sample, one to determine the cycle threshold (Ct) value for telomere (T) amplification and the other to determine the Ct value for the amplification of a single-copy (S) control gene (acidic ribosomal protein P0, RPLP0). The primer sequences for telomere amplification were TEL1B 5′-GGTTTTGGGTTGGGTTGGGTTTTGGGT-3′ and TEL2B 5′-GGTTTGCCCTACCTTTACCCCTTACCCCTTACCCCT-3′ (O’Callaghan et al, 2008) and those for RPLP0 amplification were RPLP01 5′-CAGCAAGTGGGAAGGTGTAATCC-3′ and RPLP02 5′-CCACCTCTATCATAAGGCTACAA-3′ (Boulay et al, 1999). Each PCR reaction was performed using a 10 μl sample (1 ng of DNA per μl) and a 40 μl mixture containing 1.25 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA), 150 mM 6-ROX, 0.2 × SYBRGreen I nucleic acid stain 10 000 × (Invitrogen, Milan, Italy), 50 mM KCl, 2 mM MgCl2, 0.2 mM of each deoxynucleoside triphosphate (Applied Biosystems), 5 mM dithiothreitol, 1% dimethyl sulphoxide, and 15 mM Tris–HCl pH 8.0, as well as primer pair TEL1B (300 nm) and TEL2B (900 nm) or primer pair RPL01 (300 nm) and RPL02 (500 nm). A reference curve was generated at each PCR run, consisting of reference DNA from the RAJI cell line (Nishikura et al, 1985) serially diluted from 10 to 0.41 ng μl−1. All real-time PCR reactions were carried out using the ABI Prism 7900 HT Sequence Detection System (Applied Biosystems). Telomere and RPLP0 sequences were amplified using the following conditions: 95°C for 10 min to activate the AmpliTaq Gold DNA polymerase, and then 25 cycles each at 95°C for 15 s and 54°C for 2 min for telomere; 40 cycles each at 95°C for 15 s and 58°C for 1 min for RPLP0. ABI Prism software version 2.3 was used for analysis. Intra- and inter-assay reproducibility of both telomere and RPLP0 PCR results was evaluated initially in a series of experiments using dilutions of the reference curve. The s.d. of Ct values was ≤0.189 (% coefficient of variation ≤1.13) in six replicates of samples amplified in the same PCR run, and ≤0.251 (% coefficient of variation ≤1.58) among mean values of triplicates in different PCR runs. Both reference and sample DNAs were analysed in duplicate. Variation of Ct values in the sample was ≤0.3 Ct (s.d. ≤0.021; % coefficient of variation ≤1.25) in both telomere and RPLP0 PCR runs. Mean Ct values were used to calculate the relative telomere length using the telomere/single-copy-gene ratio (T/S) according to the formula: ΔCt_sample = Ct_telomere – Ct_RPL0 control; ΔΔCt = ΔCt_sample – ΔCt_reference curve (where ΔCt_reference curve = Ct_telomere – Ct_RPL0 control) and then T/S = 2−ΔΔCt.

Telomere length measurement by Southern blotting

In an initial series of samples, telomere length was also determined by Southern blotting. Aliquots of 5 μg DNA were digested with HinfI and RsaI restriction enzymes (20 μU each) for 3 h at 37°C. Digested telomere restriction fragments were separated by 0.8% agarose gel electrophoresis. Gels were transferred to a positively charged nylon membrane and were UV cross-linked for 5 min. Hybridisation was performed with the TeloTAGG telomere length assay kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s recommendations. Briefly, filters were hybridised with a digoxigenin (DIG)-labelled probe specific for telomere, washed twice, and then incubated with a DIG-specific antibody coupled to alkaline phosphatase (AP) for 30 min. Hybridisation was finally visualised using AP-metabolising CDP-Star (Disodium 2-chloro-5-(4-methoxy-1-phenyl phosphate; Sigma, St Louis, MO, USA), a highly sensitive chemiluminescent substrate. Membranes were scanned and telomere lengths were estimated by densitometer analysis (Genescan 100, Biorad, Milan, Italy).

Quantification of hTERT transcripts

Extraction of RNA from CRC samples and reverse transcription of RNA into cDNA were carried out as previously described (Terrin...
The quantification of all hTERT transcripts (hTERT-AT) in CRC samples was carried out by real-time PCR, exactly as previously described (Terrin et al., 2007, 2008), and normalised for 18^\text{th} copies of the housekeeping hypoxanthine guanine phosphoribosyl transferase 1 (HPRT1) gene (Terrin et al., 2008).

P53 and MSI analyses

P53 gene mutations were detected by PCR single-strand conformational polymorphism (SSCP) analysis of exons 4–8, and DNA sequencing of samples with abnormal SSCP results (Bertorelle et al., 1995). The MSI status was determined by analysing five microsatellites of the Bethesda recommended panel (BAT-25, BAT-26, D2S123, D5S346, and D17S250) (Pucciarelli et al., 2003), and defining high MSI (MSI-H) tumours as those with two or more altered markers (Boland et al., 1998).

Statistical analysis

Comparisons of telomere length in normal and neoplastic tissues according to gender, site of disease, tumour stage and grade, MSI and p53 status were performed using the Kruskal–Wallis test. The relationship between telomere length and age was explored with the Spearman’s rank correlation coefficient. The diagnostic performance of T/S values was evaluated using a receiver-operating characteristic curve; the cutoff point corresponding to the maximum Youden index was selected to better discriminate between normal and cancerous tissue. Results were reported with their 95% confidence intervals (CI). Multiple linear regression analyses were used to determine the adjusted association of telomere length with tumour location, age, p53 and MSI status. All P-values were two-sided, and P-value of <0.05 was considered statistically significant. Statistical analyses were performed using SAS statistical software (SAS Institute, Cary, NC, USA).

RESULTS

Telomere length in cancer tissues and adjacent non-cancerous mucosa

In 118 tumour samples and the corresponding adjacent non-cancerous tissues, telomere length was determined by real-time PCR. For 42 cases, telomere length was also assessed by Southern blotting. A significant linear correlation was found between the T/S values provided by real-time PCR and the telomere length estimated by Southern blot (r = 0.860, 95% CI 0.77 –0.92, P < 0.0001, Figure 1). Overall, the median level of T/S values in cancer samples was 0.64 (interquartile range (IQR), 0.47 –0.99), lower than that estimated in adjacent mucosa (median 1.84 (1.47 –2.63); P < 0.0001) (Figure 2A). Conversion of T/S values in Kb, using the relationship shown in Figure 1, indicated that telomere length varied from 3.38 to 7.44 Kb with a median (IQR) of 4.10 (3.83 –4.64) Kb in tumours, and from 3.61 to 11.55 Kb with a median of 5.93 (5.36 –7.15) Kb in normal tissues.

Telomere lengths were shorter in cancers than in adjacent non-cancerous mucosa for all tumour stages (P < 0.0001; Figure 2B) and grades (median (IQR) 0.63 (0.47 –0.97) vs 2.04 (1.40 –2.41), 0.67 (0.46 –1.02) vs 1.87 (1.49 –2.78), and 0.59 (0.45 –0.78) vs 1.68 (1.45 –2.21) for grades 1, 2, and 3, respectively; tumours vs normal tissues P < 0.0001). Although no correlation was found between age and telomere length in cancers, telomere length in normal tissues inversely correlated with age (r = –0.24, 95% CI –0.42 –0.05, P = 0.017; Figure 2C). A patient-by-patient comparison of matched tissue samples showed that all but four cases had longer telomeres in non-cancerous mucosa than in CRC samples with a median difference of 1.22 (0.61 –1.81); these differences inversely correlated with age (r = –0.23, 95% CI –0.41 –0.04, P = 0.023). From the receiver-operating characteristic curve analysis, the T/S value that best discriminated between the telomere length of neoplastic and normal tissues was 1.14. By using this cutoff value, sensitivity and specificity of the assay for cancer-associated telomere were 83% (95% CI 75 –90%) and 92% (95% CI 85 –96%), respectively (Figure 2D).

Relationship between telomere length and tumour characteristics

Telomere lengths did not significantly differ with tumour stage. Median (IQR) T/S values were 0.66 (0.56 –0.66), 0.69 (0.49 –0.93), 0.55 (0.39 –1.02), and 0.64 (0.51 –0.98) in tumour stages I, II, III, and IV, respectively (Figure 2B, overall, P = 0.472). A similar observation was found by comparing T/S values with tumour grade; T/S values did not differ between well-differentiated (median 0.63, IQR 0.47 –0.97), moderately differentiated (0.67, 0.46 –1.02), and poorly differentiated tumours (0.59, 0.45 –0.78) (overall, P = 0.561).

The CRCs were then stratified according to p53 and MSI status: an equal number of MSS tumours had the wild-type (n = 45) or mutated (n = 45) p53 gene; 24 MSI-H tumours had the wild-type p53 gene, whereas four tumours showed the rare pattern of both MSI-H and the mutated p53 gene. Median (IQR) T/S values were 0.65 (0.50 –0.98) and 0.62 (0.43 –0.97) in CRCs with the wild-type (n = 69) and mutated p53 gene (n = 49), respectively, and 0.66 (0.46 –1.03) and 0.56 (0.43 –0.73) in MSS (n = 90) and MSI-H tumours (n = 28), respectively (P = 0.065) (Figures 3A and B). Furthermore, in tumours carrying the wild-type p53 gene, telomeres were significantly shorter in MSI-H (n = 24) than in MSS cases (n = 45) (0.56 (0.43 –0.73) vs 0.70 (0.50 –1.13), P = 0.027) (Figure 3C). In MSS tumours, those carrying the mutated p53 gene had shorter telomeres than those carrying the wild-type p53 gene, but this difference was not statistically significant (0.62 (0.43 –0.97) vs 0.70 (0.50 –1.13), P = 0.218) (Figure 3C).

Telomere lengths also differed according to tumour location. Median (IQR) T/S values were 0.57 (0.41 –0.82), 0.61 (0.44 –0.98), and 0.78 (0.62 –1.09) in the 53 right-colon, 30 left-colon, and 35 rectal carcinomas, respectively (overall, P = 0.03; right colon vs rectum P = 0.009) (Figure 4). Normal tissues surrounding colon and rectal tumours did not significantly differ (median (IQR) 1.82 (1.48 –2.51), 2.13 (1.49 –2.86), and 1.87 (1.25 –2.61) in right-colon, left-colon, and rectal tissues, respectively; P = 0.81). It can be noted that 49% (26 of 53) of right-colon tumours but only 3% (2 of 65) of left-colon and rectal cancers showed MSI. The four cases with both MSI and the p53-mutated gene were also right-colon cancers.
In contrast, p53 mutation occurred in a higher percentage in left-colon and rectal tumours (60 and 43%, respectively) than in right-colon cancers (23%). With multivariate analyses, telomere length remained significantly associated with tumour location after adjustment for age and p53 status ($P = 0.04$), as well as after adjustment for age and p53/MSI status ($P = 0.05$). It is interesting that even among MSS tumours, right-colon cancers had shorter telomeres than left-colon and rectal cancers (median (IQR) hTERT levels were 41(25–183), 82(60–161), 172(71–1192), and 85(37–282) copies in right-colon (26), and rectal CRCs (172), respectively ($P = 0.047$). Right-side tumours also had shorter telomere lengths than left-side tumours ($P = 0.047$), with the exception of MSS CRCs ($P = 0.195$), and moderately differentiated carcinomas ($P = 0.30$). In agreement with previous studies (Hastie et al, 1990; Gertler et al, 2004), we found that telomere length decreased with ageing in normal mucosa, but not in tumours. It has been proposed that telomere shortening with age may increase the risk of cancer (Blasco, 2005; Risques et al, 2008); this concept may be supported by our results, as telomere shortening between normal and neoplastic tissues was found to be inversely correlated with age. Although telomere length in somatic cells primarily reflects cellular proliferation, telomere length in tumour cells reflects the balance between cellular proliferation with telomere loss and telomerase activity with de novo synthesis of telomeric sequences. Evidence that telomeres were shorter in CRCs than in adjacent mucosa, even in well-differentiated tumours, strongly supports the concept that telomere erosion is a critical initial event in colorectal carcinogenesis. It is likely that stabilisation and maintenance of telomeres, essential to preventing cellular senescence and conferring unlimited replicative potential, occur after initial extensive cellular proliferation. It is noteworthy that, although several studies agree that there is a shortening of telomeres in preneoplastic lesions (O’Sullivan et al, 2006; Raynaud et al, 2008), the relationship between telomere length and tumour progression and location, as well as its relationship to specific genetic alterations, should be the subject of further investigation.

**DISCUSSION**

The role of telomere erosion in colorectal carcinogenesis is still largely undefined. Using a real-time PCR assay, which requires a considerably low quantity of biological samples, we estimated telomere length in a large number of CRCs and in corresponding adjacent non-cancerous mucosa. For the first time, we determined the relationship between telomere length and tumour progression and location, as well as its relationship to genetic instability in CRCs. Overall, our results indicated that (i) telomeres in CRCs were consistently shorter than those of adjacent non-cancerous mucosa, regardless of tumour stage and grade, site, or genetic alterations; (ii) telomeres were shorter in MSI than in MSS carcinomas, and the difference was significant between MSI and MSS tumours carrying the wild-type p53 gene; (iii) regardless of genetic alterations, telomeres were shorter in tumours arising from the right colon and were longer in rectal cancers.
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Figure 3  Relative telomere lengths, expressed as T/S values, in tumours according to (A) mutated (n = 49) or wild-type (n = 69) p53 gene, and (B) high microsatellite instability (MSI-H) (n = 28) or stable microsatellite (MSS) (90) status. (C) Relative telomere lengths, expressed as T/S values, in p53 mutated MSS (n = 45); p53 wild-type MSI-H (n = 24), and p53 wild-type MSS (n = 45) tumours. Boxes and whiskers represent the 25th–75th and 10th–90th percentiles, respectively; the median is the central line in each box.

Figure 4  Relative telomere lengths, expressed as T/S values, in tumours according to the site of origin: right colon (n = 53), left colon (n = 30), rectum (n = 35). Boxes and whiskers represent the 25th–75th and 10th–90th percentiles, respectively; the median is the central line in each box.

Progression is still controversial (Engelhardt et al., 1997; Gertler et al., 2004; O’Sullivan et al., 2006). We did not find any relationship between telomere length and tumour progression. In agreement with previous studies (Terrin et al., 2008), levels of hTERT increased with tumour progression; it is conceivable that telomere length may stabilise with tumour progression because of an increase in telomerase activity compensating for replicative telomere loss.

It should be pointed out that ~15% of CRCs presented MSI-H, whereas the p53 gene is the known major genetic alteration in CRCs with MSS (Kim et al., 2007; Ogino et al., 2009). A link between telomere shortening and MSI-H has been suggested in a previous study of 55 CRCs; all MSI tumours (n = 8) but only half of the remaining MSS tumours had shorter telomeres than those found in adjacent normal mucosa (Takagi et al., 2000). Our study conducted in a larger number of CRCs demonstrated that both MSI-H and MSS tumours had shorter telomeres than those found in adjacent mucosa and that MSI-H had shorter telomeres than MSS cancers. The MSI pathway involves a failure of the mismatch repair system (Aaltonen et al., 1993), which maintains genetic stability not only by repairing DNA replication errors but also by preventing chromosomal recombinations; a deficiency of mismatch repair helps cells overcome cellular crises caused by critical shortening of telomeres (Bechter et al., 2004). Thus, MSI cells may undergo more proliferative cycles and a more pronounced shortening of telomeres before stabilising than MSS cells. It is of interest that the difference was particularly high and significant when MSI tumours were compared with MSS tumours carrying the wild-type p53 gene. It can be noted that MSS tumours with the mutated p53 gene had slightly shorter telomeres than MSS tumours with the wild-type p53 gene. In cells with mutated p53, telomeres may protract their shortening along with cell proliferation. However, p53 is a well-known negative regulator of hTERT promoter, and mutated p53 may also result in hTERT activation (Liu et al., 2004); thus, stabilisation of telomeres may occur earlier than in MSI tumours.

We found that right-colon tumours displayed significantly shorter telomeres than tumours arising from other sites. In agreement with previous studies (Iacopetta, 2002; Gervaz et al., 2004; Walther et al., 2008), we found that MSI status was strongly associated with tumour origin in the right colon. However, this association could only partially explain the finding that telomeres were shorter in right-colon cancers, as half of these tumours were MSS. Interestingly, even in MSS tumours, those arising in the right colon had significantly shorter telomeres than those arising in the rectum. The prognosis of rectal cancers is worse than that of colon cancer and it is currently under debate whether rectal cancers are actually a distinct entity. Telomere length distinguishes colon from rectal cancers; as telomere lengths of normal tissues surrounding colon and rectal cancers did not differ, it is likely that the different telomere length in cancers is due to a different kinetics of telomere erosion/stabilisation in colon and rectal carcinogenesis. Although no association was found between hTERT level and the tumour origin site, earlier activation of hTERT may contribute to these differences between rectal and colon cancers. Future studies on these aspects may be of help in the design of anti-neoplastic strategies.

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Conflict of interest

The authors declare no conflict of interest.
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