Telomere shortening and *PCDH10* promoter methylation in colorectal cancer mucosae

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ABSTRACT

Background: telomerase activity and telomere length (TL) have important implications in several human diseases. Telomere shortening is associated with colorectal carcinogenesis. Recent studies also showed that protocadherin 10 (*PCDH10*) plays a critical role in cancer cell growth, by negatively regulating telomerase activity. *PCDH10* is frequently downregulated by promoter DNA methylation. The aim of this study was to investigate whether *PCDH10* promoter methylation was associated with TL in colorectal cancer (CRC).
Methods: DNA was extracted from 35 CRC and 35 adjacent normal tissues with Gentra Purgene Kit (Qiagen, Hilden, Germany). A quantitative methylation-specific PCR (MSP) based method was used to analyze a selected CpG site in *PCDH10* promoter. TL was evaluated with qPCR and expressed as telomere to single copy gene (T/S) ratio. Differences were assessed with Mann-Whitney test or Wilcoxon signed-ranks test when appropriate, whilst correlation analyses were performed with Spearman's test. Diagnostic performance was calculated with receiver operating characteristics (ROC) curve analysis. The level of statistical significance was set at $p < 0.05$.
Results: we found that TL was significantly lower in CRC than in adjacent non-cancerous tissues ($p=0.0005$). The area under the ROC curve (AUC) for TL was 0.759 (95% Confidence Interval: 0.643-0.875, $p=0.0002$). Aberrant *PCDH10* promoter methylation was detected in 100% of CRC tissues but in none of paired non-cancerous tissues. The median methylation rate in CRC tissues was 55.7% (range: 6.1-97.8%). TL was negatively correlated with *PCDH10* promoter methylation ($r=-0.42$, $p=0.0002$).
Conclusions: these results suggest a pivotal role of telomere shortening and *PCDH10* methylation in CRC tissues. TL may be seen as a potential biomarker in CRC diagnostics.

INTRODUZIONE

Telomeres are non-coding hexameric nucleotide repeats (TTAGGG)n at the ends of all linear eukaryotic chromosomes (1). Along with protein complex shelterin, they contribute to form protective and highly conserved nucleoprotein structures, which ultimately facilitate genomic stability and integrity (2).

With continuous shortening, telomeres eventually reach a critical length that triggers replicative senescence and apoptosis (3-4). Notably, this is thought to represent an essential tumour suppressor mechanism, that limits the cell proliferative capacity (5). Telomerase activity and telomere length (TL) have important implications in human disease and aging (6), and multiple studies have provided substantial evidence supporting their role in colorectal cancer (CRC) development and progression (7-8). Both short and long telomeres have been implicated in carcinogenesis. Many early studies indicated that

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shorter telomeres were associated with an increased risk of developing bladder, oesophageal, gastric, head and neck cancers (9). Conversely, other studies showed that longer telomeres were associated with increased risk of lung and bladder cancer, lymphoma and sarcoma (10-13). Interestingly, TL has also been found shorter in CRC tissues than in adjacent normal tissue (14).

Telomerase is a complex ribonucleic reverse transcriptase responsible for synthetizing telomeric DNA repeats at the ends of linear chromosomes. It comprises the catalytic protein subunit telomerase reverse transcriptase (*hTERT*), encoded by the *TERT* gene (located at 5p15.33), an essential RNA component (TERC) that functions as the RNA template for the addition of telomeric repeats (15-16). Elevated telomerase activity represents a hallmark of human cancer (17-18). It has been reliably demonstrated that the limiting factor for telomerase activity is *hTERT* expression, which is tightly regulated at a transcriptional level (19), through epigenetic modifications in the promoter region (20). In a recent study, Zhou et al. demonstrated that protocadherin 10 (PCDH10) negatively regulates telomerase activity and inhibits cancer cell proliferation, migration and invasion (21). Accordingly, our research group along with others, showed that *PCDH10* is frequently down-regulated by promoter DNA methylation, and functions as a tumour-suppressor gene in gastric, colorectal and lung cancer, as well as in many other carcinomas (22-24).

The aim of our study was to investigate whether *PCDH10* promoter methylation was associated with TL in colorectal cancer.

METHODS

Patients and samples

The study population consisted of 35 CRC patients (mean age 67.7 years, SD 13.8) admitted at the University Hospital of Verona (Italy) between January 2010 and December 2010 for routine colon-rectum biopsy for medical treatment and/or diagnosis. Paired tumour and adjacent normal tissue were obtained during biopsy, immediately frozen in liquid nitrogen, and stored at -80°C. Histological diagnosis and tumour stage were defined as for the 2000 World Health Organization (WHO) classification system for tumours of digestive system (25) and according to the American Joint Committee on Cancer (AJCC) staging system (26), respectively. Detailed demographic and clinical characteristics of CRC patients, including age, gender and Tumor, Metastasis, Lymph nodes (TMN) stage, are shown in Table 1. The study was carried out in accordance with the ethical standards of the revised Declaration of Helsinki and was cleared by the local Review Board.

Table 1
Demographics and clinical features of colorectal cancer patients.

Variables	CRC patients (n=35)
Mean age, years (SD)	67.7 (13.8)
male n (%)	25 (71.4)
female n (%)	10 (28.6)
AJCC stage, n (%)	
I	2 (5.7)
II	2 (5.7)
III	27 (77.1)
IV	4 (11.5)
AJCC grade, n (%)	
1	1 (2.9)
2	22 (62.8)
3	12 (34.3)

CRC, colorectal cancer; AJCC, American Joint Committee on Cancer Deviation.

Laboratory methods

For *PCDH10* methylation analysis, purified genomic DNA extracted from tissues with Gentra Purgene Kit (Qiagen, Hilden, Germany) was subjected to bisulphite treatment and purification using the Epitect Bisulfite kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Treated DNA was then subjected to methylation-specific (MSP) polymerase chain reaction (PCR) to analyze methylation status, where two specific sets of primers were used, one designed for methylated (M) and the other for un-methylated (U) *PCDH10* promoter. The following primers sequences were used, methylated primers (forward 5′-AGTTATAGGAGTTTTTACGTAGCGT-3′ and reverse 5'-ATATTCCTACTCCTCCTATACCGTA-3') and unmethylated primers (5'-GAAAGTTATAGGAGTTTTT ATGTAGTGT-3' and reverse 5'-ATATTCCTACTCCTCCTATACCATA-3').

Parallel with each set of MSP reactions, CpGenome Universal Methylated DNA (Chemicon, Millipore Billerica, MA, USA) was used as 100% methylated control DNA. The MSP products were checked on 2% agarose gel. Measurement of TL was conducted using q-PCR SYBR Green-based assay on 35 available CRC tissues and their matched normal tissue. The primers for telomeres and single copy gene 36B4 (which encodes acidic ribosomal phosphoprotein PO) were added to final concentrations of 0.9 μM and 0.3 μM, respectively. The following two sets of primers were used as previously reported by Cawthon (27):

- TeFo, 5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3' and TeRe, 5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3';
- 36B4Fo, 5'- CAGCAAGTGGGAAGGTGTAATCC-3' and 36B4Re, 5'-CCCATTCTATCATCAACGGGTACAA-3'.

The TL was obtained through calculating the ratio of telomere repeat product (T) to a single-copy gene product (S, 36B4) for each patient, using the formula T/S = 2−ΔCt, where ΔCt = Ct(telomere) − Ct(36B4). This ratio was then compared with the ratio of K562 reference DNA (Promega, Madison, USA).

Statistical analyses

Relative expression levels of methylation promoters and TL were calculated using the 2-ΔΔCt method (28). GraphPad Prism 5 (Version 5.01, GraphPad Software, Inc., USA) was used for statistical analyses and graphs drawing. Statistically significance was at p-value <0.05 for all tests. Mann-Whitney U test was used to evaluate the differences in characteristics of patients between study groups. Wilcoxon signed-ranks test was used to compare normal and tumoral tissues. Spearmen's test was used for correlation analysis. The diagnostic performance was estimated using receiver operator characteristic (ROC) curve analysis.

RESULTS

Promoter methylation of *PCDH10* was detected in 100% of CRC tissues but in none of paired non-cancerous tissues. The median *PCDH10* methylation rates in tumour tissues was 55.7% (range min-max: 6.1-97.8%). No differences in *PCDH10* methylation rate were observed between early stages (stage 1 and 2) and advanced stages (stage 3 and 4) (p=0.6594) as well as between low grades (G1 and G2) and high grade (G3) tumours (p=0.5664). The TL in CRC tissues was significantly shorter (median 1.10, range min-max: 0.25-3.91) than in normal tissues (median 1.92, range min-max: 0.37-12.73), (p=0.0005) (Figure 1). Moreover, TL was found to be non-significantly different in CRC tissues with grade 3 compared to those with grade 1-2 (p=0.875) as well as in early versus advanced stages (p=0.2762).

The area under the ROC curve (AUC) for TL was 0.759 (95% Confidence Interval: 0.643-0.875, p=0.0002) (Figure 2). A statistically significant negative correlation was found between TL and *PCDH10* methylation rate (r= -0.42, p=0.0002). A summary of main results was reported in Table 2.

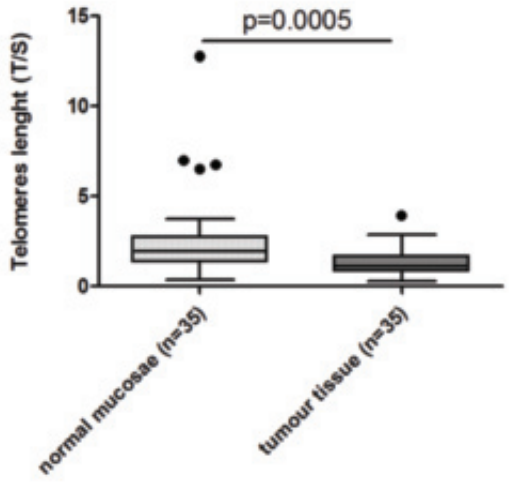

Figure 1.
Comparison of telomere length in colorectal and normal tissues. T/S= ratio of the telomere repeat product (T) and a single-copy gene product (S, 36B4).

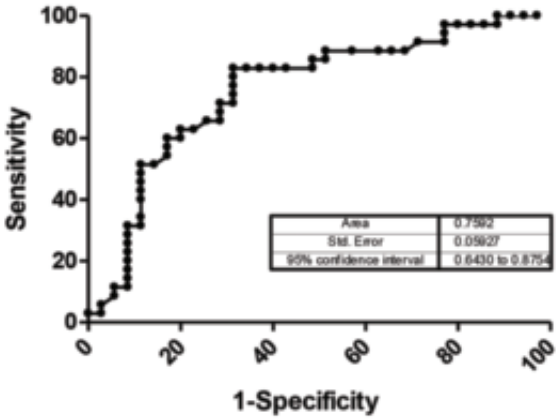

Figure 2.
Telomere length; ROC curves performed on colorectal and normal tissues).

Table 2
Results of PCDH10 methylation promoter rate and telomere length in colorectal cancer and normal tissue.

Tissue	*PCDH10* methylation rates	median telomere length	p value	correlation *PCDH10* methylation/telomere length
colorectal cancer	55.7%	1.10	0.0005	r= -0.42; p=0.0002
	(range min-max: 6.1 - 97.8)	(range min-max: 0.25 - 3.91)		
normal tissue	0%	1.92		
		(range min-max: 0.37 - 12.73)		

DISCUSSION

Recent studies revealed that cancer stem cells biology is regulated by genetic (e.g., oncogene activation and oncosuppressor gene inactivation) and epigenetic changes (e.g. miRNA targeting and promoter DNA hypo/hypermethylation), which reflect mutations in tumour microenvironment (29-30). These changes are needed for oncogenic transformation and cellular reprogramming, thus resulting in inactivation of a certain number of oncosuppressor proteins (31), differential expression of several miRNAs (32), aberrant methylation of promoters genes (33) and upregulation/reactivation of telomerase (34).

The primary hypotheses linking telomeres and cancer are based on the fact that telomeres progressively shorten after each cell division (1). Telomerase activity is strongly associated with tumour development and may hence play a critical role as a biomarker for early detection of cancer (35).

High telomerase activity or *hTERT* expression has originally been revealed in colorectal tissue (36-37). *hTERT* was then also found to be expressed in non-tumour colorectal tissues by immunohistochemistry (38).

Cancer cells have gained the ability to overcome their route toward senescence via TL maintenance mechanisms, mainly mediated by telomerase activation, but also by alternative mechanisms (alternative lengthening of telomeres – ALT) (17, 39-40). TL is also regulated by genetic and epigenetic marking, thus including hTERT alternative splicing (41), *hTERT* promoter mutations (42), *hTERT* promoter methylation (43) and *PCDH10* protein interaction (21). In particular, *PCDH10* is one of the non-clustered protocadherins encoding calcium-dependent adhesion protein, which participates in multiple molecular functions, such as cell adhesion, colony formation and signalling regulation (44,45). This gene was earlier identified as a tumour suppressor gene in many tumours, including nasopharyngeal carcinoma, gastric carcinoma (46) and multiple myeloma (47). In our previous study (48) we found that methylation of *PCDH10* promoter region is a common epigenetic event in colorectal tumours, and that this cancer-specific aberration can be frequently found into the circulation.

To the best of our knowledge, our study has been the very first to analyze TL and *PCDH10* promoter methylation in patients with CRC. A negative correlation was observed between TL and *PCDH10* promoter methylation, which hence support previous findings that *PCDH10* may play a critical role in cancer cell growth, by negative regulation of telomerase activity (21). In agreement with previous studies (49-52), our results also showed that the TL was significantly shorter in CRC tissue than in the adjacent normal counterpart, albeit we failed to demonstrate a correlation between shortened TL and higher histological grading. This apparent discrepancy with data published in other studies (53-54) can be attributed to the different study population, wherein 89% of our patients were diagnosed as having more advanced stages.

The ROC curve analysis on normal and CRC tissue seemingly reveals that TL has a good diagnostic performance. However, due to limited sample size, we could not draw definitive conclusions about its putative relationship with different tumour stages and grades, thus leading the way to performing more powered studies to assess this issue. Further studies are also needed to elucidate the diagnostic performance of TL into the circulation, as possible CRC biomarker.

TL in plasma samples was previously assessed in two studies. Wu et al. (55) found significantly shortened TL in cell-free DNA (cfDNA) in the plasma of breast cancer patients with no prior treatment. Idei et al. (56) reported similar results in patients with ovarian cancer.

In conclusion, our data suggest that abnormalities in cell-cycle regulator pathways may trigger continuous cell division and critical telomere shortening. Shortened TL may be seen as an active player in colorectal carcinogenesis, thus emerging as a potentially useful early biomarker of CRC along with *PCDH10* hypermethylation.

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