

## RESEARCH COMMUNICATION

# Reduced Telomere Length in Colorectal Carcinomas

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### Abstract

**Purpose:** Telomeres play a key role in the maintenance of chromosome integrity and stability, and telomere shortening is involved in initiation and progression of malignancies. The aim of this study was to determine whether telomere length is associated with the colorectal carcinoma. **Patients and methods:** A total of 148 colorectal cancer (CRC) samples and corresponding adjacent non-cancerous tissues were evaluated for telomere length, P53 mutation, and cyclooxygenase-2 (COX-2) mutation detected by fluorescent immunohistochemistry. Telomere length was estimated by real-time PCR. Samples with a T/S > 1.0 have an average telomere length greater than that of the standard DNA; samples with a T/S < 1.0 have an average telomere length shorter than that of the standard DNA. **Results:** Telomeres were shorter in CRCs than in adjacent tissues, regardless of tumor stage and grade, site, or genetic alterations (P=0.004). Telomere length in CRCs also had differences with COX-2 status (P=0.004), but did not differ with P53 status (P=0.101), tumor progression (P=0.244), gender (P=0.542), and metastasis (P=0.488). There was no clear trend between T/S optimal cut-off values (<1 or >1) and colorectal tumor progression, metastasis, gender, P53 and COX-2 status. **Conclusion:** These findings suggesting that telomere shortening is associated with colorectal carcinogenesis but does not differ with tumor progression, gender, and metastasis.

**Keywords:** Telomere length - colorectal cancer - COX-2 - China

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### Introduction

Telomeres are non-coding tandem repetitive DNA sequences (TTAGGG) at the end of chromosomes, and play important roles in maintaining genomic integrity and stability (Verdun et al., 2007). In dividing cells telomeres progressively shorten and, in response to short telomeres, cells normally undergo senescence, apoptosis or become genomically unstable (von Zglinicki, 2002). Telomere length has been previously reported to be associated with an increased risk of aging and related diseases including diabetes (Aviv et al., 2006; Demissie et al., 2006; ), cardiovascular disease (Brouillette et al., 2007; Fitzpatrick et al., 2007) and various cancers (Wu et al., 2003; Broberg et al., 2005; McGrath et al., 2007; Prescott et al., 2010; Kim et al., 2011).

Colorectal carcinomas (CRCs) comprise a heterogeneous complex of diseases differing in molecular pathways and biological characteristics, arising through a multi-step carcinogenic process. And these events generally follow exposure to carcinogens and result in the selection of clonal cells with uncontrolled growth (d'Adda di Fagagna et al., 2003). The earliest events are mutations, deletions or polysomy at genomic level, but these changes do not always lead to changes in cell morphology or tissue structure (Greider et al., 1989; Gorgoulis et al., 2005). Current knowledge suggests that the progression of cancer

from a pre-malignant to the malignant state is consistent with a mechanistic model, based on of the principle of natural selection. CRCs cells acquire the hallmarks of cancer during this carcinogenic selection process (Meeker et al., 2004). Cell immortality is one of the principal features acquired during this process. Immortality involves the stabilization of telomere length, which is achieved by telomerase activation in about 80% of human tumors. In normal cells, telomere length provides information regarding replication capacity. Several studies have shown that telomere length is shorter in colorectal cancer cells compared with normal mucosa (Engelhardt et al., 1997; Nakamura et al., 2000; Kim et al., 2002; Gertler et al., 2004; Garcia-Aranda et al., 2006). However, there are contradictory reports about the independent prognostic value of telomere length determination.

In order to better understand the relationship telomere length in colorectal carcinoma, we evaluated telomere length and the relationship of tumor grade, progression, metastasis, gender, P53 and COX-2 status in the multi-step process of colorectal carcinogenesis.

### Materials and Methods

#### *Patients and Sample collection*

Paraffin sections of cancer were obtained from 148 colorectal cancers who had undergone surgery at the

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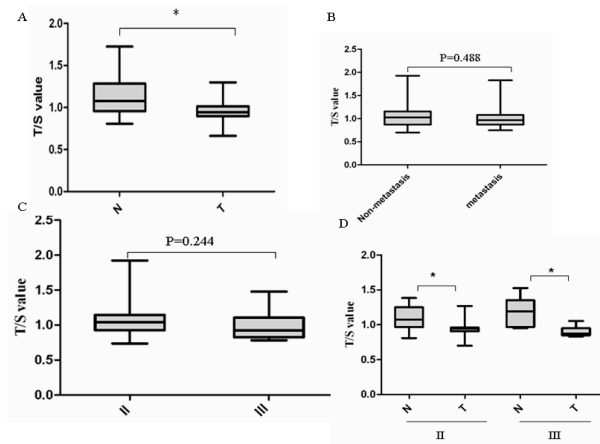
Affiliated Hospital of Nanjing Medical University, Changzhou No.2 People's Hospital, Changzhou, China, from November 2006 to March 2008. All samples were collected intraoperatively and immediately frozen and stored at -80°C until they were analysed. Specimens were examined and classified in the hospital's Department of Pathology. Tumors were staged according to the tumor-node-metastases classification of the International Union against Cancer and according to their grade of cell differentiation.

*DNA Extraction from Paraffin*

Genomic DNA was extracted from paraffin using the QIAamp DNA FFPE Tissue Kit (Qiagen, Chatsworth, CA ).We also detected the concentration and purity of DNA by eppendorf Bio-spectrophotometer. Subsequent standardization by drying down the genomic DNA and resuspending ensured accurate and uniform DNA concentrations.

*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 (the beta-globin, hbg). The primer sequences for telomere amplification were TEL-F 5'-CGGTTTGTGGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3' and TEL-R 5'-GG CTTGCCTTACCCTTACCCTT ACCCT TACCCTTACCCT-3' (O'Callaghan et al., 2008) and those for hbg amplification were HBG-F 5'-CGGCGGC GGGCGGCGCGGGCTGGGCGGCTT CATCCA CGT TCACCTTG-3' and HBG-R 5'- GCCCGGC CCGCCGCGCCCGTCCCGCCGGAGGAGAAGTCTGCCGTT-3' (Richard M et al., 2009). The final concentrations of reagents in the PCR were 0.75xSYBR Green PCR Master Mix (Applied Biosystems, Made in UK) 10mM Tris-HCl pH8.3, 50mM KCl, 3mM MgCl2, 0.2mM each dNTP, 1mM DTT and 1M betaine (U.S. Biochemicals). Each25 ml reaction received 0.625U AmpliTaq Gold DNA polymerase (Applied Biosystems, Inc). For each PCR reaction the telomere primer pair TEL-F and TEL-R (final concentrations 900nM each), were combined with the beta-globin primer pair HBG-F and HBG-R (final concentrations 500nM each) in the master mix. Telomere and hbg sequences were amplified using the follow conditions: 95°C for 10 min to activate the AmpliTaq Gold DNA polymerase, and then 35 cycles each at 95°C for 15s and 54°C for 2 min for telomere; 32 cycles each at 95°C for 15s and 62°C for 30s for hbg. After thermal cycling and raw data collection were complete, IQ5 optical system software was used for analysis. As each experimental sample was assayed in triplicate, average T/S is expected to be proportional to the average telomere length per cell. Samples with a T/S>1.0 have an average telomere length greater than that of the standard DNA; samples with a T/S<1.0 have an average telomere length shorter than that of the standard DNA. Mean Ct values were used to calculate



**Figure 1. Relative Telomere Lengths, Expression as T/S Values in Colorectal Carcinoma.** (A) the expression as T/S values, in colorectal carcinoma (T) and the adjacent tumors (N) P<0.05; (B) the median level of T/S values in non- metastasis and in metastasis was 1.015 (P>0.05); (C) the median level of T/S values in tumor stage II and in tumor stage III (P>0.05); (D) the median level of T/S values in tumor stage II and in tumor stage III ,respectively tumors and adjacent non-cancerous tissues (P<0.05)

the relative telomere length using the telomere/ single copy-gene ratio (T/S) according to the formula:  $\Delta Ct_{sample} = Ct_{telomere} - Ct_{control}$ ,  $\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{reference\ curve}$  (where  $\Delta Ct_{reference\ curve} = Ct_{telomere\ mean} - Ct_{control\ mean}$ ).

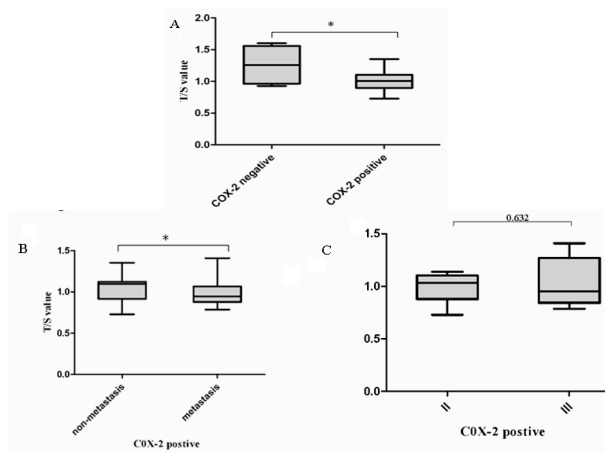
*Statistical analysis*

Comparisons of telomere length in colorectal carcinoma and adjacent tissues according to gender, tumor stage and grade, metastasis, P53 and COX-2 status were performed using the Kruskal-Wallis test, the Mann-Whitney U-test, Student's t-test and the X2 test, as appropriate.. Results were reported with their 95% confidence intervals (CI). Multiple linear regression analyses were used to determine the adjusted association of telomere length with tumor stage and grade, metastasis, P53 and COX-2 status. All P-values were two-sided, and P-value of <0.05 was considered significant. Statistical analyses were performed using SPSS 13.0 software.

**Results**

*Telomere length in the colorectal carcinomas*

In 26 tumor samples and the corresponding adjacent non-cancerous tissues, telomere length was determined by real-time PCR. Overall, the median level of T/S values in colorectal carcinoma was 0.967 (interquartile range (IQR), 0.662-1.298) lower than that estimated in adjacent carcinoma. (median1.123, (0.808-1.724); P<0.004) (Figure 1A). And in 100 colorectal carcinoma samples, including 55 tumor samples were non- metastasis and 45 tumor samples were metastasis. Overall, the median level of T/S values in non- metastasis was 1.048 (IQR, 0.698-1.925) had no differences with the median level of T/S values in metastasis (median 1.015, (0.747-1.828); P=0.488) (Figure1B). We also found that there were no differences between the median level of T/S values in tumor stage II (median 0.936, (0.735-1.922); N=38) and the tumor stage III (median1.027, (0.781-1.479); N=26; P=0.244) (Figure 1C). Telomere lengths were shorter in



**Figure 2. Relative Telomere Lengths, Expression as T/S Values in COX-2 Status.** (A) The median level of T/S values in tumor COX-2 positive was 1.003 and T/S values in tumor COX-2 negative was 1.260 ( $P < 0.05$ ); (B) The median level of T/S values in non- metastasis and in metastasis for COX-2 positive expression ( $P < 0.05$ ); (C) the median level of T/S values in tumor stage II and in tumor stage III for COX-2 positive expression ( $P > 0.05$ )

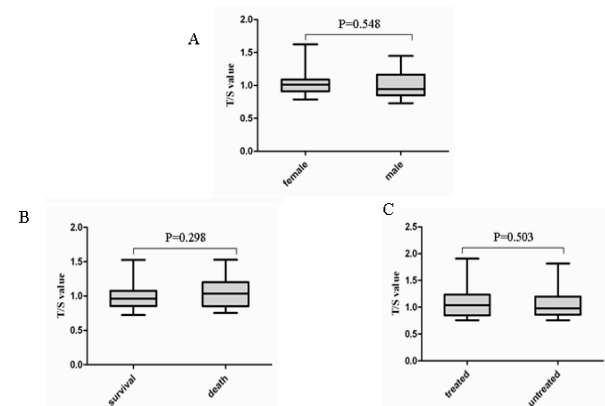
cancers than in adjacent non-cancerous for tumor stage II and stage III ( $P < 0.05$ ) (Figure 1D). The median was 1.101 (0.807-1.384); VS 0.952 (0.698-1.267);  $N = 12$ ;  $P = 0.006$ ) and 1.187 (0.950-1.925); VS 0.900 (0.831-1.053);  $N = 6$ ;  $P = 0.037$ ) for stage II and stage III, respectively tumors and adjacent non-cancerous tissues.

#### Telomere length in tumor COX-2 status

Cyclooxygenase-2 (COX-2) is expressed early in colon carcinogenesis and is known to play a crucial role in the progress of colorectal carcinomas. Here we showed the telomere length in COX-2 negative and positive of tumors. In 41 colorectal carcinoma samples, telomere length was determined by real-time PCR, including 37 tumor samples were COX-2 positive and 4 tumor samples were COX-2 negative. Totally, the median level of T/S values in COX-2 positive was 1.003 (IQR, 0.728-1.352) lower than that estimated in COX-2 negative (the median 1.260, (0.926-1.600);  $P = 0.004$ ) (Figure 2A). As the same, we also investigated the relationship between the telomere length and P53 expression pattern in colorectal cancers, we found that there was no difference in P53 positive expression and P53 negative expression ( $P > 0.05$  data were not shown). In COX-2 positive colon carcinogenesis, we detected the telomere length in metastasis and grades. We found that the median level of T/S values in non- metastasis was 1.041 (IQR, 0.728-1.352,  $N = 18$ ) and in metastasis was 0.980 (IQR, 0.785-1.407,  $N = 16$ ;  $P = 0.025$ ) (Figure 2B), and the median level of T/S values in stage II was 0.992 (IQR, 0.728-1.137,  $N = 20$ ) and in stage III was 1.024 (0.785-1.407,  $N = 8$ ;  $P = 0.632$ ) (Figure 2C).

#### Telomere length in patient's characteristics

Patient factors and telomere length were analysed using the Kruskal-Wallis test, the Mann-Whitney U-test, Student's t-test and the  $X^2$  test, as appropriate. We detected telomere length between patient's gender, survival or surgery. In 45 colorectal carcinoma samples, telomere length was determined by real-time PCR, containing 27



**Figure 3. Relative Telomere Lengths, Expression as T/S Values in Patient Factors.** (A) Telomere length in patient's gender. The median level of T/S values in male was 1.013 and the T/S values in females was 1.0524 ( $P > 0.05$ ); (B) Telomere length in patient's survival. The median level of T/S values in survival was 1.022 and the T/S values in death was 0.945 ( $P > 0.05$ ); (C) Telomere length in patient who had undergone surgery or not treated. The median level of T/S values in treated was 1.126 and the T/S values in untreated was 1.063 ( $P > 0.05$ )

**Table 1. The Relationship Between the T/S Values and Tumor Characteristics**

		Number	T<S	T>S	P
Histopath	Non-meta	55	26	29	0.702
	Metastasis	45	23	22	
P53	Negative	18	7	11	0.904
	Positive	32	13	19	
COX2	Negative	4	1	3	0.368
	Positive	37	18	19	
Differentiation	II	38	16	22	0.661
	III	26	15	11	

tumor samples were males and 18 cancer samples were females. We found there were no differences in patient's gender. The median level of T/S values in male was 1.013 (IQR, 0.729-1.447) and the T/S values in females was 1.0524 (IQR, 0.787-1.624,  $P = 0.548$ ) (Figure 3A), as the same results were found that no differences between patient's survival (median 1.022, (0.724-1.526);  $N = 24$ ) or death ((median 0.945, (0.753-1.528);  $N = 21$ ;  $P = 0.298$ ) (Figure 3B) and treatment (median 1.126, (0.758-1.907);  $N = 24$ ) or untreated ((median 1.063, (0.756-1.812);  $N = 21$ ;  $P = 0.503$ ) (Figure 3C).

#### Relationship between the T/S values and tumor characteristics

The classification and regression tree technique was used to determine optimal cut-off values ( $\leq 1$  or  $> 1$ ). Telomere length measurement using real-time PCR is rapid and simple and analysis of multiple samples can be performed in a short space of time. Average T/S is expected to be proportional to the average telomere length per cell. Samples with a  $T/S > 1.0$  have an average telomere length greater than that of the standard DNA; samples with a  $T/S < 1.0$  have an average telomere length shorter than that of the standard DNA. We detected the relationship between the value of T/S and tumor characteristics. We found the value of T/S did not significantly differ with tumor stages. The value of  $T/S < 1$  was 16 ( $N = 38$ ) in tumor stage II, and the value of  $T/S < 1$  was 15 ( $N = 28$ ,  $P > 0.05$ )

in tumor stage III. And similar observation was found by comparing tumor metastasis (the value of T/S <1 was 23, N=45) an non-metastasis (the value of T/S <1 was 26, N=55, P>0.05). As the same in tumor COX-2 and P53 stages (Table 1).

## Discussion

There has been great interest in telomere length in colorectal carcinomas and the role of telomere length in tumor is still largely unknown. In this study we demonstrated telomere length in colorectal cancer was shorter than in adjacent carcinoma. In previous studies that also focused on telomere length in colorectal cancer, a significant shortening in the tumor mucosa was also observed when compared with normal mucosa (Engelhardt et al., 1997; Nakamura et al., 2000; Kim et al., 2002; Gertler et al., 2004; Garcia-Aranda et al., 2006). COX-2 is expressed early in colon carcinogenesis and is known to play a crucial role in the progress of colorectal carcinomas. In our research, we also found that the telomere length in tumor COX-2 positive was also shorter than in COX-2 negative. Although some studies find 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 tumor progression is still controversial (Engelhardt et al., 1997). We did not find there was any relationship between telomere length and tumor grades, metastasis, P53 status. However, when we compared telomere length in COX-2 positive expression tumor, the telomere length was shorter in metastasis than in non-metastasis. We thought that COX-2 was relationship with colorectal cancers metastasis and non-metastasis. Nevertheless, we couldn't exclude the possibility that these findings may be because of chance. Further research focus on these aspects is needed to confirm these association and may be contribute to find new anti-cancer diagnostic strategies.

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