THE ROLE OF TELOMERE LENGTH IN THE RISK OF COLORECTAL CANCER INCIDENCES: A COHORT STUDY FROM THE SINGAPORE CHINESE HEALTH STUDY

by

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B.S., Peking University, China, 2015

Submitted to the Graduate Faculty of

Department of Epidemiology

Graduate School of Public Health in partial fulfillment

of the requirements for the degree of

Master of Public Health

University of Pittsburgh

2018

UNIVERSITY OF PITTSBURGH

GRADUATE SCHOOL OF PUBLIC HEALTH

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on

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ABSTRACT

Background: Telomeres are repeated DNA sequences at the end of chromosomes. Each cell division causes telomere shortening. Under normal circumstance, when telomeres are shortened to a critical level, the programmed cell death or apoptosis mechanism is invoked and results in cell death. However, some rare cells, even with extremely short telomeres, escape the apoptosis pathway with sustaining proliferation, a hall marker of cancer. Therefore, telomeres may play an important role in the development and progression of cancer. However, despite the public health significance of colorectal cancer, there is a lack of consensus about the association between telomere length and colorectal cancer.

Objective: The present study was to prospectively examine the association between telomere length and the risk of developing colorectal cancer.

Methods: We obtained blood sample from 26,761 cancer-free participants at baseline in Singapore Chinese Health Study between April 1994 and April 2005. Telomere length in leukocytes was quantified using monochrome qPCR. Until December 31, 2008, 347 subjects developed colorectal cancer (209 colon cancer and 138 rectal cancer). Multivariate Cox proportional hazard regression models were used to estimate hazard ratio (HR) and their 95% confidence interval (CI) of for risk of colon, rectal and colorectal cancer associated with longer telomeres compared with shorter telomeres after adjusting for multiple covariates.

Results: Subjects with highest quartile of telomere length had a statistically significant 55% increased risk of colorectal cancer (HR=1.55, 95% CI: 1.14-2.10) compared with the lowest quartile of telomere length after adjusting for potential confounders (p-trend=0.013). This positive association became stronger (HR=1.70, 95% CI: 1.16-2.48) after excluding patients who were diagnosed with colorectal cancer within the first two years post blood collection (p-trend=0.022). A statistically significant and stronger association between telomere length and risk of colorectal cancer was observed among never smokers and participants without history of diabetes.

Conclusion: This large cohort study built on Singapore Chinese population provides new evidence that longer telomere is associated with a higher risk of colorectal cancer, particularly among those who never smoked cigarettes or had no history of diabetes. Further studies are needed to explain the involved biologic mechanisms.

TABLE OF CONTENTS

LIST OF TABLES

1.0 INTRODUCTION

1.1 TELOMERES

Telomeres are repeated TTAGGG sequences at the end of eukaryotic chromosomes, coated by shelterin proteins (1). These proteins are involved in the process of telomere homeostasis to maintain chromosomal integrity and stability. Telomere repeat-binding factor 1 (TRF1), one of the shelterin proteins, can be bound to long telomeres to prevent excessive lengthening of telomeres by regulating telomerase access and stabilize telomere (2). Telomerase is composed of human telomeric reverse transcriptase (h-TERT) and human telomeric RNA component (h-TERC). As the main positive regulator of telomere length, telomerase can use h-TERC as a template and h-TERT as an enzyme to add telomeric repeats (3). In the process of cell division and aging, telomere length (TL) become shorter due to the end replication problem in most somatic cells that are lack of telomerase. The end replication problem is the shorten on the 5' end of the new DNA due to the lack of RNA primer in each round of cell duplication. Longer TL indicates higher cell duplication capacity, and telomere length erosion, in turn, leads to cell senescence and aging. Thus, telomere shortening was thought to be an important tumor-suppressive mechanism by trigging cell-cycle arrest to stop cell division (4). Many rare monogenic disorders indicate that both short and long telomere length are associated with cancers (5). However, the association between telomere length changes and sporadic cancers onset and progression among general population is still not well known. This association may be distorted by factors affecting telomere length, so it is critical to assess those factors.

Age is the strongest predictor of TL. A longitudinal study conducted by Chen et al. indicated that age-dependent telomere attrition is the major pattern of telomere dynamics in peripheral blood leukocytes (6). There is also a consistent finding that males have significantly

shorter TL than females (6-8). Von Zglinicki reported that oxidative stress accelerates TL erosion (9). However, the findings on the relationship between TL and unhealthy life style, including smoking, obesity, alcohol intake and lack of exercise, which may cause oxidative stress are controversial. One longitudinal study measuring the TL at three-time points among 4576 individuals reported no relationship between telomere attrition and unhealthy life style. In contrast, another longitudinal study conducted among 8074 participants at three-time points indicated that smoking accelerates telomere shortening (7, 8). To assess an unbiased relationship between telomere length and cancer, we may need to adjust for the above factors.

1.2 EPIDEMIOLOGY OF COLORECTAL CANCER

Colorectal cancer (CRC) is the second and third most common cancer among females and males worldwide (age-standardized incidence rates: 34.9 per 100,000 individuals) (10). CRC was also listed among the top 20 causes of death globally and this rank was predicted to increase with time (11). According to patients' family history, colorectal cancer can be divided into sporadic and hereditary. Around three forth of cancer cases are sporadic, and the majority of them are in patients over 50 years old (10). Men have a higher risk of developing CRC than women in most parts of the world (12). Incidence of CRC varies by regions; more-developed countries have a higher morbidity than less-developed countries. In the United States, the average lifetime risk for colorectal cancer is 4.7% in females and 5.0% in males (13). Singapore is a multiethnic country, which consists of 75% Chinese, 13% Malays, 9% Indians and 3% others. Based on the Singapore Cancer Registry Annual Report 2015, colorectal cancer is the most common cancer in Singapore, and Chinese population had the highest risk (age-standardized incidence rates: 41.7 per 100,000 individuals) of developing CRC compared with other ethical groups (14). More than 75% of CRC cases were diagnosed among individuals over 55 years old. Consistent with the global epidemiologic trend, men in Singapore had substantially higher age-standardized incidence rates of CRC (14). In Singapore, the estimated lifetime probability of colorectal cancer is 2.9% for women and 3.9% for men (15).

The global burden of CRC emphasizes the importance of risk factors assessment. Known environmental risk factors included intake of red meat and processed meat, smoking, alcohol intake, low physical activity, obesity and type 2 diabetes. Studies about ethnic disparity in CRC incidence were mainly conducted in the United States (13, 16, 17). Researchers found substantially higher CRC rates among African Americans compared with other racial groups. The above studies concluded that low socioeconomic status, prevalence of obesity, diabetes, and less likely to undergo routine screening are responsible for the racial disparity, which indicates that there is no major difference in CRC risk caused by race itself. Further research about new risk factors will provide us new etiology and potentially new ways of prevention, diagnosis, prognosis or even treatment to the disease.

Singapore has a higher incidence of CRC compared with the worldwide incidence, but a lower lifetime risk than the United States. Overall, the epidemiology of CRC in Singapore is close to the world level, and the high number of cases in Chinese population can enable the study with an appropriate sample size. More importantly, the Singapore Cancer Registry is one of the oldest and well-established registries in the world, and thus, using data from this registry system can reduce bias. Therefore, Singapore can act as a representative population to analyze risk factors of CRC.

As we all know, the endless division of cells is one of the hall markers of cancers, including colorectal cancer (18). The interaction between cell division and telomere length in non-cancer cells from general population can be concluded that: 1) Cells with longer telomere length have stronger potential of duplication compared with shorter telomeres, and, 2) Programmed cell death or apoptosis mechanism is invoked and leads to cell death, when telomeric structure is lost to a critical level during somatic cell division, and, 3) telomere shortening was thought to be an important tumor-suppressive mechanism by trigging cell-cycle arrest to stop cell duplication. With that being the case, extremely long TL is likely to promote cell replication and result in cancerous. Therefore, the patterns of telomere length regulation might be an important mechanism and potential risk factor for cancers. Several studies consistently reported on the level of TRF1 protein and Telomerase activity in tumor tissue during

CRC progression (19-23). However, the association between TL in leukocytes and CRC is still ambiguous.

1.3 PREVIOUS EPIDEMIOLOGICAL STUDIES

We performed a literature search to review all published papers about the relationship between TL and CRC, and summarized them in **Supplemental Table 1**. Telomere lengths were shorter in cancer tissue than in adjacent mucosa, regardless of cancer stage, site and genetic alternation (24) (25). Telomere lengths among different normal tissues including skin, blood, synovium and colon mucosa were significantly positively associated with each other and they performed similar telomere shortening patterns with age in non-cancer individuals (26, 27). However, this common pattern of change in TL is lost among normal colon mucosa, tumor tissue and peripheral blood leukocytes (PBL) in CRC patients (27). In this case, the association between TL from blood cells and CRC may be affected by cancer.

There is a lack of consensus about the relationship between CRC and TL from PBL in retrospective studies. One study based on a Chinese population reported that shorter TL was associated with CRC after adjusting for age, sex, smoking status and alcohol use (28). Pellatt et al. observed shorter TL among colon cancer patients after adjusting for age and sex. However, this association was attenuated in further adjustment for BMI and smoking (29). Boardman el al. analyzed 598 cases and 2212 healthy controls. They found younger individuals (<50 years old) with longer TL or older individuals (>50 years old) with shorter TL were under higher risk of CRC after adjusting for diet, alcohol, tobacco, hormone therapy (among females only), diabetes, family history of CRC, age at blood draw, BMI and medicine use (30). The above case-control studies recruited participants without history of receiving chemotherapy or radiotherapy. One study concluded that the association between shorter TL and CRC risk in retrospective studies becomes weaker in prospective studies, which indicated that CRC occurrence affected telomere length in PBL (31). Therefore, prospective study designs are superior to retrospective study designs to investigate changes in TL before cancer onset.

Lack of consensus also exists among four prospective studies. All published prospective studies used a nested case control design. Two studies were conducted on White females and males separately. Conditional on age, smoking, BMI, alcohol use, exercise and length of follow up, no evidence of association between TL and risk of CRC was found in both genders (32) (33). The other two studies were conducted among Chinese. The Shanghai women's health study reported a higher CRC risk among individuals with longer and shorter TL after adjusting for age, date and time of sample collection, menopausal status, antibiotic use in past week and time interval since last meal (34). Zhang et al. detected an association between shorter TL and CRC risk adjusting for age, sex, smoking status and alcohol use (35). Naing el al. conducted a meta-analysis using the above mentioned seven studies (28-34) in order to measure the relationship between TL in PBL and CRC risk (36). This meta-analysis found no evidence on the association based on a pooled analysis.

It is possible that TL shortening usually occurs after cancer diagnosis, based on the above cross-sectional and retrospective studies. However, the prospective studies on the change of TL prior to CRC onset are still limited according to the inconsistent findings from the above prospective studies. Thus, the primary aim of this study is to prospectively assess the role of TL in the risk of CRC, and we hypothesize that longer TL is associated with a higher risk of CRC. In addition, this study will hopefully shed some light on the role of telomere length as a marker of cancer progression by clearing the TL dynamic patterns in different stages of colorectal cancer.

2.0 MATERIALS AND METHODS

2.1 STUDY POPULATION

A residential cohort of 63257 middle-aged and older (45-74 years) Singapore Chinese men and women was assembled between 1993 and 1998. At recruitment, each study subject was interviewed in person by a trained interviewer using a structured questionnaire that asked for information on demographics, body weight and height, lifetime use of tobacco (cigarettes and water-pipe), current physical activity, menstrual/reproductive history (women only), occupational exposure, medical history, and family history of cancer. Body mass index (BMI) was calculated as the current weight in kilograms divided by height in meters squared. Information on current diet and consumption of beverage was assessed via a 165-item food frequency questionnaire that had been violated against a series of 24-hour dietary interview (37).

Beginning in April 1994, a random 3% of cohort participants were asked to provide blood. Eligibility for this biospecimen subcohort was extended to all surviving cohort participants starting in January 2000. By April 2005, all surviving cohort participants had been contacted for biospecimen donation. 28,346 subjects donated baseline blood samples, representing a consent rate of about 60%. Between July 1999 and December 2003, all surviving cohort subjects were recontacted for a telephone interview to update information on alcohol use, tobacco use, diabetes states, current physical activity, and body weight.

2.2 ASSESSMENT OF COLORECTAL CANCER CASES

Identification of incident colorectal cancer cases and deaths (International Classification of Disease-Oncology codes, 2nd edition-ICD-O2) was accomplished by annual record linkage of all surviving cohort participants with the database of the nationwide Singapore Cancer Registry (38) and the Birth and Death Registry that have complete records of incident cancer and death cases, respectively. The ascertainment of cancer and death incidences among all study participants was virtually complete as to date, only 56 (<0.1%) of entire cohort participants were known to be lost to follow-up due to migration out of Singapore.

By December 31, 2008, 314 developed colorectal cancer among 26,540 subjects after excluding 1,585 participants with a history of cancer at the time of blood collection and 221 subjects without valid genomic DNA results, with a median follow-up of 6.36 years after their donation of blood sample, ranging from 2 days to 14.52 years.

2.3 MEASUREMENT OF LEUKOCYTE TELOMERE LENGTH

Genomic DNA was extracted from peripheral blood using QIAamp 96 DNA Blood kits (Qiagen, Valencia, CA) according to the manufacturer's protocol. Telomere length was measured using a validated monochrome multiplex qPCR method, as described elsewhere (39). Briefly, this method measures the relative average telomere length in genomic DNA by determining the ratio of telomere repeated copy number (T) to single (albumin) gene copy number (S) in experimental samples relative to a reference sample. The DNA sample for the standard curve was composed of an equimolar pool of 77 samples selected from participants of the Singapore Chinese Health Study who were identified in a prior study; the telomere length values of all the 77 samples were within 10% of the population mean. This pooled DNA sample was run on all qPCR plates: 8 replicates for each of four concentrations (4, 0.9, 0.16 and 0.032 ng/µl). Thermal cycling was carried out on an Applied Biosystem 7900 HT instrument, using PCR cycling conditions as described (39). Real-time PCR cycle thresholds, determined

independently for albumin gene (ALB) and telomere (TEL) amplification traces for all wells (experimental and standard DNA samples), were used to calculate telomere length with the 384-well plate-based normalization of telomere length, which was more robust than the overall standard-curve based normalization. All experimental DNA samples were assayed in duplicate, and the average value of the two replicates was used for final analysis for each subject. The mean coefficient of variation, as a measure of reproducibility, of all technical sample duplicates for telomere length in the present study was 3.5%.

2.4 STATISTICAL ANALYSIS

Chi square test and t-test (for non-normally distributed variables, we used non-parametric test) were used to compare the distribution of selected variables between cancer cases and non-cases. The analysis of covariance (ANCOVA) method was used to assess the difference in geometric mean 95% confidence intervals (CI) of telomere length by selected characteristics.

For each participant, person-years at risk was computed from the date of blood draw to the date of colorectal cancer diagnosis, death, migration out of Singapore, or December 31, 2015. We initially stratified age into 8 groups (46-50, 51-55, 56-60, 61-65, 66-70, 71-75, 76-80 and 81-86) to examine the association between age and cancer. Due to small number of cases in the younger age groups, the first four groups were combined and resulted in the following age groups: 46-65, 66-70, 71-75, 75-80 and 81-86 years. Participants were categorized into following body mass index (BMI) groups: underweight (BMI<18.5), normal weight (18.5≤ BMI<23.0), overweight (23.0≤BMI<27.0) and obese (BMI≥27), according to specific BMI cutoff for Asians (40). A new variable-smoking index was created to define participants as: never smokers, light smokers, whose onset smoking age ≥15 years old and cigarettes per day <13, and heavy smokers, whose onset smoking age ≥15 years old and cigarettes per day >13. We categorized telomere length by dividing it into quartiles: Q1 (relative length: 0.19-0.87), Q2 (relative length: 0.87-1.00), Q3 (relative length: 1.00-1.15), and Q4 (relative length: 1.15-3.24).

Multivariate Cox proportional hazard regression models were used to calculate hazard ratio (HR) and the 95% CI of colon, rectal and colorectal cancer. To assess the potential interaction effect of risk factors on the association between TL and hazard of colorectal cancer, we calculated the HR of colon, rectal and colorectal cancer stratified by sex, smoking index, and diabetes status. We subtracted 2 years from the original years of follow-up to conduct sensitivity analysis.

Statistical analyses were carried out by using SAS software 9.4. All P-values are two sided, and those that were less than 0.05 were considered being statistically significant.

3.0 RESULTS

3.1 COHORT CHARACTERISTICS AND THE CHOICE OF CONFOUNDING FACTORS

The distribution of baseline characteristics of cancer cases and non-cancer cases is shown in **Table 1**. There were significant associations between colorectal cancer and older age ($p<0.0001$), male gender ($p<0.0001$), lower education levels ($p=0.0136$), former and current smoking status ($p<0.0001$), heavy smoking ($p<0.0001$), longer years of smoking ($p=0.0049$), higher alcohol intake per day ($p=0.0097$) as well as diabetes ($p=0.0004$). Since age and gender are the two major factors associated with TL erosion, we assessed the relationship between TL with demographics and lifestyle factors conditional on age and sex. The geometric mean of telomere length was inversely associated with age at sample collection (p-trend<0.0001), male gender (p-trend<0.0001), and higher education levels (p-trend=0.0130). Subjects with diabetes ($p=0.0139$) and family history of colorectal cancer (p-trend=0.0381) had a longer geometric mean of TL compared with their counterparts (**Table 2**).

In a multivariate Cos regression model (**Supplemental Table 2**), the hazard ratio of colorectal cancer was significantly associated with age (p-trend<0.0001), sex (p-trend<0.0001), and diabetes (p-trend=0.0049). The number of years of smoking was only significantly associated with rectal cancer (p-trend=0.0423).

Based on the above results, we included the following covariates into fully adjusted models to assess the association between TL and risk of colorectal cancer: age, gender, BMI levels (<18.5, 18.5-<23.0, 23.0-<27.0, ≥27.0), education levels (no formal education, primary,

secondary, and above), smoking index (never smokers, light smokers, and heavy smokers), numbers of alcohol drinks per week (non-drinkers, <7 drinks/week, and ≥7 drinks/week), weekly physical activity, family history of CRC, and diabetic status.

3.2 TELOMERE LENGTH AND COLORECTAL CANCER RISK

There was a significant (p-trend=0.0129) association between colorectal cancer and TL in quartile after adjusted for all potential confounders described above (**Table 3**); participants with the longest telomere length in quartile had a higher risk of developing colorectal cancer (HR=1.55, 95% CI: 1.14-2.10) compared with those in the lowest quartile of TL. The associations for TL with risks of colon and rectal cancer were similar **(Table 3)**. The positive association was present for TL with both colon and rectal cancer, although their trends tests did not reach statistical significant level due to relatively small sample size for each subsite of cancer.

Given that the underlying disease progression might have impact on TL, we conducted sensitivity analysis after excluding patients who were diagnosed with colorectal cancer within the first two years post enrollment. The positive association was present for TL with risk of colorectal cancer (p-trend=0.0220), and colon or rectal cancer individually (**Table 4**).

The hazard ratio of colorectal cancer by TL in quartile stratified by risk factors are shown in **Table 5**. The longest TL in quartile was associated with a higher risk of colorectal cancer among never smokers (HR=1.68, 95% CI: 1.110-2.527, p-trend=0.0148) and subjects with no history of diabetes (HR=1.68, 95% CI: 1.204-2.331, p-trend=0.0052). Similar associations were present between TL and risk of colon or rectal cancer individually among never smokers or participants without history of diabetes, the tests for trend did not reach statistical significance level. The associations between TL and colorectal cancer among diabetics or ever smokers. We examined potential interaction effect of these variables on colorectal cancer risk, but did not find

any significant effect of history of diabetes or smoking with TL on risk of colorectal cancer (both p's for interaction >0.325).

4.0 DISCUSSION

4.1 INTERPRETATION OF CONFOUNDING FACTORS

Similar to previous studies (6-8), we observed that a shorter TL is associated with advanced age and male gender. A higher education level in this population may be a representation of other upstream unmeasured factors such as stress, social economic level and diet to name a few. These factors may participate in the process of accelerating TL shortening (41, 42). The longer telomere length observed among participants with diabetes and family history of CRC may be results of selection bias, that more healthier diabetic patients or subjects with family history of CRC were recruited to the study compared with their inpatient or dead companion, and the healthier individuals may have longer TL. However, we cannot deny the probability that this phenomenon is true.

In the fully adjusted model, we observed older age, male gender and diabetes history to be independent risk factors for the development of colorectal cancer. Interestingly, longer years of smoking was only associated with rectal cancer. Aging is one of the main risk factors of CRC. There are a few mechanisms possibly underlying the link between aging and cancer development (43) : 1) Age-related proliferation of opportunistic bacteria destroying the function and composition of gut microbiome, and 2) Aging-related chronic inflammation in luminal environment. Overall, we found a significant association between male gender and a higher CRC risk in this population. Male gender is a risk factor for many chronic diseases, which may be caused by sex-specific exposure to risk factors and a protective effect of sex hormone (17). However, sex disparities in CRC are complicated. Males and females have different risks for different sites of cancer occurrence and women had worse prognosis compared with age-matched men due to gender-specific differences in cancer screening (44).

A meta-analysis which combined data from case-control and cohort studies reported an increasing colorectal cancer risk among diabetic patients (45). Our study also observed that diabetes was an independent risk factor for colon and rectal cancer. Hyperinsulinemia was proposed to be one of the explanations for a higher CRC risk in diabetes patients (46). Insulin has growth-promoting function, which may facilitate the growth of cancer cells. Unhealthy dietary habits leading to diabetes may also be responsible for the development of colorectal cancer.

Our study found a significant p-trend value between longer years of smoking and rectal cancer in the fully adjusted model, which is consistent with two previously published studies among men (47, 48) . Both studies indicated that smoking was associated with rectal cancer. Two prospective studies in men and women reported a significant association between more than 35 years of smoking and CRC risk (49, 50) . However, based on our data, the HR of rectal cancer among subjects smoking over 40 years was 1.590 and the 95% CI included zero. Our cohort study had smaller total sample size, number of cases and fewer smokers compared with the two prospective studies, which may explain our results. All previous research indicates that years of smoking is a more important indicator of cancer risk rather than a smoking status.

Based on the above results, we can conclude that age, gender, smoking and diabetes acted as strong confounding factors between the association of telomere length with colorectal cancer. In contrast, alcohol intake, weekly physical activity, education levels, family history of CRC, and BMI were not associated with CRC and TL in this data set, indicating that there may be a complicated pathway among the above variables. Therefore, it is important to take all potential confounding factors into consideration.

4.2 RESULTS AND INTERPRETATION

In the present study, we found subjects with the longest TL in quartile had a higher risk of developing CRC in the fully adjusted model. This association became stronger after inhibiting the effect of CRC on TL. The stratification analyses results indicate that the prediction role of longer TL in CRC incidence was stronger among the low risk participants who never smokers or without a history of diabetes. One possible reason might be the bigger sample size and more cases among never smokers and participants without diabetes. In addition, among diabetic patients and smokers, diabetic status and smoking may play more important roles in the process of cancer development compared with the effect of longer TL, which could be another explanation for our observations.

Some studies show that the expression levels of telomeric proteins are altered in tumors and cancer cells. Some low grade gastrointestinal tumors expressed more TRF1 protein (51). Increased telomerase activity (TA) associates with the histological grade and staging of CRC (52). Many studies investigated the association between TL with TA and their prognosis role in CRC patients not receiving chemotherapy or radiation therapy (19-22). They consistently reported: 1) The shorter TL in early-stage tumor mucosa and a longer TL in advanced cancer mucosa, and, 2) Longer TL and positive TA in tumor mucosa is associated with the worse prognosis among CRC patients. Overall, tumor samples showed higher TA and higher TRF1 protein levels compared with adjacent normal mucosa (23). Based on the above results, we can hypothesize that tumors in the early stage have higher division capacity and relatively less telomerase, so the speed of replacing lost telomeric repeats during division cannot catch up with the speed of cell duplication. Therefore, the previous studies observed shorter TL in tumor tissue compared with adjacent mucosa. The second hypothesis is that increased TA with cancer progress leads to a longer TL in higher stage colorectal cancer. In this case, longer TL observed in more advanced stage of CRC can predict a worse prognosis.

This TL dynamic pattern before cancer onset and during cancer progression may partially explain the lack of consensus among published papers, even though telomere dynamics are different in PBL and tumor mucosa after cancer onset. Different patterns of association

between TL and CRC may be caused by the timing of cancer diagnosis in both (and mainly in) retrospective and prospective studies. In prospective studies, the appropriate length of follow-up time and reducing the effect of cancer on TL are two critical factors affecting the assessment of association between TL and CRC incidence. Besides, there are differences in confounding adjustment in the four prospective studies. Some of the previous studies did not adjust for important confounding factors such as diabetes, smoking, and physical activities. This may distort the true association between TL and CRC risk.

4.3 PUBLIC HEALTH IMPORTANCE

Based on the two-stage model of carcinogenesis, Aviv et al. proposed a hypothesis about the role of longer telomeres in development of cancers (53).We would like to use this hypothesis to explain the causal link between TL and CRC. The first stage is the occurrence of single or multiple mutations during stem cells' replication in colon or rectum, which results mutated clones with higher capacity of duplication than adjacent cells. This telomere independent stage may happen in every individual, and the mutations accumulate with age. Individuals with longer telomeres possess stronger potential of cell division, so the mutated clones have higher chance to expand and undergo malignance transformation among subjects with longer TL during the second stage. Since telomere lengths were positively associated among different normal tissues before cancer onset (26, 27), the longer TL in PBL reflects a longer TL in colonic tissue cells. Therefore, longer TL is associated with a higher risk of developing CRC. Based on our findings and the hypotheses built on the previous paper about the change of TA, TRF1 and TL, we may use the combination of changes in expression of TRF1 protein and TA, as well as the TL dynamic patterns as a marker of CRC progression. Individuals with longer TL have a higher chance of developing CRC. Tumor tissue expresses more TRF1 and TA compared with adjacent mucosa. We will observe a shorter TL because of higher levels of TRF1 expression in the early cancer stage and telomere elongation with the increase of TA during cancer progression.

Our study also emphasizes the importance of smoking cessation and diabetes in CRC prevention. Our finding that longer years of smoking is a more significant risk factor for rectal cancer than smoking status should be used for strategies to encourage smokers to quit smoking early on rather than give up trying. Healthy dietary habits and life style actually can also reduce a proportion of new cases of CRC among smokers and diabetes patients, because some of them do not have genetic susceptibility for a longer telomere.

4.4 STRENGTH AND LIMITATION

Our data is derived from a cohort study with a big sample size and long-term follow-up. In the fully adjustment model, we controlled for many common causes of telomere length dynamic and colorectal cancer and tried to meet the condition of exchangeability between cancer cases and non-cases. These are the two major advantages of our analysis compared with the four other prospective studies. However, telomere length is not consistent during the life span of humans. It will change because of cell duplication in every individual. In this case, using the measurement of telomere length at one time point to describe the association between TL and cancer seems not convincing. As previously mentioned, Chen et al. (6) and Huzen et al. (7) reported a similar pattern of leukocytes telomere length (LTL) dynamic that is age-dependent telomere shortening. LTL elongation with age is unlikely to happen, which may be a result of measurement error (6). Therefore, we believe that the observed relationship between TL and CRC is not spurious given that we used reliable TL measurement procedures.

This study has a few limitations. First of all, more cases are needed to increase the power of our study. Secondly, the dynamic of TL indicates that longitudinal study design with multiple measurements of TL over time would be a better choice to fully examine the association between TL and CRC. Third, our cohort population had the oldest mean age of blood extraction compared with other published prospective papers. Even though all studies adjusted for age, there may be unknown downstream confounding factors of age distorting the association between TL and

CRC. Lastly, the Singapore Chinese Health Study is based on a Chinese population, so our result may lack of external validity to different ethnic groups due to unknown racial/ethical differences. As an observational study, this research project still had many unmeasured confounding factors which may distort our interpretation of the relationship between TL and CRC incidence.

In conclusion, we observed that longest telomere length in peripheral blood leukocytes is associated with higher risk of colorectal cancer in the Singapore Chinese Health Study cohort. The increasing trend of developing CRC among longer TL participants is more significant among never smokers and individuals without diabetes.

APPENDIX: TABLES

Table 1. Characteristics of colorectal cancer cases and non-cases: The Singapore Chinese Health Study 1993-2008

Characteristics	Cancer cases	Non-cases	P-value
Number of subjects	347	26193	
Mean age (SD), years	67.15 (7.94)	62.76 (7.62)	<.0001
Gender, %			<.0001
Female	40.63	54.08	
Male	59.37	45.92	
Mean BMI (SD), kg/m^2	23.05 (3.44)	23.26 (3.51)	0.2725
Level of education, %			0.0136
No formal education	23.92	20.78	
Primary (1-6 years)	49.57	45.28	
Secondary and above	26.51	33.94	
Smoking Index, %			<.0001
Never smokers	55.91	68.06	
Light smokers	12.97	9.18	
Heavy smokers	31.12	22.76	
Median cigarettes/day (25%, 75%)*	15 (8, 20)	15 (9, 20)	0.7213
Median years of smoking (25%, 75%)*	38 (28, 48)	36 (25, 44)	0.0049
Median pack-years of smoking (25%, 75%)*	25 (12, 43)	24 (10.5, 42)	0.4010
No. of alcohol drinks per week, %			0.5018
Non-drinkers	79.83	81.32	
<7 drinks/week	14.41	14.21	
≥7 drinks/week	5.76	4.47	
Median alcohol intake (g) per day (25%, 75%)*	5.76 (1.92, 13.50)	3.85 (0.90, 10.33)	0.0097
Weekly physical activity, %			0.5043
No	65.99	64.27	
Yes	34.01	35.73	
Family history of CRC cancer, %			0.2171
No	96.25	97.33	
Yes	3.75	2.67	
Diabetes, %			0.0004
No	87.32	92.38	
Yes	12.68	7.62	

* Only among smokers and alcohol drinkers

Table 2. Distribution of telomere length by selected demographics factors of study participants: The Singapore Chinese Health Study 1993-2008

Characteristics	N	Geometric means of TL (95% CI)*	P for trend*
Age (year)			
46-50	312	1.131 (1.105, 1.158)	***<.0001***
51-55	5062	1.061 (1.055, 1.067)	
56-60	6002	1.034 (1.029, 1.0410)	
61-65	5882	0.992 (0.987, 0.997)	
66-70	4523	0.959 (0.952, 0.965)	
71-75	2916	0.925 (0.918, 0.932)	
76-80	1540	0.900 (0.891, 0.910)	
81-86	300	0.866 (0.845, 0.887)	
Gender			
Female	14306	1.017 (1.013, 1.020)	***<.0001***
Male	12234	0.970 (0.967, 0.974)	
BMI levels			
Underweight (<18.5)	960	0.988 (0.978, 0.997)	0.3713
Normal weight (18.5-<23.0)	12034	0.995 (0.992, 0.999)	
Overweight (23.0-<27.0)	9945	0.996 (0.992, 1.001)	
Obese (27.0+)	3601	0.995 (0.988, 1.002)	
Levels of education			
No formal education	5526	1.001 (0.995, 1.008)	***0.0130***
Primary (1-6 years)	12032	0.995 (0.992, 0.999)	
Secondary and above	8982	0.991 (0.987, 0.996)	
Smoking Index, %			
Never smokers	18021	0.996 (0.992, 0.999)	0.3683
Light smokers	2449	0.999 (0.990, 1.007)	
Heavy smokers	6070	0.992 (0.986, 0.998)	

Table 2 Continued

Number of alcohol drink per week			
Non-drinkers	21577	0.994 (0.992, 0.997)	0.8229
<7 drinks/week	3772	1.003 (0.996, 1.010)	
≥7 drinks/week	1191	0.985 (0.973, 0.997)	
Weekly physical activity			
No	17062	0.995 (0.992, 0.998)	0.8580
Yes	9478	0.996 (0.991, 0.999)	
Family history of CRC			
No	25828	0.995 (0.992, 0.997)	***0.0381***
Yes	712	1.012 (0.996, 1.027)	
Diabetes			
No	24499	0.994 (0.992, 0.997)	***0.0139***
Yes	2041	1.006 (0.997, 1.016)	

*Adjusted for age and gender except for age, which was adjusted for sex only, and for sex, which was adjusted for age only.

Table 3. Hazard ratio of colorectal cancer by telomere length in quartile: The Singapore Chinese Health Study 1993-2008

Relative telomere length in quartile	Person years	Colorectal cancer		Colon cancer		Rectal cancer	
		Cancer cases	HR (95% CI)*	Cancer cases	HR (95% CI)*	Cancer cases	HR (95% CI)*
Q1 (0.19-0.87)	40198.76	89	1.00	58	1.00	31	1.00
Q2 (0.87-1.00)	40986.18	93	1.257 (0.938, 1.684)	52	1.081 (0.742, 1.575)	41	1.594 (0.997, 2.549)
Q3 (1.00-1.15)	42376.24	77	1.179 (0.865, 1.607)	46	1.082 (0.730, 1.603)	31	1.365 (0.824, 2.262)
Q4 (1.15-3.24)	43012.7	88	***1.549 (1.140, 2.104)***	53	1.452 (0.985, 2.139)	35	***1.742 (1.054, 2.878)***
P for trend*			***0.0128***		0.0847		0.0649

*Adjusted for: age, gender, BMI levels (<18.5, 18.5-<23.0, 23.0-<27.0, ≥27.0), education levels (no formal education, primary, secondary, and above), smoking index (never smokers, light smokers, and heavy smokers), numbers of alcohol drinks per week (non-drinkers, <7 drinks/week, and ≥7 drinks/week), weekly physical activity, family history of CRC, and diabetic status.

Table 4. Hazard ratio of colorectal Cancer by telomere length in quartile after excluding cases in the first two years: The Singapore Chinese Health Study 1993-2008

Relative telomere length in quartile	Person years	Colorectal cancer		Colon cancer		Rectal cancer	
		Cancer cases	HR (95% CI)*	Cancer cases	HR (95% CI)*	Cancer cases	HR (95% CI)*
Q1 (0.19-0.87)	27202.2	54	1.00	34	1.00	20	1.00
Q2 (0.87-1.00)	270906.39	67	1.454 (1.014, 2.085)	38	1.310 (0.823, 2.087)	29	1.702 (0.960, 3.019)
Q3 (1.00-1.15)	28293.43	49	1.186 (0.801, 1.756)	31	1.194 (0.728, 1.958)	18	1.175 (0.616, 2.240)
Q4 (1.15-3.24)	29909.39	64	***1.701 (1.166, 2.484)***	35	1.526 (0.934, 2.493)	28	***2.004 (1.103, 3.642***)
P for trend*			***0.0220***		0.1324		0.0745

*Adjusted for: age, gender, BMI levels (<18.5, 18.5-<23.0, 23.0-<27.0, ≥27.0), education levels (no formal education, primary, secondary, and above), smoking index (never smokers, light smokers, and heavy smokers), numbers of alcohol drinks per week (non-drinkers, <7 drinks/week, and ≥7 drinks/week), weekly physical activity, family history of CRC, and diabetic status.

Table 5. Hazard ratio of colorectal cancer by telomere length in quartile stratified by selected characteristics: The Singapore Chinese Health Study 1993-2008

Relative telomere length in quartile	Person years	Colorectal cancer			Colon cancer			Rectal cancer		
		Cancer cases	HR (95% CI)*	P for trend*	Cancer cases	HR (95% CI)*	P for trend*	Cancer cases	HR (95% CI)*	P for trend*
Female										
Q1 (0.19-0.87)	18864.53	29	1.00	0.0610	22	1.00	0.4083	7	1.00	0.0425
Q2 (0.87-1.00)	21812.32	33	1.141 (0.691, 1.882)		21	0.957 (0.525, 1.743)		12	1.724 (0.677, 4.391)	
Q3 (1.00-1.15)	24124.01	37	1.315 (0.804, 2.151)		22	1.031 (0.567, 1.877)		15	2.212 (0.894, 5.476)	
Q4 (1.15-3.24)	27221.46	42	1.541 (0.947, 2.506)		26	1.262 (0.703, 2.267)		16	2.419 (0.976, 5.992)	
Male										
Q1 (0.19-0.87)	21334.24	60	1.00	0.0975	36	1.00	0.1197	24	1.00	0.4548
Q2 (0.87-1.00)	19173.86	60	1.327 (0.925, 1.902)		31	1.166 (0.719, 1.891)		29	1.574 (0.913, 2.714)	
Q3 (1.00-1.15)	17252.23	40	1.069 (0.713, 1.604)		24	1.097 (0.650, 1.852)		16	1.037 (0.546, 1.969)	
Q4 (1.15-3.24)	15791.24	46	1.539 (1.034, 2.291)		27	1.607 (0.959, 2.695)		19	1.469 (0.787, 2.742)	
Never smokers										
Q1 (0.19-0.87)	25108.77	43	1.00	***0.0148***	31	1.00	0.1079	12	1.00	0.0520
Q2 (0.87-1.00)	27806.54	47	1.185 (0.782, 1.796)		29	1.030 (0.619, 1.713)		18	1.593 (0.765, 3.317)	
Q3 (1.00-1.15)	29655.02	47	1.265 (0.831, 1.925)		31	1.189 (0.717, 1.972)		16	1.473 (0.690, 3.144)	
Q4 (1.15-3.24)	32272.77	57	***1.675 (1.110, 2.527)***		35	1.478 (0.894, 2.442)		22	**2.188 (1.056, 4.532)**	
Ever smokers										
Q1 (0.19-0.87)	15089.99	46	1.00	0.3070	27	1.00	0.4303	19	1.00	0.5079
Q2 (0.87-1.00)	13179.64	47	1.364 (0.904, 2.059)		23	1.178 (0.673, 2.062)		23	1.650 (0.895, 3.042)	
Q3 (1.00-1.15)	11721.22	47	1.083 (0.680, 1.724)		15	0.937 (0.495, 1.772)		15	1.303 (0.657, 2.587)	
Q4 (1.15-3.24)	10739.93	57	1.402 (0.876, 2.243)		18	1.449 (0.783, 2.683)		13	1.354 (0.654, 2.804)	
No history of diabetes										
Q1 (0.19-0.87)	37021.18	75	1.00	***0.0052***	50	1.00	0.0542	25	1.00	***0.0367***
Q2 (0.87-1.00)	38082.02	82	1.324 (0.966, 1.815)		45	1.088 (0.725, 1.632)		37	1.807 (1.085, 3.012)	
Q3 (1.00-1.15)	38314.48	68	1.267 (0.907, 1.768)		41	1.135 (0.745, 1.727)		27	1.540 (0.887, 2.675)	
Q4 (1.15-3.24)	40058.82	78	***1.675 (1.204, 2.331)***		48	1.538 (1.017, 2.326)		30	***1.963 (1.131, 3.407)***	
Had a history of diabetes										
Q1 (0.19-0.87)	3177.58	14	1.00	0.8299	8	1.00	0.8185	6	1.00	0.9512
Q2 (0.87-1.00)	2904.16	11	0.797 (0.360, 1.765)		7	0.924 (0.333, 2.562)		4	0.651 (0.181, 2.347)	
Q3 (1.00-1.15)	3061.77	9	0.702 (0.300, 1.643)		5	0.720 (0.230,2.249)		4	0.676 (0.187, 2.438)	
Q4 (1.15-3.24)	2953.88	10	0.927 (0.405, 2.124)		5	0.922 (0.295, 2.883)		5	0.947 (0.281, 3.197)	

*Adjusted for: age, gender, BMI levels (<18.5, 18.5-<23.0, 23.0-<27.0, ≥27.0), education levels (no formal education, primary, secondary, and above), smoking index (never smokers, light smokers, and heavy smokers), numbers of alcohol drinks per week (non-drinkers, <7 drinks/week, and ≥7 drinks/week), weekly physical activity, family history of CRC, and diabetic status.

Supplemental Table 1. Summary of results from previous epidemiological studies on telomere length and risk of colorectal cancer up to November 2017

Study Design	#Cases/ #Control (Follow-up time)	DNA Sources	Methods of TL measurement	Findings	Reference
Case-control	148 tumor tissue/ adjacent non-cancer tissue	Tumor and adjacent mucosa	PCR	Telomeres were shorter in CRCs than in adjacent tissues, regardless of tumor stage and grade, site, or genetic alternations.	(24)
Case-control	118 tumor tissue/ adjacent non-cancer tissue	Tumor and adjacent mucosa	Real-time PCR	Telomeres were significantly shorter in CRCs than in adjacent tissues, regardless of tumor stage and grade, site, or genetic alternations.	(25)
Case-control	628 newly diagnosed CRC cases/ 1256 age and sex frequently matched controls	PBL*	Real-time PCR	Shorter telomere was significantly associated with CRC risk. Adjusted for: age, sex, smoking status and alcohol use.	(28)
Case-control	249 colon cancer cases/ 371 controls; 276 rectal cancer cases/ 372 controls	PBL*	qPCR	Longer TL was significantly associated with reduced colon cancer risk after adjusting for age and sex. This significant association attenuated in further adjustment for BMI and smoking.	(29)
Case-control	598/2212	PBL*	qPCR	Younger (<50) individuals with longer TL or older (>50) individuals with shorter TL at higher risk for CRC. Adjusted for: fruit, vegetable, and red meat consumption, alcohol, tobacco and hormone therapy, diabetes, family history of CRC, age at blood draw, BMI and medicine use.	(30)
Prospective nested case-control	134/357 matched White females (1993-Dec. 2005)	PBL*	qPCR	No evidence for an association of $\log_e$-TSRs with CRC risk. Adjusted by BMI, randomized treatment group, presence of colorectal polyps, alcohol use, exercise, postmenopausal status, and hormone therapy use.	(32)
Prospective nested case-control	191/ 306 matched White males (1982-Dec. 2005)	PBL*	qPCR	No evidence for an association of the observed T/S ratio with CRC risk. Adjusted by randomized treatment group, BMI, alcohol use, and exercise.	(33)

Supplemental Table 1 Continued

Prospective nested case-control	441/ 549 matched Chinese females (Enrolled between: Dec.1996-May 2000; Ended in Dec. 2009)	PBL*	qPCR	A U-shaped association: the lowest risk was observed in women with TSR in the third quintile, and risks were elevated with a shorter or longer TL. Adjusted by matched sets: age, date and time of sample collection, antibiotic use in past week and time interval since last meal.	(34)
Prospective nested case-control	300 cases/ 900 controls (June 2010-Jan 2015)	PBL*	Real Time PCR	Shorter TL was associated with CRC risk. Adjusting for age, sex, smoking status and alcohol use.	(35)
Meta-analysis	3 prospective studies, 3 retrospective studies. 1 mix design	PBL*	PCR	The current analysis is insufficient to provide evidence on the relationship between PBL telomere length and the risk of CRC.	(36)

*PBL: Peripheral Blood Leukocytes

Supplemental Table 2. Hazard ratio of colorectal cancer by selected characteristics: The Singapore Chinese Health Study 1993-2008

Characteristics	Person years	Colorectal Cancer		Colon Cancer		Rectal Cancer	
		Cancer cases	HR (95% CI)*	Cancer cases	HR (95% CI)*	Cancer cases	HR (95% CI)*
Age							
46-65	110108.17	142	1.00	81	1.00	61	1.00
66-70	28029.03	82	2.042 (1.543, 2.701)	51	2.319 (1.617, 3.326)	31	1.697 (1.088 2.648)
71-75	17413.11	67	2.628 (1.942, 3.556)	43	3.124 (2.125, 4.593)	24	2.029 (1.242, 3.316)
76-80	8552.32	46	3.754 (2.664, 5.289)	28	4.252 (2.732, 6.626)	18	3.180 (1.848, 5.469)
81-86	1471.26	10	4.686 (2.449, 8.969)	6	5.247 (2.266, 12.146)	4	3.180 (1.448, 11.22)
P trend*			***<.0001***		***<.0001***		***<.0001***
Gender							
Female	92022.31	141	1.00	91	1.00	50	1.00
Male	73551.57	206	1.668 (1.264, 2.202)	118	1.565 (1.101, 2.223)	88	1.857 (1.182, 2.919)
P trend*			***0.0001***		***0.0080***		***0.0051***
BMI levels							
Underweight (<18.5)	5923.81	13	1.154 (0.782, 1.704)	4	0.973 (0.570, 1.659)	9	1.448 (0.817, 2.567)
Normal weight (18.5-<23.0)	75273.67	160	1.00	99	1.00	61	1.00
Overweight (23.0-<27.0)	61857.05	129	1.010 (0.803, 1.295)	78	1.004 (0.739, 1.364)	51	1.044 (0.712, 1.530)
Obese (27.0+)	22519.36	45	1.046 (0.747, 1.465)	28	1.058 (0.690, 1.623)	17	1.026 (0.594, 1.771)
P trend*			0.8495		0.8151		0.5429
Levels of education							
No formal education	34636.89	83	1.00	49	1.00	34	1.00
Primary (1-6 years)	74977.82	172	0.988 (0.744, 1.311)	105	1.101 (0.763, 1.587)	67	0.839 (0.536, 1.314)
Secondary and above	55959.18	92	0.862 (0.615, 1.207)	55	0.953 (0.616, 1.473)	37	0.740 (0.436, 1.258)
P trend*			0.3403		0.7639		0.2549
Smoking Index							
Never smokers	114843.10	194	1.00	126	1.00	68	1.00
Light smokers	14611.81	45	1.153 (0.815, 1.633)	25	0.995 (0.629, 1.573)	20	1.442 (0.842, 2.468)
Heavy smokers	36118.97	108	1.103 (0.836, 1.455)	58	0.925 (0.643, 1.329)	50	1.425 (0.923, 2.200)
P trend*			0.4487		0.7089		0.1044
Years of smoking							
Never smoker	14843.10	194	1.00	126	1.00	68	1.00
0-<20	8958.92	18	0.859 (0.522, 1.415)	11	0.821 (0.435, 1.552)	7	0.927 (0.415, 2.072)
20-<40	23467.63	66	1.242 (0.911, 1.693)	36	1.066 (0.708, 1.604)	30	1.558 (0.963, 2.520)
≥40	18304.24	69	1.104 (0.804, 1.518)	36	0.884 (0.580, 1.349)	33	1.518 (0.930, 2.479)
P trend*			0.2841		0.7688		***0.0423***

Supplemental Table 2 Continued

Pack-year of smoking							
Never smoker	114848.14	194	1.00	126	1.00	68	1.00
<20	21728.18	61	1.163 (0.853, 1.584)	36	1.064 (0.715, 1.582)	25	1.344 (0.820, 2.202)
20-<40	15378.11	46	1.110 (0.780, 1.580)	23	0.867 (0.536, 1.404)	23	1.555 (0.917, 2.639)
40-<60	7801.65	25	1.039 (0.663, 1.629)	11	0.706 (0.368, 1.355)	14	1.647 (0.872, 3.111)
≥60	5817.8	21	1.074 (0.661, 1.744)	13	1.023 (0.553, 1.893)	8	1.166 (0.530, 2.565)
P trend*			0.6598		0.5728		0.1792
No. of alcohol drink per week							
Non-drinkers	134724.65	277	1.00	168	1.00	109	1.00
<7 drinks/week	23661.23	50	1.045 (0.769, 1.420)	30	1.071 (0.720, 1.591)	20	1.009 (0.621, 1.640)
≥7 drinks/week	7188	20	1.182 (0.742, 1.882)	11	1.177 (0.630, 2.198)	9	1.185 (0.590, 2.379)
P trend*			0.4877		0.5597		0.7024
Weekly physical activity							
No	106195.96	229	1.00	140	1.00	89	1.00
Yes	59377.92	118	0.870 (0.693, 1.093)	69	0.835 (0.621, 1.123)	49	0.923 (0.646, 1.320)
P trend*			0.2172		0.2246		0.6404
Family history of CRC							
No	161100.32	334	1.00	201	1.00	133	1.00
Yes	4473.56	13	1.612 (0.924, 2.812)	8	1.664 (0.818, 3.381)	5	1.562 (0.637, 3.831)
P trend*			0.0931		0.1610		0.3219
Diabetes							
No	153476.5	303	1.00	184	1.00	119	1.00
Yes	12097.39	44	1.580 (1.149, 2.172)	25	1.459 (0.958, 2.221)	19	1.784 (1.095, 2.905)
P trend*			***0.0049***		0.0783		***0.0201***

*Adjusted for: age, gender, BMI levels (<18.5, 18.5-<23.0, 23.0-<27.0, ≥27.0), education levels (no formal education, primary, secondary, and above), smoking index (never smokers, light smokers, and heavy smokers), numbers of alcohol drinks per week (non-drinkers, <7 drinks/week, and ≥7 drinks/week), weekly physical activity, family history of CRC, and diabetic status

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