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ORIGINAL ARTICLE

Loss of the Association between Telomere Length and Mitochondrial DNA Copy Number Contribute to Colorectal Carcinogenesis

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Abstract Positive association between telomere length and mitochondrial DNA (mtDNA) copy number were introduced in healthy and patients with psychiatric disorder. Based on frequent genetic changes of telomere and mitochondria in colorectal carcinomas (CRC), we studied their clinical characteristics and their association in colorectal carcinogenesis. DNA was extracted from 109 CRCs, 64 colorectal tubular adenomas (TAs), and 28 serrated polyps (SPs), and then, telomere length and mtDNA copy number were analyzed in these legions by using a real-time PCR assay. Telomere length and mtDNA copy number (mean ± S.D) in CRCs was 1.87 ± 1.52 and 1.61 ± 1.37, respectively. In TAs and SPs, relative mtDNA copy number was 0.92 ± 0.71 and 1.84 ± 1.06, respectively, shoing statistical difference ($p = 0.017$). However, telomere length was similar in these precancerous legions. Telomere length and mtDNA copy number did not show clinical and prognostic values in CRCs, however, positive correlation between telomere length and mitochondrial DNA copy number were found in CRC ($r = 0.408$, $p < 0.001$). However, this association was not shown in precancerous lesions ($r = -0.031$, $p = 0.765$). This result suggests that loss of co-regulation between telomeres and mitochondrial function may induce the initiation or play a role as trigger factor of colorectal carcinogenesis.

Keywords Colorectal cancer · Mitochondria DNA copy number · Serrated polyps · Telomere · Tubular adenomas

Introduction

Colorectal cancer (CRC) is common throughout the world, and two pathways of its carcinogenesis have been identified as chromosomal instability and microsatellite instability (MSI or nMSI) [1–3]. These steps were initiated from tubular adenoma (TA) to CRC, however, recent proposals have suggested that serrated polyp (SP) may be precursors of CRC [4, 5]. It may progress through a serrated neoplastic pathway characterized by frequent *BRAF* mutation. Though details on the molecular mechanism of such progression remain unclear, precancerous legions of CRC as TA and SP may have different genetic background and carcinogenesis process.

Telomeres, composed of 6-bp TTAGGG repeat sequences, are the nucleoprotein complexes capping the each end of the eukaryotic chromosome [6]. Mitochondrial DNA (mtDNA) differs from nuclear DNA, and multiple copies of mtDNA are present in each mitochondrion. Previous studies showed that telomere length (TL) and copy number of mtDNA (mtCN) was associated with many disease especially cancers [7–12]. In some cancers, their changes included clinical or prognostic significances suggesting their early and important effect on carcinogenesis. Interestingly, recent study showed that telomere length and mitochondrial DNA copy number were positively correlated in healthy people and pregnant women [13–15]. However, their

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relationship has not been studied in cancerous or precancerous legions until now. In our previous study [16], we demonstrated the characteristics of mtCN in colorectal precancerous legions, however, mtCN and telomere in CRC were not studied.

In the present study, TL and mtCN was evaluated in CRCs and their precancerous legions comprising of TAs and SPs. To contribute to a better understanding of colorectal carcinogenesis, not only the association of TL and mtCN but also clinicopathological and molecular genetic characteristics were analyzed. This study may help in establishing better treatment of CRCs by identifying the role of mtDNA and telomere in colorectal carcinogenesis.

Materials and Methods

Patients and DNA Extraction

Altogether, 109 patients with CRC were included by the Keimyung Human Bio-Resource Bank at Dongsan Medical Center in this study. Preoperative chemoradiotherapy, previous history of surgical resection for CRCs, HNPCC (hereditary nonpolyposis coli cancer) and FAP (familial polyposis coli) patients were excluded from the study.

To obtain data on the precancerous lesions, pathology records and histological slides of colonoscopic polypectomies between 1999 and 2003 were reviewed. All of the pathologic specimens were reviewed by two gastrointestinal pathologists (Hwang and Kang). As a result, our study was consisted of 64 TAs and 28 SPs. SPs included serrated adenomas or hyperplastic polyps. Among SPs, mixed form and traditional serrated adenomas were excluded. The study was approved by the Institutional Review Board at Dongsan Medical Center (IRB No.10–157).

Tumor area and adjacent normal mucosa were selected from slide by microdissection under a light microscope. Then, the selected paraffin embedded tissues were used for DNA extraction. Using DNA extraction Kit (BioSewoom, Korea).

Telomere Length and Mitochondrial Copy Number

The relative telomere length (TL) and mitochondrial copy number (mtCN) were analyzed by quantitative real-time PCR. For the quantitative determination of TL and mtCN (T) relative to ß-globin (nDNA, S), primers for specific amplification of telomere and COX I were selected according to previous study, respectively [16, 17]. Real-time PCR was then carried out on a LightCycler 480 II system (Roche Diagnostics, Germany). Relative TL and mtCN were calculated using T/S values as following formula: $T/S = 2^{\Delta Ct}$, where ΔCt = average $Ct_{telomere\ or\ COX\ I}$ - average $Ct_{ß\text{-}globin}$. Each measurement was repeated in triplicate and 5 serially diluted control samples with standard curve were included in each experiment.

KRAS and BRAF Mutations

KRAS mutations (codons 12 and 13) and the *BRAF* V600E mutation were analyzed by pyrosequencing (PyroMark Q24, Sweden). The primers for pyrosequencing were used as described previously [18]. The pyrosequencing reaction was performed on a PyroMark Q24 instrument using Pyro Gold Q24 reagents (Qiagen, Netherlands). The pyrosequencing primers were used at a final concentration of 0.3 μmol/L. The resulting data were analyzed and quantified using PyroMark Q24 software version 2.0.6 (Qiagen, Netherlands), which identifies the presence of a specific mutation and its percentage, was used for analyzing of pyrosequencing results.

Statistical Analysis

The SPSS statistical package, version 19.0 for Windows, was used for all statistical analyses. Telomere length and mitochondrial copy number are presented as mean ± standard deviation (SD). To further explore the correlation between these markers and the clinicopathological variables, we calculated the ratio of telomere length and mitochondrial copy number in tumors to that in paired normal tissues. And then, patients were categorized into two subgroups according to their median value. Chi-square, Fischer' exact tests, Mann Whitney U test, and Spearman correlation analysis were used to analyze the relationship between variables. Pearson correlation coefficients were calculated to evaluate the relationships between TL and mtCN.

Survival curves, estimated with the Kaplan–Meier method (Univariate analysis), were compared by log-rank test. Overall survival (OS) was defined as the time between diagnosis and either death from disease or death from other causes. A two-tailed probability <0.05 was required for statistical significance.

Results

TL and mtCN were successfully analyzed in 109 CRCs (67.9 ± 13.11 years of age), 64 TAs (60.71 ± 11.67 years of age), and 28 SPs (58.52 ± 11.84 years of age) by using real time PCR. Relative mtCN and TL (mean ± S.D) were

Table 1 Summary of molecular genetic analysis in CRCs, TAs, and SPs

	CRCs	Precancerous legion	
		TAs	SPs
Telomere length (mean ± S.D.)	1.87 ± 1.52	1.18 ± 0.94	1.37 ± 1.13
MtCN (mean ± S.D.)*	1.61 ± 1.37	0.92 ± 0.71	1.84 ± 1.26

MtCN, mitochondrial copy number

*TAs vs. SPs, $p = 0.017$

Table 2 Clinicopathological characteristics of CRCs according to telomere status

	Total (n)	Telomere length (%, n)			Mitochondrial content (%, n)		
		Short	Maintain	P	Lower	High	P
Total	109	37.6 (41)	62.4 (68)		40.4 (44)	59.6 (65)	
Gender				0.14			0.50
Male	62	43.5 (27)	56.5 (35)		40.3 (25)	59.7 (37)	
Female	47	29.8 (14)	70.2 (33)		34.0 (16)	66.0 (31)	
Age				0.39			0.82
< 69 (median)	58	41.4 (24)	58.6 (34)		41.4 (24)	58.6 (34)	
> 69 (median)	51	33.3 (17)	66.7 (34)		39.2 (20)	60.8 (31)	
Location				0.19			0.10
Colon	42	45.2 (19)	54.8 (23)		50.0 (21)	50.0 (21)	
Rectal	67	32.8 (22)	67.2 (45)		34.3 (23)	65.7 (44)	
T stage				0.49			0.76
T1/2	39	33.3 (13)	66.6 (26)		38.5 (15)	61.5 (24)	
T3/4	70	40.0 (28)	60.0 (42)		41.4 (29)	58.6 (41)	
Differentiation				0.47			0.26
Well/Moderate	101	36.6 (37)	63.4 (64)		38.6 (39)	61.4 (62)	
Poor/Undifferentiated	8	50.0 (4)	50.0 (4)		62.5 (5)	37.5 (3)	
KRAS mutation				0.72			0.97
(+)	20	35.0 (7)	65.0 (13)		40.0 (8)	60.0 (12)	
(−)	89	39.3 (35)	60.7 (54)		40.4 (36)	59.6 (53)	
BRAF mutation				1.00			0.22
(+)	6	50.0 (3)	50.0 (3)		66.7 (4)	33.3 (2)	
(−)	103	44.7 (46)	55.3 (57)		38.8 (40)	61.2 (63)	
Vascular invasion				0.35			0.015
(+)	34	44.1 (15)	55.9 (19)		23.5 (8)	76.5 (26)	
(−)	75	34.7 (26)	65.3 (49)		48.0 (36)	52.0 (39)	
CEA				0.17			0.79
< 13.5 ng/ml (median)	71	42.2 (30)	57.8 (41)		39.4 (28)	60.6 (43)	
> 13.5 ng/ml (median)	38	28.9 (11)	71.1 (27)		42.1 (16)	57.9 (22)	

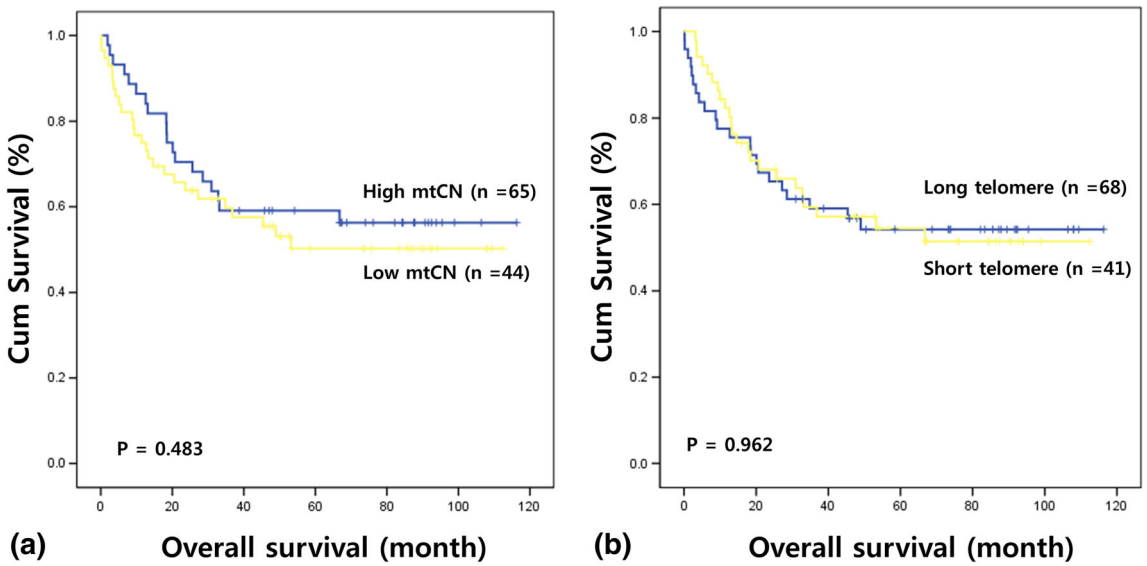

Fig. 1 Kaplan-Meier plots showing overall survival analysis in colorectal cancers according to mitochondrial copy number (**a**) and telomere length (**b**)

summarized in Table 1. mtCN was significantly higher in SPs than TAs, (p = 0.017), however, there was no difference between CRCs and precancerous legions (TAs and SPs). Shortening of TL was found in precancerous legions compared to CRCs, however, it did not have significant difference (p = 0.237).

Clinicopathological characteristics of TL and mtCN in CRCs were presented in Table 2. These markers were not associated with gender, age, location, invasion depth, pathological differentiation, and CEA. However, higher frequency of vascular invasion was shown in CRCs with high mitochondrial content (p = 0.015). CRCs with higher TL and mtCN were found more in colon than rectum, however, it did not get statistical significance (p = 0.10 and 0.19, respectively). Other clinicopathological characteristics were not associated with TL and mtCN. In precancerous legions, KRAS and BRAF mutations were analyzed. KRAS mutation was more frequent in TAs (18.7%, 12/64) than SPs (3.6%, 1/28). However, BRAF mutation was found in only SPs (21.4%, 6/28), therefore, KRAS and BRAF mutations were mutually exclusive in TAs and SPs (p < 0.0001).

We then assessed the survival analysis in CRCs to assess the prognostic value of TL and mtCN. The median follow-up of patients for survival analysis was 47.5 months (1–113). Kaplan-Meier curve revealed OS was not associated with both mtCN (p = 0.483) and TL (p = 0.962, Fig. 1). When stratifying for the variables, they seemed to confer no significant prognostic value statistically.

And then, correlation between TL and mtCN were analyzed in CRCs and their precancerous legions (Fig. 2). In agreement with previous studies, TL and mtCN was correlated with each other in normal tissues of CRCs and their precancerous legions (r = 0.271, p = 0.005). Interestingly, a positive association was also found in CRCs (r = 0.408, p < 0.001). However, it did not found in precancerous legions (r = −0.031, p = 0.765). When stratifying them into TAs and SPs, mtDNA copy number and telomere length did not show a correlation statistically.

Discussion

This study showed that telomere length (TL) was positively correlated with mtDNA copy number (mtCN) in the tissue from normal and colorectal cancers (CRCs), and it was in agreement with previous studies in healthy peoples. This association was firstly introduced by Kim et al. [13] in 129 women over the age of 60 years. Recent studies also demonstrated an association between TL and mtCN in healthy adults and pregnant women [14, 15]. And neuropsychiatric conditions were associated with shorter TL and higher mtCN, however, detail data about their association was not shown [19]. Recently, Bao et al. [20] also demonstrated this correlation in 250 hepatocellular carcinoma patients (r = 0.165, p = 0.029). Based on these previous reports and our results, we assumed that there are positive correlation between telomeres and mitochondria not only in healthy peoples and but also in malignant condition.

To clarify the role of telomere and mitochondrial DNA in colorectal carcinogenesis, we also investigated their content

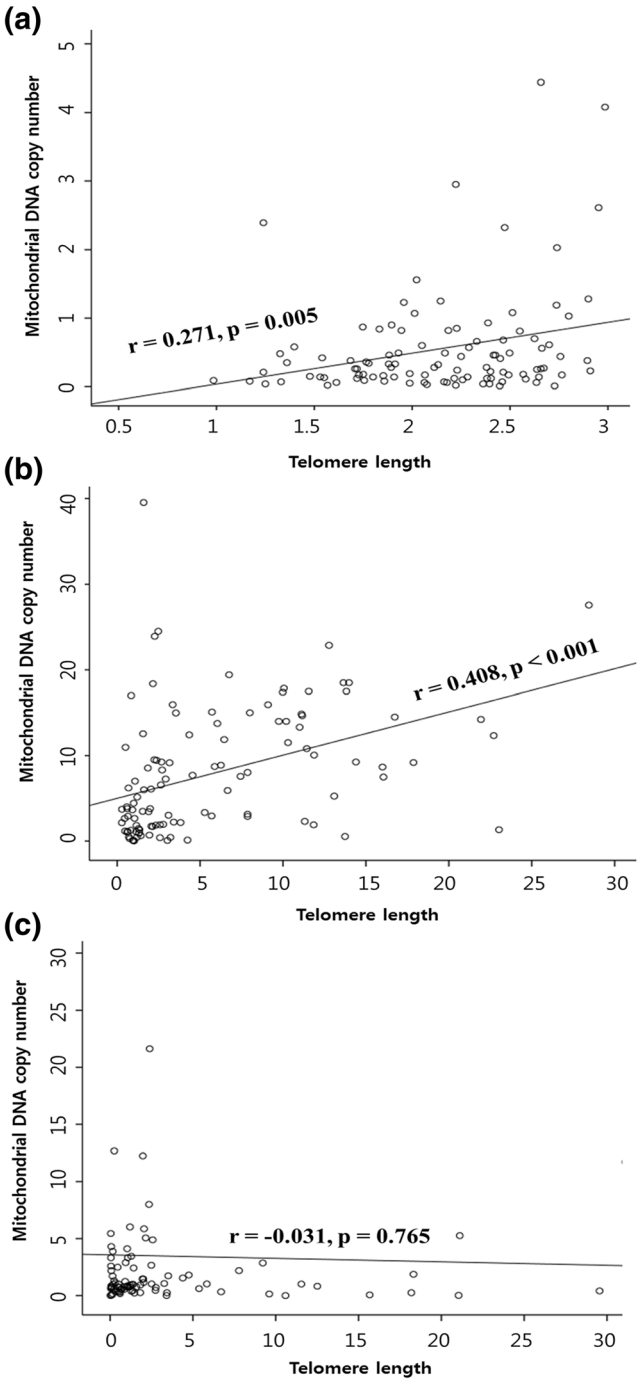

Fig. 2 Association of mitochondrial DNA copy number and telomere length. Positive correlations in non-tumorous tissues (**a**) and CRC (**b**); No association in precancerous legions (**c**)

and association in colorectal precancerous legions. We had already reported clinicopathological characteristics of mtCN in precancerous legions [16]. Lower mtCN was found in TAs than SPs, and the level of mtCN in TA was different according to the presence of microsatellite instability of mtDNA. And mutually exclusiveness of KRAS and BRAF mutations in these legions were in agreement with previous studies [4, 5], however, they did not have an association with mtCN or TL. Though clinical value of TL was studied in colorectal precancerous legions for the first time, there was no significant implication. Interestingly, TL in both precancerous legions did not correlate with mtCN. These results suggested that normal correlation between TL and mtCN was broken in early stage of colorectal carcinogenesis, but it was reinstated in CRC by changing its biological characteristics. In other words, loss of co-regulation between telomeres and mitochondria may induce the initiation or play a role as trigger factor of colorectal carcinogenesis.

For a long time, both mitochondria and telomere is considered to be key instigators of natural ageing, however, its detail mechanism was still unclear. Aged tissue showed mtDNA instability or change causing respiratory chain deficiency and reactive oxygen species (ROS). Therefore, mitochondrial dysfunction may be related to the aging process by the increase of ROS production and the decrease of ATP generation. Disorder of mitochondrial biogenesis and decreased energy production were shown in telomerase-deficient mice with severe telomere dysfunction compared to that in telomerase-deficient mice with largely intact telomeres [21]. And additional studies supported this theory that telomere dysfunction-induced p53 represses PGCs and induces metabolic and mitochondrial compromise [22, 23]. Therefore, this telomere-p53-PGC-mitochondria axis may explain why shortened telomeres lead to metabolic deterioration related to biological aging. And our result supports this theory suggesting that disorder of telomere-mitochondria axis by aging or other factors occurring physiological stress may be an important and early event in carcinogenesis.

In summary, TL and mtCN was correlated in normal and cancerous legions of colorectum, however, irregular telomere-mitochondria axis was shown in precancerous legions. Their precise mechanism remains unclear, maintenance of the regulation between mitochondria and telomere may an important for colorectal carcinogenesis, which suggests that this pathway could be targeted for cancer prevention. Therefore, we would like to mention that further study with molecular mechanism and larger clinical cases should be needed to clarify this hypothesis.

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Compliance with Ethical Standards

Conflict of Interest The authors have no conflict of interest to declare.

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