Int J Clin Exp Pathol 2017;10(2):1946-1953
www.ijcep.com /ISSN:1936-2625/IJCEP0025983

Original Article

Mitochondrial D-loop polymorphism in tubular adenomas and serrated polyps of colorectal lesions

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Received February 15, 2016; Accepted May 20, 2016; Epub February 1, 2017; Published February 15, 2017

Abstract: Colorectal cancer is a heterogeneous disorder than arises via multiple distinct pathways, from tubular adenomas (TAs) and serrated polyps (SPs), which are clinically, morphologically, and molecularly different. We examined mitochondrial D-loop polymorphism in colorectal precancerous lesions, including TAs and SPs. DNA was isolated from paired normal and tumoral tissues in 78 TAs and 34 SPs. Mitochondrial D-loop polymorphism (D146, D150, D152, D310, and D514), *KRAS* and *BRAF* mutations, and microsatellite instability (MSI) were analyzed by direct sequencing and pyrosequencing. D146, D310, and D514 were polymorphic in these patients and their distributions were significantly different between TAs and SPs. D146 and D310 polymorphism was associated not with *KRAS* and *BRAF* mutations, but with MSI and other mitochondrial polymorphism each other. Our data suggest that mitochondrial D-loop polymorphism may play an important role in development of colorectal precancerous lesions and contribute to regulate their progression to TAs or SPs.

Keywords: Colorectal cancer, mitochondria, polymorphism, serrated polyps, tubular adenomas

Introduction

Colorectal cancer (CRC) is the third most common cancer in the world and its incidence rate has increased seriously every year in Korean populations [1, 2]. After the discovery of the oncogenes and tumor suppressor genes, various genetic studies have been carried out in CRCs to clarify their carcinogenesis [3-5]. As a result, it is accepted that from the tubular adenomas (TAs) to carcinoma sequence underlies the colorectal carcinogenesis, and *APC*, *KRAS,* and p53 mutations and microsatellite instability (MSI) were associated with this sequence [3-7]. Serrated polyps (SPs) are histologically classified into hyperplastic polyp (HP), traditional serrated adenoma (TSA), sessile serrated adenoma (SSA), and mixed hyperplastic/adenomatous polyp [8]. For many years, SPs have been regarded as little neoplastic potential lesions. However, recent proposals suggested that SPs have been reported to be the precursor of CRC with MSI via the serrated neoplastic pathway [9-13]. Although this pathway is characterized by frequent *BRAF* mutation and infrequent *KRAS* mutation, the details molecular mechanism of this progression remains unclear [14, 15].

Mitochondrial DNA (mtDNA) has different genetic system from nuclear DNA, and high frequencies of mitochondrial mutations were found in various cancers independently with MSI [16-19]. Most of mutations were found in D-loop region, especially in the D310 region, which is a polymorphic C-tract sequence and it was associated with poor prognosis of CRC [20-24]. Recent studies have showed the T146C, C150T, T152C, and D524 mutations or polymorphism in several tumors [25-27].

In present study, mitochondrial D-loop polymorphisms were investigated in colorectal precursor lesions, comprising of TAs and SPs. To contribute to better understanding on colorectal carcinogenesis, *KRAS* and *BRAF* mutations and MSI, as key markers in CRCs, were also studied in these lesions. Clinicopathological character-

Table 1. Clinicopathological characteristics of TAs and SPs in present study

	TA		SP (N, %)	*P*
	LTA (N, %)	HTA (N, %)		
Total	49	29	34	
Age (mean ± SD)	61.94 ± 8.93	58.83 ± 12.76	58.41 ± 9.78	0.21
Sex				0.69
Male	35 (71.4)	18 (62.1)	23 (67.6)	
Female	14 (28.6)	11 (37.9)	11 (32.4)	
Region				0.93
Right	13 (26.5)	8 (27.6)	8 (23.6)	
Left	36 (73.4)	21 (72.4)	26 (76.5)	
KRAS[a]				0.012
(+)	9 (18.4)	9 (31.0)	1 (2.9)	
(-)	40 (81.6)	20 (69.0)	33 (97.1)	
BRAF[b]				0.001
(+)	0 (0)	0 (0)	7 (20.6)	
(-)	49 (100)	29 (100)	27 (79.4)	
MSI				0.86
(+)	4 (8.2)	3 (10.3)	4 (11.8)	
(-)	45 (91.8)	26 (89.7)	30 (88.2)	

LTA, low-grade tubular adenoma; HTA, high-grade tubular adenoma. [a]$P = 0.009$ between TA and SP; [b]$P < 0.001$ between TA and SP.

istics in these patients were analyzed according to their genetic status.

Patients and methods

Patients and DNA extraction

To obtain the precancerous lesions, the records of colonoscopic polypectomy performed at Dongsan Medical Center between 1999 and 2003 were reviewed retrospectively. Exclusion criteria were: previous history of surgical resection for CRCs, and evidence of hereditary nonpolyposis colorectal cancer (Amsterdam criteria) or familial adenomatous polyposis. As a result, precancerous lesions were comprised of 78 TAs and 34 SPs. The institutional regional review board (IRB) approved the research proposal, and informed consent was obtained from all individuals involved in the study.

Tumor area and adjacent normal mucosa were selected from slide according to hematoxylin and eosin stained sections. Subsequently, the selected areas from paraffin embedded tissues were used for DNA extraction. DNA was isolated by using DNA extraction Kit (Absolute™ DNA extraction Kit, BioSewoom, Korea) according to the manufacturer's instructions.

Amplification and sequencing of the D-loop of mitochondrial DNA

A 501-bp fragment containing the D-loop region of mtDNA was amplified via semi-nested polymerase chain reaction (PCR). The primer sequences were as follows: Forward (5'-CCT CAG ATA GGG GTC CCT TG-3') and reverse (5'-TTT GGT TGG TTC GGG GTA TG-3') for the first PCR amplification and forward (5'-GAG CTC TCC ATG CAT TTG GT-3') for the second PCR amplification. PCR was performed by using a thermal cycler (Applied Biosystems, USA) in the order as follows: 40 cycles of 40 sec at 94°C for denaturation, 40 sec at 56°C for annealing, and 60 sec at 72°C for extension. Final extension was performed at 72°C for 10 min. For amplification, a 50 μL of mixture containing DNA from normal mucosa (50 ng), dNTPs (200 μM), primary primers (25 pmol), Taq DNA polymerase (2.5 U; Blend Taq-plus, Toyobo, Japan) and 10× buffer (5 μL) was used. The PCR products were electrophoresed on 1.5% of agarose gel with ethidium bromide to confirm the size of the bands. Then, direct DNA sequencing was performed using the ABI 3730 DNA sequencer (Bionics Inc, Korea).

Microsatellite instability

A recommended method for MSI analysis by National Cancer Institute is the Bethesda panel, however, recent studies described that BAT25 and BAT26 analysis can accurately detect MSI without additional markers [28, 29]. Therefore, the MSI were analyzed with two microsatellite markers, BAT25 and BAT 26. PCR amplification was performed by same method as described previously.

KRAS and BRAF mutations

KRAS mutations in codons 12 and 13, and *BRAF* V600E mutation were analyzed by pyrosequencing (PyroMark Q24, Sweden). Primers for amplification and pyrosequencing were designed as previously described [30]. The pyrosequencing reaction was performed on a PyroMark Q24 instrument using the Pyro Gold Q24

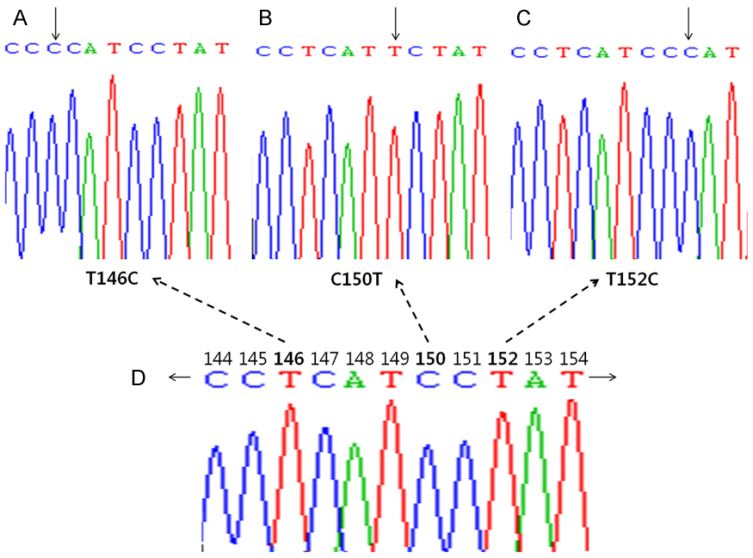

Figure 1. Representative results of mitochondrial polymorphism of D146, D150 and D152 by direct sequencing. A: T146C; B: C150T; C: T152C; D: Normal sequences.

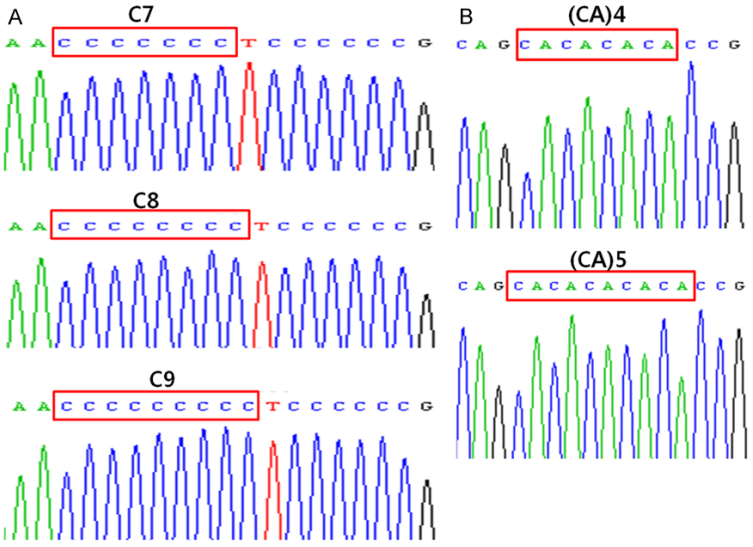

Figure 2. Representative results of D310 and D514 polymorphism by direct sequencing. A: D310 varied from C7 to C9; B: D514 showed (CA)5 and (CA4).

Reagents (Qiagen, Netherlands). The pyrosequencing primers were used in a final concentration of 0.3 μmol/L. Resulting data were analyzed and quantified with the PyroMark Q24 software version 2.0.6 (Qiagen, Netherlands).

Statistical analysis

SPSS software for Windows was used. Chi-square, Fischer' exact tests and Mann Whitney U test were used to analyze the relationship between variables. A p value < 0.05 was considered statistically significant.

Result

Precursors of CRCs were comprised of 78 tubular adenomas (TAs) and 34 serrated polyps (SPs). TAs were classified into low and high grades (LTA and HTA) according to histological feature and SPs were only comprised of hyperplastic polyps and sessile serrated adenomas. Clinicopathological characteristics of TAs and SPs were presented in **Table 1**. Higher frequency of *KRAS* mutation was found in LTA and HTA than that in SP (P = 0.009). However, *BRAF* mutation was shown only SPs, therefore, *KRAS* and *BRAF* mutations were mutually exclusive in TAs and SPs. Other clinicopathological characteristics were not associated with their classifications.

Mitochondrial polymorphism in tubular adenomas and serrated polyps

The sequences of amplified PCR products containing D-loop were successfully analyzed in all patients (**Figures 1**, **2**). Among various polymorphic sites in D-loop, five loci (D146, D150, D152, D310, and D514) were selected and their frequencies in 78 TAs and 34 SPs were presented in **Table 2**. The allele frequency of D146, D310, and D514 differed significantly between TAs and SPs. The frequency of the C allele of D146 in LTAs and HTAs was about 80%, much higher than that (41.2%) in SPs (P < 0.001). According to the revised Cambridge Reference Sequence for human mtDNA (GI: 251831106), reference sequence was (C)7 in D310 loci [31]. However, (C)9 was most common type in present study. In SPs, the number of repeats of D310 and D514 were lower than that in TAs (P = 0.009 and 0.035,

Table 2. Distribution of mitochondrial D-loop polymorphism in TAs and SPs

	TA		SP (N, %)	*P*
	LTA (N, %)	HTA (N, %)		
D146[a]				< 0.001
T	7 (14.3)	3 (10.3)	12 (35.3)	
C	39 (79.6)	26 (89.7)	14 (41.2)	
T/C	3 (6.1)	0 (0)	8 (23.5)	
D150				0.52
C	48 (98.0)	29 (100)	34 (100)	
T	1 (2.0)	0 (0)	0 (0)	
D152				0.74
T	44 (89.8)	27 (93.1)	33 (97.1)	
C	3 (6.1)	1 (3.4)	1 (2.9)	
T/C	2 (4.1)	1 (3.4)	0 (0)	
D310[b]				0.046
(C)7	8 (16.3)	5 (17.2)	14 (41.2)	
(C)8	6 (12.2)	5 (17.2)	0 (0)	
(C)9	34 (69.4)	19 (65.5)	20 (58.8)	
(C)10	1 (2.0)	0 (0)	0 (0)	
D514[c]				0.06
(CA)5	41 (83.7)	27 (93.1)	24 (70.6)	
(CA)4	8 (16.3)	2 (6.9)	10 (29.4)	

[a]*P* < 0.001 between TA and SP; [b]*P* = 0.009 between TA and SP; [c]*P* = 0.035 between TA and SP.

respectively). The polymorphism in D150 and D152 locus did not show significant difference between TAs and SPs.

Clinicopathological characteristics of D146 and D310 polymorphisms in tubular adenomas and serrated polyps

Among the mitochondrial polymorphic locus, D146 and D310 showed the association with other markers statistically. D146 polymorphism had significant relationship with other D-loop polymorphism and MSI in LTA (**Table 3**). The frequency of the T allele of D146 was higher in the patients with recessive type of D150 (T allele), D152 (C allele), and D310 (C7). In the patients with MSI (+), T allele of D146 was also significantly higher than C allele. In HTA and SPs, the frequency of the T allele of D146 was also higher in the patients with recessive type of D152 (C allele) and D310 (C7), respectively.

D310 polymorphism had also significant relationship with other polymorphism or MSI (**Table 4**). In LTA, (C)7 of D310 was also associated with MSI (+) and (CA)4 of D524, as recessive types. Deep relationship between (CA)4 of D524 and (C)7 of D310 was also found in SP. Other variables were not associated with D146 and D310 polymorphism significantly except the variables describe above.

Discussion

This study suggests that mtDNA D-loop polymorphism play a significant role in the etiology of colorectal precancerous legions. Though the mutation of mtDNA has been recently reported in these tumors [32, 33]. Polymorphism of mtDNA has been not studied. This analysis of mtDNA in Korean populations, as a homogeneous population, is valuable, because the confusion by the heteroplasmy may be lowers [34].

Colorectal cancer (CRC) is a heterogeneous disease because CRCs and their precursors displayed distinct pathological features and molecular signatures. The predominant chromosomal instability (CIN) pathway accounted for up to 85% of cases [3-5]. A minority of CRCs, less than 5%, developed via the nucleus microsatellite instability (MSI) pathway [6, 7]. However, the mechanism of colorectal carcinogenesis has not been fully identified because there were some troubles in acquirement of precancerous legions and various genes, such as *KRAS*, *BRAF*, and p53, and MSI affect their development. To contribute to better understanding on colorectal tumorigenesis, this study investigated mitochondrial polymorphism with MSI, *BRAF* and *KRAS* mutations in various kinds of colorectal precancerous legions.

Clinicopathogical characteristics of low-grade and high grade tubular adenomas (LTAs and HTAs) and serrated polyps (SPs) in present study were in agreement with previous results [12-14]. Additionally, we found that mitochondrial D-loop polymorphism in T146C, poly C in D310, and dinucleotide repeat (CA)n in D514 were significantly different between TAs and SPs. In D146 polymorphism, SPs showed a similar frequency of T and C alleles, however, about 80% of LTAs and HTAs had the C allele. Especially in LTAs, C allele was associated with MSI (-), C allele of D150, T allele of D152, and (C)9 of D310, which were entirely recessive types. In D310 polymorphism, LTAs with dominant type showed a significantly association with MSI (-) and (CA)4 of D524, which were entirely recessive types. On the other hand, this

Table 3. Clinicopathological characteristic of D146 polymorphism in LTAs, HTAs, and SPs

D146	LTA			HTA		SP		
	T	C	T/C	T	C	T	C	T/C
Sex								
Male	4 (11.4)	28 (80.0)	3 (8.6)	2 (11.1)	16 (88.9)	8 (34.8)	12 (52.2)	3 (13.1)
Female	3 (21.4)	11 (78.6)		1 (9.1)	10 (80.8)	4 (36.4)	2 (18.2)	5 (45.5)
Region								
Right	4 (30.8)	9 (69.2)	0 (0)	0 (0)	8 (100)	3 (37.5)	3 (37.5)	2 (25.0)
Left	3 (8.3)	30 (83.3)	3 (8.3)	3 (13.6)	18 (86.4)	9 (34.6)	11 (42.3)	6 (23.1)
KRAS								
(+)	2 (22.2)	6 (66.7)	1 (11.1)	2 (22.2)	7 (77.8)	1 (100)	0 (0)	0 (0)
(-)	5 (11.6)	33 (76.7)	5 (11.6)	1 (5.0)	19 (95)	11 (33.3)	14 (42.4)	8 (24.2)
BRAF								
(+)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	3 (42.9)	2 (28.6)	2 (28.6)
(-)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	9 (33.3)	12 (44.4)	6 (22.2)
MSI		$P = 0.018$						
(+)	2 (50.0)	1 (25.0)	1 (25.0)	0 (0)	3 (100)	2 (50.0)	1 (25.0)	1 (25.0)
(-)	5 (11.1)	38 (84.4)	2 (4.4)	3 (11.5)	23 (88.5)	10 (33.3)	13 (43.3)	7 (23.3)
D150		$P = 0.047$						
C	6 (12.5)	39 (81.3)	3 (6.3)	3 (10.3)	26 (89.7)	12 (35.3)	14 (41.2)	8 (23.5)
T	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
D152		$P < 0.001$			$P < 0.001$			
T	4 (9.1)	39 (88.6)	1 (2.3)	1 (3.7)	26 (96.3)	11 (33.3)	14 (42.4)	8 (23.5)
C	3 (100)	0 (0)	0 (0)	1 (100)	0 (0)	1 (100)	0 (0)	0 (0)
T/C	0 (0)	0 (0)	2 (100)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)
D310		$P = 0.002$					$P = 0.007$	
(C)7	3 (37.5)	3 (37.5)	2 (25.0)	1 (20.0)	4 (80.0)	9 (64.3)	2 (14.3)	3 (21.4)
(C)8	1 (16.7)	4 (66.7)	1 (16.7)	0 (0)	5 (100)	0 (0)	0 (0)	0 (0)
(C)9	2 (5.9)	32 (94.1)	0 (0)	2 (10.5)	17 (89.5)	3 (15.0)	12 (60.0)	5 (25.0)
(C)10	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
D524								
(CA)5	5 (12.2)	34 (82.9)	2 (4.9)	2 (7.4)	25 (92.6)	8 (33.3)	11 (45.8)	5 (20.8)
(CA)4	2 (25.0)	5 (62.5)	1 (12.5)	1 (50.0)	1(50.0)	4 (40.0)	3 (30.0)	3 (30.0)

type of D310 was associated with (CA)5 of D524 in SPs. Considering its significance in LTA, mitochondrial D-loop polymorphism may have early and important role in the progression of tubular adenomas by interaction with MSI and each other. Interestingly, these polymorphisms were not associated with *KRAS* and *BRAF* mutations, as distinctive genetic markers for TAs and SPs. So, mtDNA polymorphism may be an important pathway of colorectal carcinogenesis independently with *KRAS* and *BRAF* mutations.

There are some limitations because of deficient knowledge about mitochondrial D-loop polymorphism. Previous studies showed that D146 and D150 polymorphisms showed the association with prognosis of hepatocellular carcinomas and cervical cancer risk, respectively [27, 35]. Zhang et al. [36] described that C150T polymorphism had a replicative advantage to the mtDNA by changing the binding site for mitochondrial transcription factor A, and D146 and D152 polymorphisms were fibroblast-specific site. D524 has been suggested a hallmark for breast cancer risk, however, their reason and exact mechanism was unclear [37]. High frequency of D310 mutation was found in various cancers. This mutation may alter mtDNA transcription because D310 sequence is located in essential element for mtDNA replication containing the H-strand replication origin [22-24]. Lièvre et al. [38] presented that prevalence of D310 mutations increased significantly with

Table 4. Clinicopathological characteristic of D310 polymorphism in LTAs, HTAs, and SPs

D310	LTA				HTA			SP	
	(C)7	(C)8	(C)9	(C)10	(C)7	(C)8	(C)9	(C)7	(C)9
Sex									
Male	6 (75.0)	5 (83.3)	23 (67.6)	1 (100)	4 (80.0)	3 (60.0)	11 (57.9)	9 (64.3)	14 (70.0)
Female	2 (25.0)	1 (16.7)	11 (32.4)	0 (0)	1 (20.0)	2 (40.0)	8 (42.1)	5 (35.7)	6 (30.0)
Region									
Right	2 (25.0)	1 (16.7)	10 (29.4)	0 (0)	1 (20.0)	3 (60.0)	4 (21.1)	2 (14.3)	6 (30.0)
Left	6 (75.0)	5 (83.3)	24 (70.6)	1 (100)	4 (80.0)	2 (40.0)	15 (78.9)	12 (85.7)	14 (70.0)
KRAS									
(+)	2 (25.0)	1 (16.7)	5 (14.7)	1 (100)	1 20.0)	3 (60.0)	5 (26.3)	1 (7.1)	0 (0)
(-)	6 (75.0)	5 (83.3)	29 (85.3)	0 (0)	4 (80.0)	2 (40.0)	14 (73.7)	13 (92.9)	20 (100)
BRAF									
(+)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	3 (21.4)	4 (20.0)
(-)	8 (100)	6 (100)	34 (100)	1 (100)	5 (100)	5 (100)	19 (100)	11 (78.6)	16 (80.0)
nMSI			*P* = 0.011						
(+)	3 (37.5)	0 (0)	1 (2.9)	0 (0)	1 (20.0)	1 (20.0)	1 (5.3)	1 (7.1)	3 (15.0)
(-)	5 (62.5)	6 (100)	33 (97.1)	1 (100)	4 (80.0)	4 (80.0)	18 (94.7)	13 (92.9)	17 (85.0)
D150									
C	0 (0)	1 (16.7)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
T	8 (100)	5 (83.3)	34 (100)	1 (100)	5 (100)	5 (100)	19 (100)	14 (100)	20 (100)
D152									
T	5 (62.5)	5 (83.3)	33 (97.1)	1 (100)	4 (80.0)	5 (100)	18 (94.7)	14 (100)	19 (95.0)
C	2 (25.0)	0 (0)	1 (2.9)	0 (0)	1 (20.0)	0 (0)	0 (0)	0 (0)	1 (5.0)
T/C	1 (12.5)	1 (16.7)	0 (0)	0 (0)	0 (0)	0 (0)	1 (5.3)	0 (0)	0 (0)
D524			*P* = 0.036			*P* = 0.027			
(CA)5	4 (50.0)	6 (100)	30 (88.2)	1 (100)	4 (80.0)	5 (100)	18 (94.7)	7 (50.0)	17 (85.0)
(CA)4	4 (50.0)	0 (0)	4 (11.8)	0 (0)	1 (20.0)	0 (0)	1 (5.3)	7 (50.0)	3 (15.0)

the number of cytosines in the sequence in CRCs. D310 mutation rate was 9%, 49.5%, and 73% in C7, C8, and C9 alleles, respectively. Therefore, the relations between mtDNA polymorphism and mitochondrial mutations or copy numbers should be studied further. This polymorphism study should be performed by case-control study, however, the acquirement of the control without previous TAs or SPs history in matched age was difficult. And reference Sequence and previous studies suggested that most common type in D310 was (C)7 located between nucleotides 303 and 315 and interrupted by a T at position 310 [22, 24, 31, 35]. However, (C)9 of D310 was most common type in present study and its distribution was significantly different between TAs and SPs. This racial difference of mtDNA polymorphism should be confirmed with larger case in Korean population.

In summary, our study suggests that the mtDNA D-loop polymorphism may be important and distinctive role in tumorigenesis of TAs and SPs. They could explain the tumorigenesis of TAs and SPs independently with *KRAS* and *BRAF* mutations. This is the first study to show mtDNA polymorphism in colorectal precancerous legions, and further research is needed to study the potential biological mechanism of these polymorphisms.

Acknowledgements

This work was supported by grants of the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2014R1A6A3A04058057) and by the Korean Government (MSIP) (No. 2014R1A5A2010008).

Disclosure of conflict of interest

None.

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