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Mitochondrial DNA D-loop lesions with the enhancement of DNA repair contribute to gastrointestinal cancer progression

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Abstract. Gastrointestinal cancer (GIC) is a worldwide public health problem with a high mortality rate. Mitochondrial DNA (mtDNA) mutations in the displacement loop (D-loop) region are quite common in various types of primary human cancers; however, their role in the pathogenesis of GIC is controversial. In the present study, tumor and para-tumor tissues were selected from 18 patients with gastric cancer (GC), 21 patients with colon cancer (CC) and 30 patients with rectal cancer (RC). The mtDNA D-loop was analyzed by sequencing and reverse transcription-quantitative polymerase chain reaction. Furthermore, DNA oxidative damage and DNA repair functioning were detected by immunohistochemistry. The results demonstrated that increased mtDNA deletion was not evident in GIC; however, significant DNA oxidative damage was significant in RC by detecting 8-hydroxyguanine expression. In addition, over-activated DNA repair was identified in CC and RC through the detection of 8-oxo-20-deoxyguanosine glycosylase 1 expression. The mtDNA D-loop had a specific mutation hotspot region, and the level of mtDNA D-loop mutations was correlated with the progression of the GIC. The mutations of the mtDNA D-loop were primarily homoplasmic in GIC and often transitioned at pyrimidine sites. Mitochondrial microsatellite instability, including the formation of poly-cytidine stretches, was common in GIC. These results demonstrate the occurrence of mtDNA D-loop mutations in GIC in Chinese patients and support the correlation of these mutations with carcinoma progression. Over-activated DNA repair function possibly repairs the GIC mtDNA lesions.

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Introduction

Gastric cancer (GC) and colorectal cancer (CRC) are worldwide public health problems; GC is the fourth most common type of carcinoma and the second most common cause of carcinoma-associated mortality, and CRC is the second most common cause of carcinoma-associated mortality with an annual incidence of 1,000,000 cases and an annual mortality of >500,000 cases (1). In China, the incidence of GC ranks third among all malignant tumors and the mortality rate was 26.3/100,000 in 2005 (2); the incidence of CRC ranked fourth of all cancer types and the estimated mortality rate was ranked the fifth leading cause of cancer-associated mortality in all cancer types in 2011 (3). However, the pathogenesis of these diseases remains to be fully elucidated. Previous studies have focused on the induced oncogenes and inhibited tumor suppressor genes, as well as the dysfunction of mismatch base repair in nuclear DNA, which does not fully explain the pathogenesis and development of these diseases. Mitochondria can generate adenosine triphosphate via oxidative phosphorylation and in turn control essential cellular activities. The displacement loop region (D-loop) is the main noncoding area of mitochondrial DNA (mtDNA). mtDNA mutations in the D-loop region and somatic mtDNA mutations have been described to be common in various types of primary human cancer types including hepatocellular carcinoma, and bladder, breast and lung cancers (4,5); however, their role in the pathogenesis of gastrointestinal cancer (GIC) is controversial. In the present study, whether somatic mtDNA and D-loop mutations

occurred in Chinese patients with GIC and their association with disease progression were investigated.

Materials and methods

Patients and tissues. Tumor and para-tumor tissues were obtained from GC and CRC patients who underwent surgical tumor resection at the Fourth General Surgery Division, Shandong Cancer Hospital (Jinan, China) between February 2012 and August 2012. Tumor tissues were pathologically diagnosed as GC or CRC, and para-tumor tissues were confirmed to be non-cancerous by experienced pathologists. The inclusion criteria included: i) Preoperative pathological biopsy and postoperative histopathology confirmed the diagnosis of GIC; ii) patients without other diagnosed tumors or diseases; and iii) patients or their families all provided signed informed consent. The exclusion criteria included: ii) Patients with incomplete clinical data available; ii) patients diagnosed with other types of tumors and diseases; and iii) patients and their families who refused to provide informed consent. Patient demographics and clinical characteristics are listed in Table I. Among the participants, there were 18 patients with GC, 21 patients with colon cancers (CC) and 30 patients with rectal cancer (RC); their average ages were 55.1, 54 and 57.4 years, respectively. Men appeared to be over-represented, accounting for 13/18 GC patients, 13/21 CC patients and 20/30 RC patients. The majority of these patients were diagnosed with tumor node metastasis (TNM) stages II-III and grades I-III. The present study was approved by the Ethics Committees of Shandong Cancer Hospital. Written informed consent was obtained from all study participants.

Immunohistochemistry staining. GI tissues were fixed with 10% formalin at 4˚C for 24 h and then paraffin-embedded; sections 4-μm-thick were cut and immunohistochemical staining was performed. Tissue sections were first deparaffinized and rehydrated, followed by heat-induced epitope retrieval at 95-100˚C, washing with 100% Ethanol 2 for 5 min, 90% Ethanol 1 for 5 min, 70% Ethanol 1 for 5 min and ddH_2O 1 for 5 min, and then treated with a 10 mmol/l citrate buffer (pH 6.0). Next, 3% H_2O_2 was used to block endogenous peroxidase and sections were blocked with PBS containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at room temperature for 1 h. Then, sections were incubated with anti-8-hydroxyguanine (oxo-G; cat. no. 4354-MC-050; Trevigen, Inc., Gaithersburg, MD, USA) and anti-8-oxo-20-deoxyguanosine glycosylase 1 (OGG1; cat. no. NBP2-52724; Novus Biologicals, LLC, Littleton, CO, USA) antibodies, diluted with blocking reagent (1:1,000), overnight at 4˚C, followed by incubation with a biotin-free horseradish peroxidase-conjugated secondary antibody (cat. no. pv9005; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China); diluted with blocking reagent (1:1,000) for 1 h at room temperature. Visualization was performed with 3,3'-diaminobenzidine. The slides were viewed and photographed under a fluorescent inverted microscope (Olympus IX71; Olympus Corporation, Tokyo, Japan), and positively stained cells were counted using Image Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

DNA isolation, and cloning and sequencing of the mtDNA D-loop. Total DNA from the tumor and para-tumor tissues was isolated using a DNA extraction kit (Qiagen China Co., Ltd., Shanghai, China) following the manufacturer's protocol, and polymerase chain reaction (PCR) was performed to amplify the mtDNA D-loop region using high-fidelity Platinum *Taq* polymerase (Invitrogen; Thermo Fisher Scientific, Inc.). The primer pairs and PCR procedure for the D-loop in the present study have been described in our previous study (6). The pGEM-18T vector (Takara Biotechnology Co., Ltd., Dalian, China) was used to clone the PCR products, and 10-12 randomly selected clones/samples were sequenced on an ABI 3730 genetic analyzer (Thermo Fisher Scientific, Inc.). The D-loop nucleotide sequences from each clone were analyzed and manually adjusted using NCBI BLAST(blast.ncbi.nlm.nih.gov/Blast.cgi) and free BioEdit software (version 7.1.3; Ibis Therapeutics, Carlsbad, CA, USA). All sequences have been submitted to GenBank (accession nos. KY402468-KY403500).

Reverse transcription-quantitative PCR (RT-qPCR). The RT-qPCR assay for mtDNA deletion quantification was performed using the SYBR Green (Beijing Biomed Biotechnology Co., Ltd., Beijing, China)method based on the relative nicotinamide adenine dinucleotide hydride dehydrogenase subunit 1 (ND1)/ND4-quantification method as well as $2^{-\Delta\Delta Cq}$ as previously reported (6,8-11), and was performed using the TaqMan 7900HT system (Thermo Fisher Scientific, Inc.). The primers used in the present study were as follows: Reference gene, *Homo sapiens* mitochondrion complete genome (Gen-Bank NC 012920); ND1 forward, 5'-CCCTAAAACCCGCCACATCT-3' and reverse, 5'-TGGAATCGAGAGTGGTAGCGAG-3'; ND4 forward, 5'-CCATTCTCCTCCTATCCCTCAAC-3' and reverse, 5'-TTTATATCAAATTGGTTTTGTAGTCTAACAC-3' (synthesized by Invitrogen; Thermo Fisher Scientific, Inc.). The procedures were similar to those described in our previous report (7,12). Briefly, 250 nM probe and 300 nM primer were used in the PCR reaction mix. The qPCR thermocycling conditions were as follows: 5 min at 95˚C, followed by 50 cycles of 15 sec at 95˚C and 1 min at 60˚C. qPCR reactions were performed in triplicate for each sample. Double-distilled water was used as a control reaction and was subjected to the same conditions as the test reactions.

Sequence analysis. The sequences were analyzed as previously described (13-15). Briefly, the obtained nucleotide sequences from each clone were assembled and error checked using the Vector NTI suite 7.0 ContigExpress software package (Thermo Fisher Scientific, Inc.). The sequences were then aligned to the reference sequence (Gen-Bank NC 012920) using the Clustal W multiple sequence alignment program (www.ebi.ac.uk/Tools/msa/clustalw2/), then the nucleotide mutations were identified and calculations were performed. Shannon entropy (www.hiv.lanl.gov/content/sequence/ENTROPY/entropytwo.html), as a measure of variation in mtDNA D-loop sequence alignments, was used to determine whether there were more highly variable areas in tumors when compared with the para-tumors. The sequences were then aligned to the reference sequence using the MITOMASTER web tool in the Mitomap database (www.mitomap.org/) to check for mtDNA mutations. Microsatellite instability (MSI), a simple repetitive sequence

Table I. Demographics and clinical characteristics of the patients recruited to the present study.

Characteristics	Carcinoma type		
	Gastric cancer (n=18)	Colon cancer (n=21)	Rectum cancer (n=30)
Sex (male)	13	13	20
Age (years)			
Mean	55.1	54.0	57.4
Range	40-68	30-71	34-75
TNM stage (n)			
I	0	0	4
II	2	10	9
III	14	5	14
IV	2	6	3
Grade (n)			
I	2	7	5
II	6	12	20
III	10	1	5
IV	0	1	0
Type of surgery (n)			
Local resection	10	17	16
Organ resection	8	4	14
Multiorgan resection	0	0	0
CEA (n ≥5 μg/l)	5	11	10
CA19-9 (n ≥37 U/ml)	6	10	3
CA72-4 (n ≥6 U/ml)	6	N/A	N/A
Risk factors			
Tobacco use (n)			
Current	7	7	3
Former	3	2	3
Never	8	12	24
Alcohol use (n)			
Current	8	4	3
Former	1	3	1
Never	9	14	26
Family history (n)	1	3	1
Polyps (n)	1	3	1
Inflammatory disease (n)[a]	3	2	0
Unhealthy diet (n)[b]	1	0	0

[a]Inflammatory diseases including Crohn's disease, ulcerative colitis or long-term stomach inflammation. [b]As determined by the dietary guidelines for Chinese residents (2016) (6). N/A, not applicable.

change caused by a mismatch repair gene mutation, was also examined in 69 Chinese patients via alignment of sequences to the reference sequence.

Statistical analysis. The results are presented as the mean ± standard error of the mean. Statistical significance was determined by one-way analysis of variance with post hoc correction using the Tukey's multiple comparison test. Nonparametric Mann-Whitney, Chi-square, or Fisher's exact tests were used to compare nonparametric data. All statistical analyses were performed using SPSS software (version 16.0; SPSS Inc., Chicago, IL, USA), and $P<0.05$ was considered to indicate a statistically significant difference.

Results

No increased mtDNA loss was observed in GIC. In the present study, whether mtDNA deletions occurred in GIC were assessed via qPCR. The ND1 gene is located in the minor arc of mtDNA and is rarely deleted; however, the ND4 gene is located at the major arc of mtDNA and is frequently deleted. ND1/ND4 relative qPCR was used to detect mtDNA deletions via the $2^{-\Delta\Delta Cq}$ method in the present study. The results demonstrated that the relative amount of mtDNA copies were 99.44% in GC tumors and 99.58% in GC para-tumors, 99.44% in CC tumors and 99.35% in CC para-tumors, and 99.32% in RC tumors and 99.13% in RC para-tumors (Fig. 1). No significant mtDNA deletions were noted in the GC, CC or RC tumor tissues when compared with para-tumor tissues. Furthermore, the relative amount of mtDNA copies between the different clinical carcinoma stages were compared; however, no mtDNA deletions in stage III-IV GC, CC or RC were identified (data not shown).

DNA oxidative damage and over-activated DNA repair in GIC. Oxidative damage has been reported in the mtDNA of tumor cells (16). DNA oxidative damage commonly produces oxo-G, and OGG1initiates base excision repair, which removes oxo-G damaged DNA (17). In the present study, the expression of oxo-G and OGG1 was determined in GIC tumor and para-tumor tissues using immunohistochemistry staining, as described in our previous studies (7,18). No significant increases were observed in oxo-G (92±1 vs. 90±2%) or OGG1 (94±1 vs. 92±2%) expression in GC tissues when compared with para-GC tissues (Fig. 2A and B). In addition, no significant difference in oxo-G expression was observed between CC tissues (94±1%) and para-CC tissues (84±4%), but OGG1 expression was higher in CC tissues (97±1%) when compared with para-CC tissues (83±1%; Fig. 2C and D). However, oxo-G (94±4 vs. 83±1%) and OGG1 (97±1 vs. 41±7%) expression levels were increased in RC tissues when compared with para-RC tissues (Fig. 2E and F). These results indicated that oxidative damage and over-activated DNA repair functioning occurred in GIC.

Somatic mtDNA D-loop mutations in GIC. To identify somatic mutations in the D-loop of mtDNA in GIC, Shannon entropy was used to identify highly variant regions in the D-loop of mtDNA. The results revealed that GC had two highly variant regions located at nucleotide position (np) 75-173 and np 314-447; CC had a highly variant region located at np 307-476, which was similar to the second highly variant region of GC; and RC had two highly variant regions located at np 16069-16177 and np 517-576, which were significantly different from the locations in GC and CC (Fig. 3A). Then, the sequences in the mtDNA D-loop region from tumor and

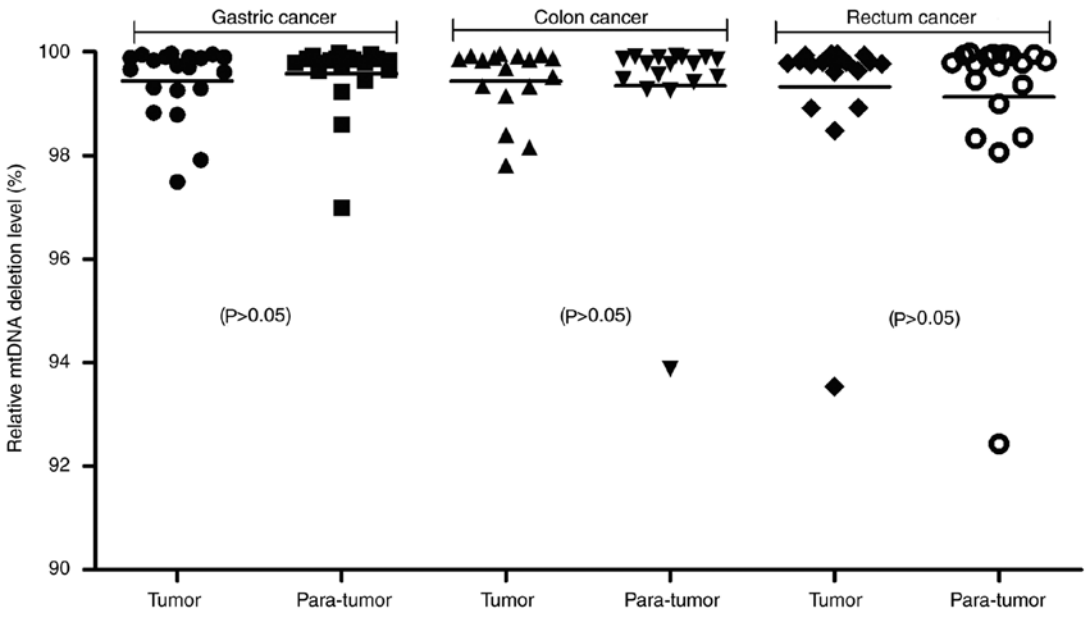

Figure 1. No increases in mtDNA loss were observed in gastrointestinal cancer. The calculated percentage of mtDNA copies are presented for tumor and para-tissues from patients with gastric, colon and rectum cancer. mtDNA, mitochondrial DNA.

para-tumor tissues from 18 GC, 21 CC and 30 RC patients were directly analyzed. The results demonstrated a total of 221 mutations in 196 sequences from the tumor tissues of the 18 patients with GC; this number was significantly higher than that observed in the paired para-tumor tissues, which contained 141 mutations in 179 sequences (P<0.05). Similarly, a high frequency of mutation(s) was identified in the 21 patients with CC (153 mutations in 155 sequences from the tumor tissues vs. 107 mutations in 154 sequences from the para-tumor tissues) and the 30 patients with RC (166 mutations in 183 sequences from the tumor tissues vs. 104 mutations in 168 sequences from the para-tumor tissues; both P<0.05; Table II). These results indicated that all three types of GIC contained more somatic mutations than the normal para-tumor tissues. Based on the delineation of the functional regions of the mtDNA D-loop in previous reports (19,20), the mutation sites were analyzed, and it was revealed that these mutations clustered in the replication origin of the H-strand (P<0.05) and conserved sequence block 2. Furthermore, GC and RC also possessed somatic mutation(s) in an unknown functional region (P<0.05; Fig. 3B; Table II).

More severe mtDNA D-loop mutations are observed in advanced stages of GIC. It is well known that tumor staging is an important prognostic factor for malignant neoplasms (21). In the present study, the mutation rate of the mtDNA D-loop region between tumors of different stages and para-tumor tissues were compared. The results demonstrated that the mean mutation rate of the mtDNA D-loop region was significantly higher in stage III-IV GC tissues when compared with in para-tumor tissues (1.46±1.02 vs. 0.75±0.38; P<0.05); however, the increase in the D-loop mutation rate was not significant in stage I-II GC (1.04±0.22 vs. 0.84±0.05; P>0.05; Fig. 4A). Similar analysis revealed that early and advanced stages of CC and RC had a significantly higher D-loop mutation rate when compared with para-tumors: 1.01±0.43 vs. 0.55±0.16 in stage I-II CC; 1.62±1.39 vs. 0.66±0.41 in stage III-IV CC; 0.94±0.39 vs. 0.72±0.32 stage I-II RC; and 1.39±1.56 vs. 0.73±0.37 stage III-IV RC (all P<0.05). The mutation rates of stage III-IV tumors were greater when compared with those of stage I-II tumors (Fig. 4B and C). These results indicated that the later the GC stage, the more severe the mtDNA D-loop mutations.

Homoplasmic mutations of the mtDNA D-loop in GIC. Certain studies have indicated that mtDNA mutations in the coding regions of CRC are primarily transitions; A-T and G-C are common in the D-loop region (22). In the present study, the mutation types in the mtDNA D-loop in GIC were also analyzed. The results revealed that of the single-base mutations, transitions accounted for 83.86% in GC, 96.74% in CC and 93.9% in RC. The T-C base substitution was the most common (44.11% in GC, 52.29% in CC and 45.12% in RC), followed by C-T (20.10% in GC, 22.88% in CC and 18.29% in RC); and the G-A transition was relatively rare (2.94% in GC, 5.23% in CC and 2.44% in RC; Table III). These results suggested that the mutations of the mtDNA D-loop were primarily homoplasmic in GIC and were often transitions at pyrimidine sites.

Mitochondrial MSI (mtMSI) in GIC. mtMSI has been reported to frequently occur in GC and CRC (23,24). In the present study, the mtMSI in 69 Chinese patients with GIC were analyzed. It was revealed that polynucleotide stretches were common and resulted from a 1-3 cytosine insertion or cytosine deletion. Among them, the percentage of sequences containing a continuous 10-cytosine stretch was 11.7% in GC, 13.8% in CC 11.7 and 20.9% in RC. The percentage of sequences containing a continuous 7-cytosine stretch was 40.3% in GC, 68.6% in CC 11.7 and 47% in RC (Table IV). Furthermore, repeated AC stretches were observed in GC, which were caused by a 1-2 CA insertion or CA deletion, and the total

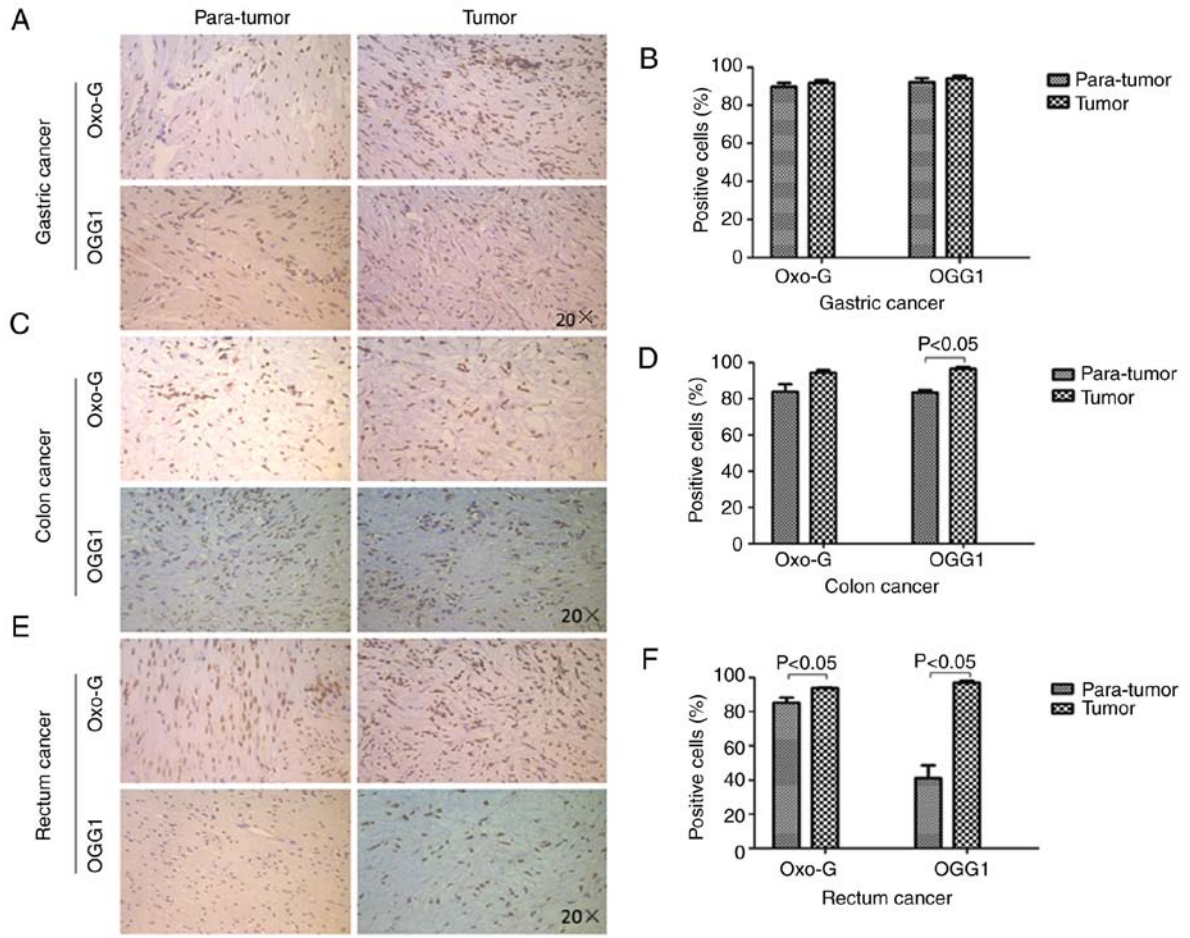

Figure 2. DNA oxidative damage and over-activated DNA repair in gastrointestinal cancer: (A and B) Gastric cancer, (C and D) colon cancer and (E and F) rectum cancer. (A, C and E) Representative immunohistochemical staining demonstrating the morphology of tissues (magnification, x20). (B, D and F) Graphs comparing the number of immunohistochemically-positive cells between the tumor and para-tumor groups. P<0.05, as indicated. oxo-G, 8-hydroxyguanine; OGG1, 8-oxo-20-deoxyguanosine glycosylase 1.

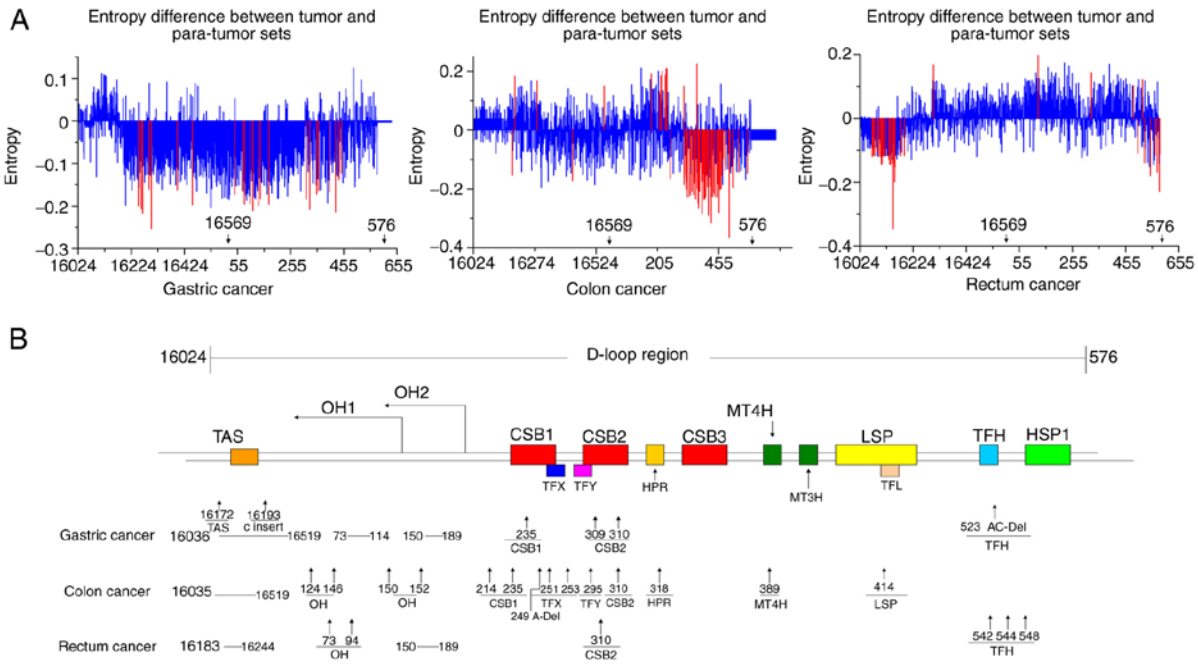

Figure 3. Somatic mitochondrial DNA D-loop mutations in gastrointestinal cancer. (A) Difference in Shannon entropy between tumor and para-tumor tissues in gastrointestinal cancer. Residue-specific entropy was computed and significant sites (P<0.05) are presented in red on the plots. (B) A diagrammatic sketch presenting the mtDNA D-loop mutations clustered in the replication OH1, the CSB2 and the unknown functional regions. OH1, origin of the H-strand; CSB2, conserved sequence block 2.

Table II. Distribution of mtDNA D-loop mutations in gastrointestinal cancer.

	Gastric cancer			Colon cancer			Rectum cancer		
Mitomap	Tumor (n[a]=196)	Para-tumor (n=179)	P-value	Tumor (n=153)	Para-tumor (n=154)	P-value	Tumor (n=183)	Para-tumor (n=168)	P-value
OH1	135	87	<0.05	68	40	<0.05	85	48	<0.05
OH2	-	-	-	16	12	>0.05	18	12	>0.05
CSB1	1	1	-	6	4	-	-	-	-
TFX	-	-	-	3	0	-	-	-	-
TFY	-	-	-	1	0	-	-	-	-
CSB2	17	8	>0.05	17	16	>0.05	16	11	>0.05
HPR	-	-	-	1	1	-	-	-	-
CSB3	-	-	-	-	-	-	-	-	-
MT4H	-	-	-	-	-	-	-	-	-
MT3H	1	0	-	15	11	>0.05	-	-	-
LSP	-	-	-	1	0	-	-	-	-
TFL	-	-	-	-	-	-	-	-	-
TFH	9	7	-	-	-	-	3	0	-
HSP1	-	-	-	-	-	-	-	-	-
TAS	7	6	-	1	1	-	-	-	-
UNKNOW	51	32	<0.05	25	22	>0.05	44	33	<0.05
Sum	221	141	<0.05	154	107	<0.05	166	104	<0.05

The data were statistically analyzed using a nonparametric paired Chi-square test. n[a] refers to the sequence number. The value in the table equals the base mutation number. OH, replication origin of H-strand; CSB, conserved sequence block; LSP, L-strand promoter; mt-/mtDNA, mitochondrial DNA; TF, mtTF1 binding site; TFY, mtTF1 binding site; HPR, Replication primer; MT4H, mt4 H-strand control element; MT3H, mt3 H-strand control element; TFL, mtTF1 binding site; TFH, mtTF1 binding site; HSP1, Major H-strand promoter; TAS, Termination-associated sequence; UNKNOW, unknown functional area.

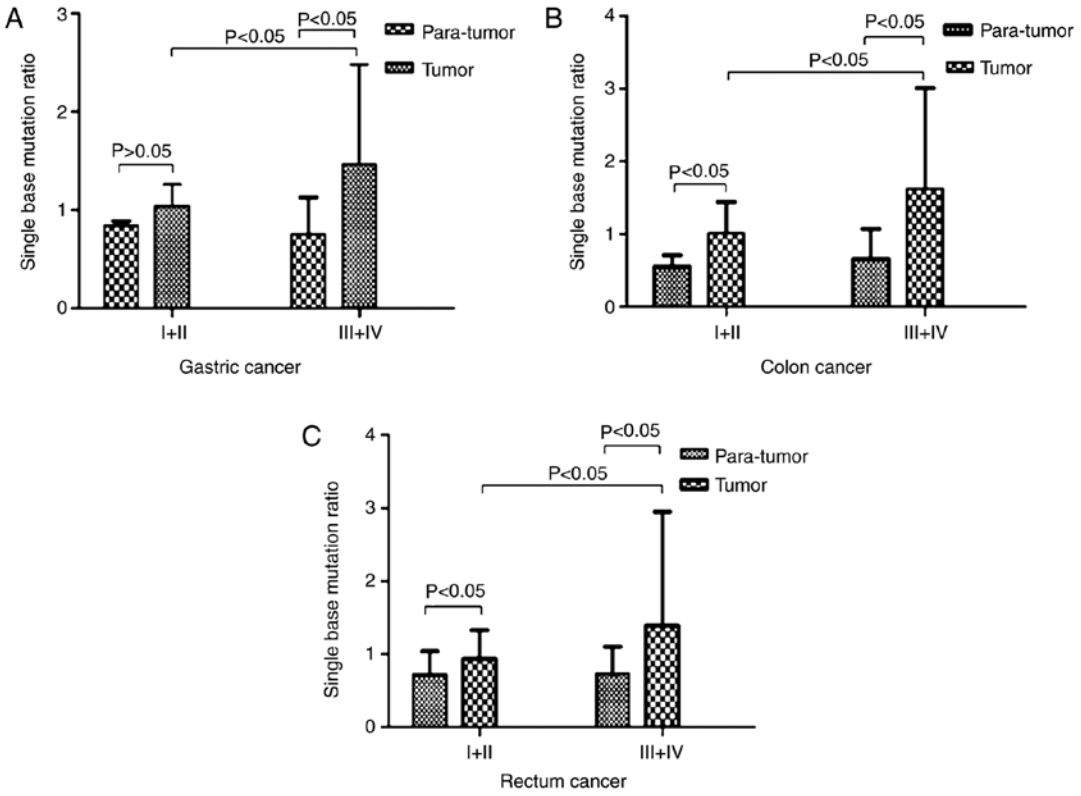

Figure 4. More severe mitochondrial DNA D-loop mutations are present in the advanced stages of gastrointestinal cancer: (A) Gastric, (B) colon and (C) rectum cancer. Stages I-II were defined as early cancer stages and stages III-IV as advanced cancer stages. P<0.05, as indicated.

Table III. Subtypes of single base mutation in gastrointestinal cancer.

	Gastric cancer			Colon cancer			Rectum cancer		
Mutation subtype	Sites	Tumor	PCT (%)	Sites	Tumor	PCT (%)	Sites	Tumor	PCT (%)
Transition									
A-G	12	34	16.67	6	25	16.34	6	46	28.05
G-A	4	6	2.94	6	8	5.23	3	4	2.44
C-T	14	41	20.10	10	35	22.88	8	30	18.29
T-C	18	90	44.11	18	80	52.29	13	74	45.12
Sum			83.86			96.74			93.9
Transversion									
A-C	2	11	5.39	1	4	2.61	1	8	4.88
A-T	1	1	0.49	0	0	0	0	0	N/A
C-A	0	0	0	1	1	0.65	1	1	0.61
C-G	0	0	0	0	0	0	1	1	0.61
G-C	1	1	0.49	0	0	0	0	0	N/A
T-G	1	20	9.80	0	0	0	0	0	N/A
Sum			16.14			3.26			6.1

Where 'Sites' denotes the number of mutation sites; 'Tumor' denotes the absolute counts of base mutations in a tumor; and 'PCT (%)' denotes the percentage of base mutations in tumors. N/A, not applicable; PCT, percentage.

Table IV. Mitochondrial microsatellite instability in gastrointestinal cancer.

	CCCCCTCCCC (16184-16193[a], T→C,(C)10[b]) (%)				CCCCCCC (303-309, (C)7) (%)				CACACACA CA (515-524 (CA) 5) (%)
Type	Gastric cancer	Colon cancer	Rectum cancer	Type	Gastric cancer	Colon cancer	Rectum cancer	Type	Gastric cancer
C1 insert	7.1	7.2	7.7	C1 insert	34.7	45.1	35.0	CA1 insert	4.1
C2 insert	3.6	3.9	4.4	C2 insert	5.6	15.0	10.9	CA2 insert	1.5
C3 insert	1.0	2.0	2.2	C3 insert	0	5.9	1.1	CA1 deletion	31.6
C1 deletion	0	0.7	6.6	C3 deletion	0	2.6	0	-	-
Sum	11.7	13.8	20.9	Sum	40.3	68.6	47	Sum	37.2

[a]Refers to the mutation site; [b]Refers to the repeat base number.

percentage of this sequence was 37.2% (Table IV). The formation of the polynucleotide stretch is presented in Fig. 5. These results suggested that mtMSI occurs in Chinese patients with GIC.

Discussion

It is thought that the inactivation of mitochondrial energy metabolism does not occur in cancer cells with mutations in mitochondrial genes; however, the mitochondrial bioenergetic and biosynthetic state may be altered through a series of modulations of signal transduction pathways between the nucleus and mitochondria (25,26). Although certain studies do not support the idea of D-loop alterations of the mtDNA genome and their carcinogenic role in CRC (27,28), increased mtDNA mutations, deletions and even mitochondrial dysfunction have been identified in GIC (29-33), and even in certain precancerous lesions, including ulcerative colitis lesions and adenomatous polyps (34). Mitochondrial dysfunction is associated with tumor development and progression (35). Various mtDNA mutations have been observed to modify tumor progression depending on the level of respiratory complex I (36), and defective mitochondrial respiration may be restored and tumor-forming ability regained via mitochondrial acquisition (37). In GC, the mtDNA repair system does not appear to be disrupted (38). The present study supports the

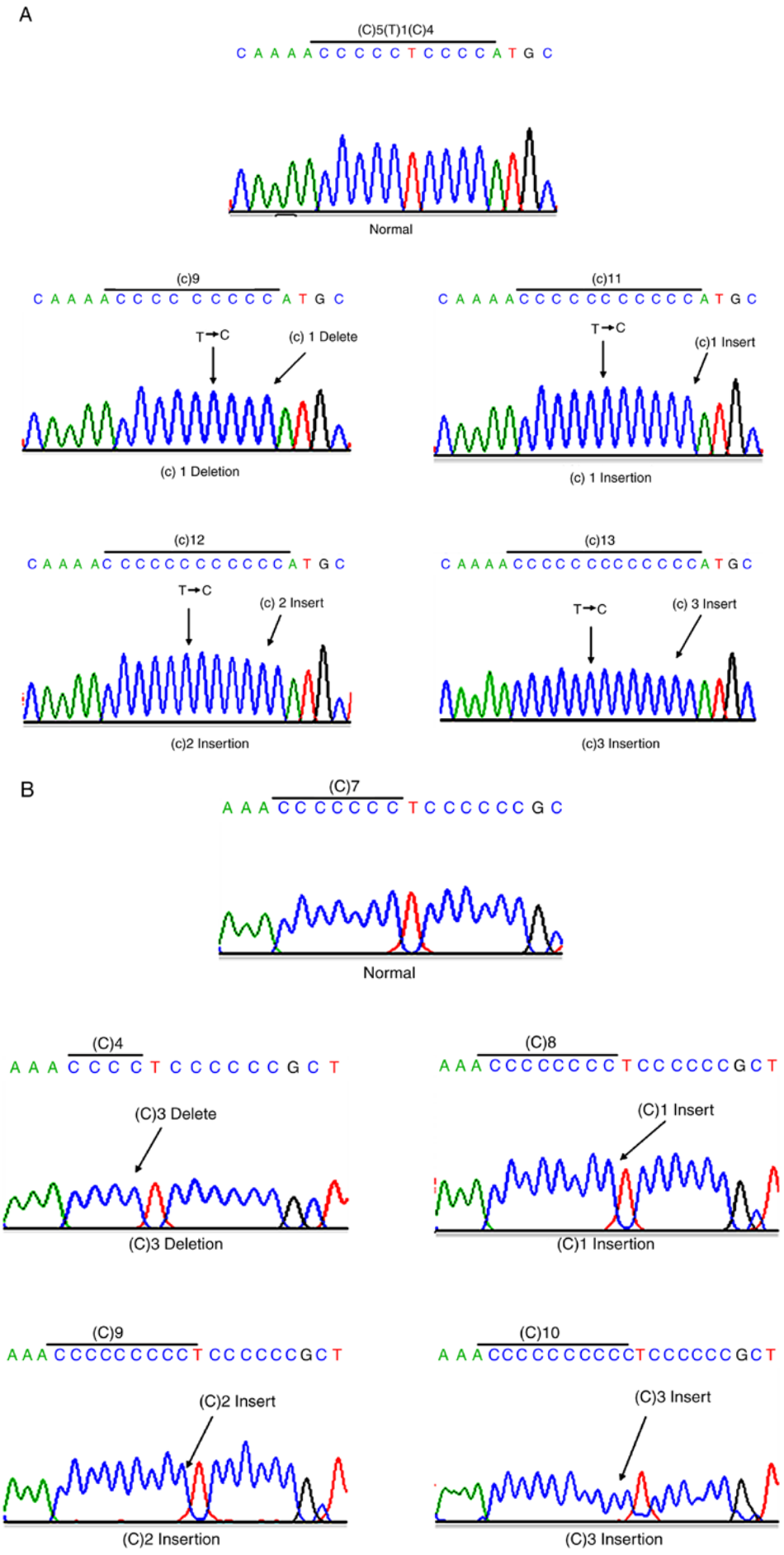

Figure 5. Formation of polynucleotide stretches. (A and B) The formation of polynucleotide C stretches.

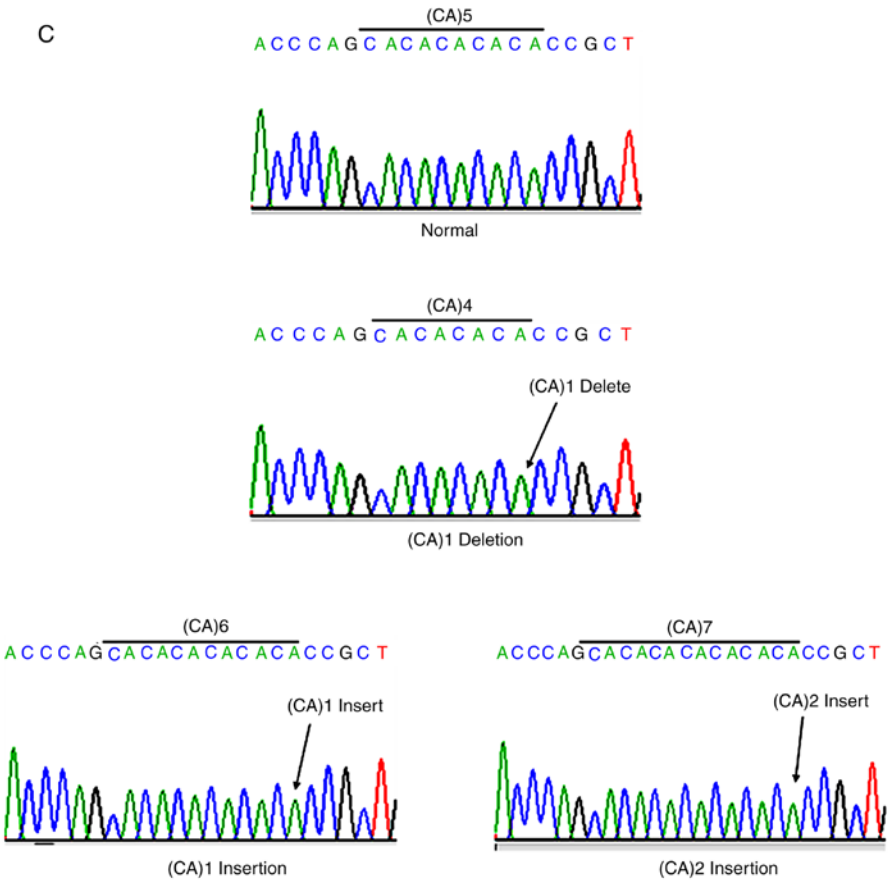

Figure 5. Continued. (C) Formation of repeated CA stretches. The letters in parentheses indicate the base type and the numbers following the parentheses indicate the number of bases.

occurrence of mtDNA D-loop mutations, but does not provide evidence of mtDNA deletions in Chinese patients with GIC; more severe mtDNA D-loop mutations may be identified in the advanced stages of GIC.

A previous study focused on the location of the tumorigenic mtDNA D-loop mutations, and identified carcinogenesis-specific nucleoside sites and poly-C variations (39). In addition, one study indicated that the np 16189 T-C transition of the mtDNA D-loop may contribute to polyC instability in GC (19). Furthermore, another previous study reported that the minor haplotype of nucleotide 16290T and the frequent haplotype of nucleotide 16298T in the hypervariable segment 1 region were associated with a high survival rate of CRC, and the nucleotide site of 16290 was an independent predictor of CRC (40). However, a controversial opinion is that site-specific nucleotide mutations may result from mtDNA heterogeneity, and may not contribute to carcinogenesis and/or tumor progression (41). Thus, single nucleotide polymorphisms in the D-loop of mtDNA have been reported to be associated with an increased risk of GC and CC, including the frequent alleles of 73G/A, 146T/C, 195T/C, 324C/G, 16261C/T and 16304T/C; additionally, the majority of mtDNA mutations are transitions (42-44). In the present study, the T-C transition was the most common and clustered in specific areas in GIC; poly-C variations were evident in GIC.

It has been reported that mtMSI is a frequent occurrence in CRC (45), and mtDNA D-loop mutations and mtMSI appear to be associated with reactive oxygen species, apoptosis and proliferation in GC (46). To the best of our knowledge, no association has been identified between mtDNA mutations and mtMSI status, and no mtMSI-positive GC cases have exhibited large deletions in mtDNA (44). Furthermore, mtMSI appears to be particularly frequent at the D310 locus; however, the high prevalence of mtMSI was not associated with the prognosis of patients with CRC (23,47). Notably, a previous study reported that stromal mtMSI may have possibly served an independent role in the pathogenesis of CC (48). In the present study, in addition to the poly-C stretch, a CA repeat sequence that was involved in GC-associated mtMSI may have been identified in GC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

BW collected the clinical data, analyzed the sequencing results and was a major contributor in writing the manuscript. LQ and JZ performed RT-qPCR. YW and DC conducted immunohistochemistry staining. HG and YZ assisted with the experiments and with writing the article. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committees of Shandong Cancer Hospital (Shandong, China). Written informed consent was obtained from all study participants.

Patient consent for publication

All patients agreed to the publication of their results and signed informed consents.

Competing interests

The authors declare that they have no competing interests.

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