Correlation between increased ND2 expression and demethylated displacement loop of mtDNA in colorectal cancer

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Abstract. Change in cellular glucose metabolism is considered to be a biochemical hallmark in cancer cells. The mitochondrion is the key organelle in which glucose metabolism occurs. However, whether DNA methylation at the displacement loop (D-loop) region of mitochondrial DNA (mtDNA) has an effect on the expression of the rate-limiting enzyme, and, therefore, on oxidative phosphorylation in colorectal cancer remains to be determined. Quantitative change in ND2 (a subunit of NADH) and the methylation status of the D-loop were observed during the initiation and progression of colorectal cancer. Furthermore, the possible correlations with clinicopathological stage were also investigated. Tumor and corresponding non-cancerous tissues were surgically resected from 44 colorectal cancer patients between 2008 and 2009. Cox IV expression was quantified in all of the specimens, and the ND2 expression was calculated. Quantitative changes in ND2 expression exhibited a significant increase. The average relative ratios of ND2 content were 1.67±0.44 in the tumor tissues and 0.89±0.44 in the corresponding non-cancerous tissues ($p<0.01$). In addition, the D-loop of most corresponding non-cancerous tissues was methylated and the percentage was 79.5%, while this percentage was much smaller in the tumor tissues (11.4%). Following correlation with clinicopathological data, changes in the ND2 expression in the colorectal cancer exhibited a significant association with clinicopathological stage. This increase was significant as early as in stage I. Furthermore, the ratios of unmethylated D-loop cases were increased in both tumor and corresponding non-cancerous tissues, and the ND2 expression was also increased from stages I to IV. Our results indicate that demethylation of the D-loop plays a key role in regulating ND2 expression during the initiation and/or progression of colorectal cancer.

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Introduction

Colorectal cancer is one of the most widespread malignant tumors in humans. It is also the third most common cause of cancer-related death in the Western population (1). Following the research of Otto Warburg, the Noble laureate of 1931, an ever-growing number of researchers have studied the metabolic activity of cancer cells. In 2006, Shaw concluded that a change in cellular glucose metabolism is the first biochemical hallmark in cancer cells (2). Initially, researchers believed that cancer cell energy is supplied by glycolysis. Nowadays, accumulating evidence indicates that the energy supply of cancer cells is not provided by glycolysis alone. In 2004, Zu and Guppy asserted that there is no evidence that cancer cells are inherently glycolytic, but that some tumors might indeed be glycolytic *in vivo* as a result of their hypoxic environment (3). Furthermore, a large number of reports have shown that the process of oxidative phosphorylation in cancer is altered (4-7).

Several studies have already proven that genetic and epigenetic alterations play an important role during the initiation and progression of colorectal cancer. Human mitochondrial DNA (mtDNA) exists inside the mitochondrion within the cytoplasm. It is the only genetic material that exists outside the cellular nucleus. mtDNA is a closed circular duplex species of 16,569 bases, which encodes 13 respiratory chain polypeptides, as well as 2 ribosomal and 22 transfer RNAs (8). In addition, mtDNA contains a noncoding region called the displacement loop (D-loop), which controls the replication and the transcription of mtDNA (9,10). DNA hypermethylation occurs at the CpG islands and is typically associated with gene silencing. Numerous studies have demonstrated that genes with high levels of methylcytosine in their promoter region are usually transcriptionally silent. Such hypermethylation has been reported to be associated with a large number of human malignancies, other non-neoplastic diseases and aging (11). Moreover, DNA hypermethylation also regulates DNA replication (12). Furthermore, as previously described, the D-loop region controls transcription of mtDNA; however, whether DNA hypermethylation of the D-loop region is involved in the regulation of mtDNA expression in colorectal cancer remains unknown.

The metabolic level is associated with the expression level of the rate-limiting enzyme. The NADH is the rate-limiting enzyme of oxidative phosphorylation, and its subunit, ND2,

is encoded by mtDNA. In order to detect the potential link between oxidative phosphorylation and the hypermethylation status in the D-loop region of mtDNA in colorectal cancer, we investigated the hypermethylation status in the D-loop region and the relative ND2 content of 44 colorectal cancer cases. The *in vitro* demethylation experiment was carried out on primary rat colon cells.

Materials and methods

Patients and specimens. Tumor and corresponding non-cancerous tissues from 24 colon and 20 rectal cancer patients were surgically resected at West China Hospital, Sichuan University, during the years of 2008-2009. Of these 44 colorectal patients, 25 were males and 19 were females, aged between 33-74 years (51.3±9.9) (Table I). All specimens were immediately fresh frozen after resection and stored in liquid nitrogen. Tumor tissue specimens were cut from the edge of the tumors, and their corresponding non-cancerous tissues were taken from at least 5 cm away from the tumors.

mtDNA extraction. A freezing microtomy was used for methylation-specific PCR (MSP). Each 10-μm section was stained with haematoxylin. A dissecting needle was used under a microscope to dissect tumorous cells from tumor tissues and corresponding cells from non-cancerous tissues. For each specimen, 4000-5000 cells were needed. Then, a cell/tissue Mito isolation kit (GenMed Scientifics Inc., USA) was used to extract the mtDNA, according to the manufacturer's instructions.

Methylation-specific PCR. Following extraction, mtDNA was subjected to bisulfite modification, and was then immediately analyzed using the MSP amplification kit (GenMed Scientifics Inc.), following the manufacturer's instructions. Furthermore, modified mtDNA was used for MSP for the detection of the methylation status of the D-loop region. Primer sequences were: forward, 5'-TGTTTCGGTTTTAGCGTTTC-3' and reverse, 5'-TACTACTCTCCTCGCTCCGA-3' (methylated); forward, 5'-GGGTGTTTTGGTTTTAGTGTTTT-3' and reverse, 5'-ATACTACTCTCCTCACTCCAAAC-3' (unmethylated). The PCR cycling conditions were: an activation step at 95˚C for 5 min, then 35 cycles of 30 sec at 95˚C, 30 sec at 56˚C and 40 sec at 72˚C. Universal methylated DNA was used as a positive control and ddH_2O was used as a negative control.

Western blotting. ND2 levels were quantified by standard western blotting procedures, using the rabbit anti-human ND2 antibody (Abcam; dilution 1:400). The Cox IV protein was used as an internal control, using the rabbit monoclonal anti-Cox IV antibody (Bioss; dilution 1:400).

Primary rat colon cell culture and the demethylation experiment. Primary rat colon cells were cultured as previously described (13). 5-Aza-2'-deoxycytidine (5-Aza, Sigma, USA) was used to cause mtDNA demethylation. Cells ($5x10^5$) were seeded into 6-well plates in 2 ml of medium. After 24 h of incubation, the medium was removed and cells were incubated in 2 ml of fresh medium containing a final concentration of 5 μM 5-Aza for 96 h. After treatment, the medium was removed and the cells were subjected to an additional 24-h incubation in 2 ml of fresh medium without 5-Aza. Cells were collected for MSP and western blotting prior to and following the treatment with 5-Aza.

Table I. Relationship between D-loop region methylation and clinicopathological parameters in 44 colorectal cancer cases.

		Methylated D-loop (%)	
	No. of cases	T	C
Total	44	5 (11.4)	35 (79.5)
Gender			
Male	25	2 (8)	21 (84)
Female	19	3 (15.8)	14 (73.7)
Mean age (years)			
>50	24	2 (8.3)	21 (87.5)
≤50	20	3 (15)	15 (75)
Clinicopathological stage			
I	10	2 (20)	10 (100)
II	16	2 (12.5)	14 (87.5)
III	9	1 (11.1)	6 (66.7)
IV	9	0 (0)	6 (66.7)

D-loop, displacement loop; T, tumor tissue; C, corresponding non-cancerous tissue.

Statistical analysis. Statistical calculations were performed with the statistical package SPSS version 13.0. The relative ND2 expression of each specimen was calculated. Student's t-test was used to analyze the quantitative data. All quantitative data are expressed as the mean ± standard deviation. $p<0.05$ denoted a statistically significant difference.

Results

Methylation of the D-loop in patients and correlation with clinicopathological stage. The MSP results are shown in Fig. 1A, whereas the correlation between D-loop methylation and clinicopathological parameters are presented in Table I. The MSP results showed that in all 44 colorectal cancer cases the D-loop of most tumor tissues was demethylated, while in the corresponding non-cancerous tissues most of the D-loops were partially methylated. Clinical features, age and gender, were not correlated with the methylation status at the D-loop. However, the percentage of methylated cases for different clinicopathological stages suggest that the D-loop was particularly prone to demethylation status in stage IV cases. The D-loop was methylated in 2 out of 10 (20%) stage I cases, although none of the 9 stage IV cases were methylated, and this difference was significant ($p<0.05$). In corresponding non-cancerous tissues, the D-loop of all 10 stage I cases was methylated (100%), but in stage VI the percentage was 66.7%; $p<0.05$.

Relative ND2 content of the colorectal cancer tissues. To identify the manner in which ND2 expression was altered

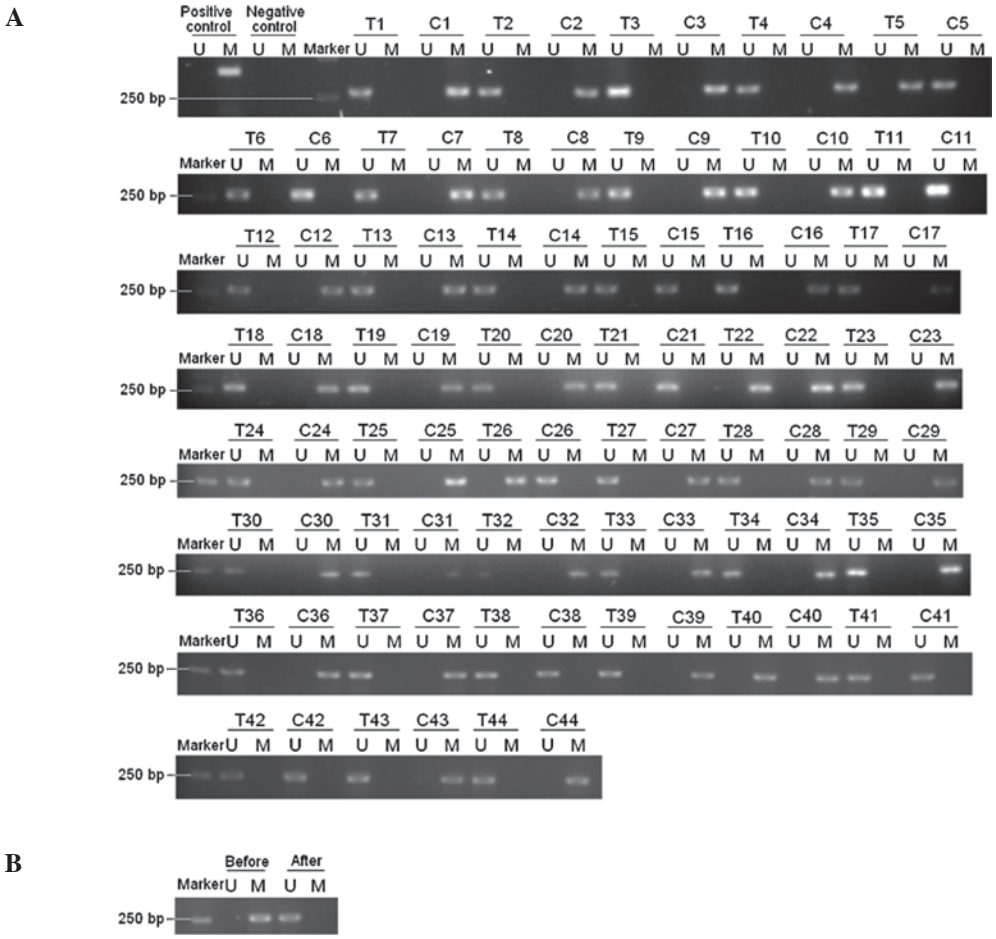

Figure 1. Methylation-specific PCR (MSP) results of the displacement loop (D-loop) region of the (A) 44 colorectal cancer cases and (B) the demethylation experiment on primary rat colon cells. T, tumor tissue; C, corresponding non-cancerous tissue; U, unmethylated; M, methylated.

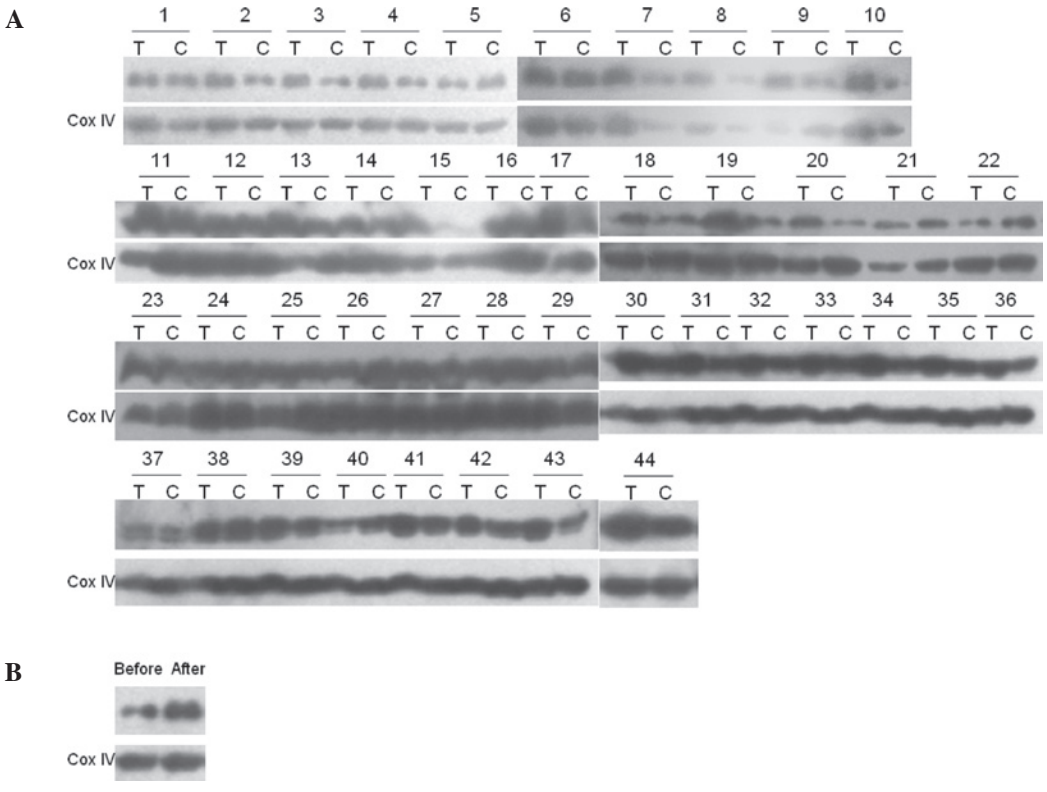

Figure 2. Western blotting results of the relative ND2 content in the (A) 44 colorectal cancer cases and (B) the demethylation experiment on primary rat colon cells. T, tumor tissue; C, corresponding non-cancerous tissue. Cox IV protein was used as the internal control.

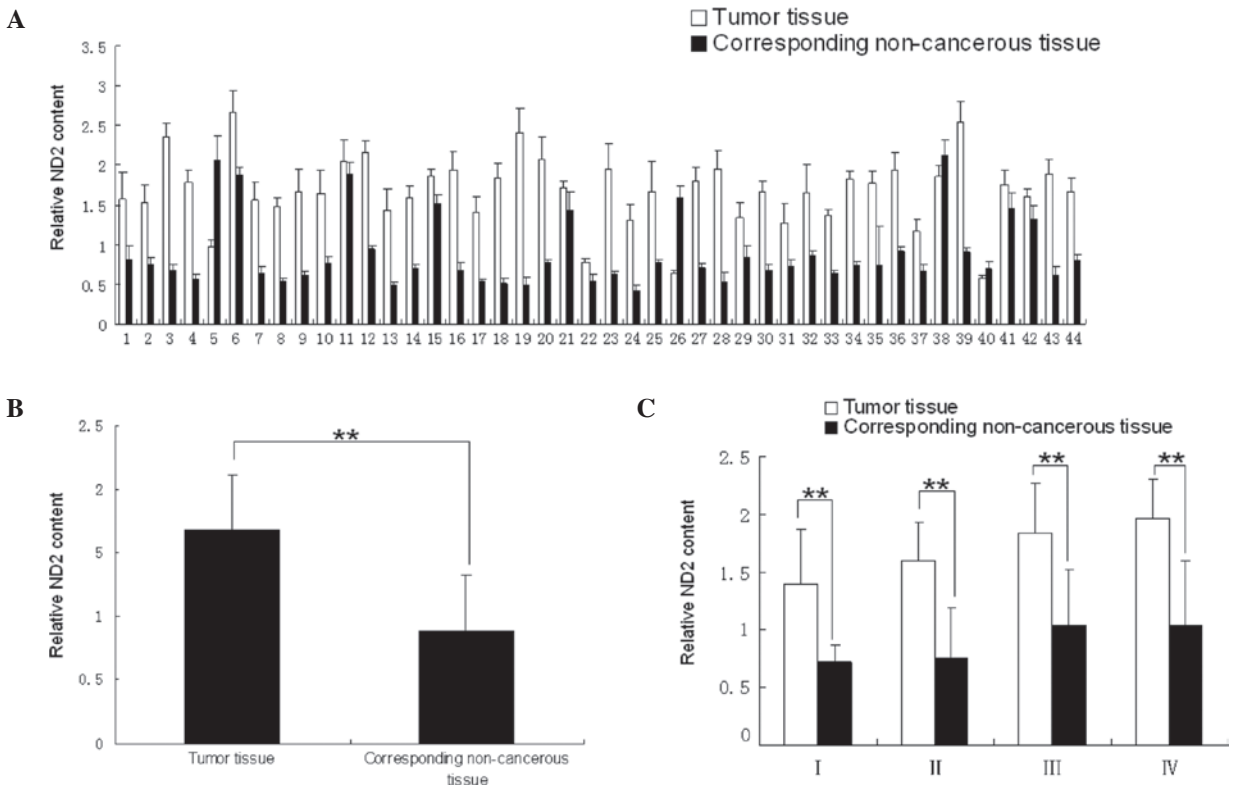

Figure 3. (A) Comparison of the relative ND2 content between the tumor and corresponding non-cancerous tissues of colorectal cancer in each case. Each bar represents the mean values ± SD from three independent experiments. (B) Comparison of the average relative ND2 content between the 44 cases of tumor tissues and their corresponding non-cancerous tissues. The ratio of tumor tissues was significantly higher in comparison to the corresponding non-cancerous tissues ($p<0.01$, Student's t-test), $^{**}p<0.01$. (C) Comparison of the average relative ND2 content of each clinicopathological stage. I-IV, clinicopathological stage; T, tumor tissue; C, corresponding non-cancerous tissue.

within the tumor tissue of the colorectal cancer, ND2 levels of the tumor and the corresponding non-cancerous tissues were evaluated by western blotting (Fig. 2A). Following statistical analysis, the relative ND2 content was markedly increased in 40 colorectal cancer cases (90.1%), when compared to the non-cancerous tissues (Fig. 3A). The average relative ND2 content ratios were 1.67±0.44 in the tumor tissues and 0.89±0.44 in the corresponding non-cancerous tissues. This difference was significant ($p<0.01$) (Fig. 3B).

Correlation between relative ND2 content and clinicopathological stage. The correlation between the clinicopathological stage and relative ND2 content was analyzed. Fig. 3C shows the relationship between the relative ND2 content of the tumor and corresponding non-cancerous tissues in each stage. Stages I and II were defined as group 1, and stages III and IV as group 2. The relative ND2 content of the tumor tissues was markedly higher compared to that of the corresponding non-cancerous tissues in both groups 1 and 2. A significant difference was noted between the two groups regarding the relative ND2 content between the tumor tissues ($p<0.01$). Moreover, this difference between the corresponding non-cancerous tissues was significant ($p<0.05$) (Table II).

Demethylation experiment. Prior to and following the treatment with 5-Aza, the primary rat colon cells were collected for MSP and western blot analysis. The MSP results confirmed that the 5-Aza treatment changed the D-loop region from a methylation to demethylation status (Fig. 1B). The average relative ND2 content ratios prior to and following the demethylation treatment were 0.55±0.14 and 1.31±0.36, respectively ($p<0.01$, Fig. 2B).

Table II. Average relative ND2 content of groups 1 (stages I and II) and 2 (stages III and IV).

	Group 1	Group 2
T	1.49±0.4[a,c]	1.90±0.41[c]
C	0.74±0.33[b]	1.03±0.52

Values are expressed as the mean ± standard deviation (SD). T, tumor tissue; C, corresponding non-cancerous tissue. [a]$P<0.01$ between groups 1 and 2; [b]$p<0.05$ between groups 1 and 2; [c]$p<0.01$ between T and C within each group.

Discussion

All human cells, except erythrocytes, contain mitochondria which produce almost 95% of the cell energy through the process of oxidative phosphorylation. In addition, the D-loop region controls the replication and the transcription of mtDNA. Several studies have already noted that epigenetic changes on the DNA control region could lead to the functional disorder of DNA replication and expression (14,15). Furthermore, the

existence of DNA methyltransferases inside the mitochondria has been confirmed (16). All these data indicate that DNA methylation might be a method by which cells regulate their mtDNA expression.

In the present study, MSP was carried out to detect the methylation status of the D-loop region in tumor and the corresponding non-cancerous tissues. Our results showed that in the tumor tissues the D-loop was prone to have a demethylation status, while in the corresponding non-cancerous tissues it was prone to have a methylation status (Table I). Table I also shows that in the tumor tissues the percentage of methylated D-loop cases was decreasing from the clinicopathological stage I to IV, until the percentage was 0% in stage IV, while in the corresponding non-cancerous tissues, although the percentage also decreased from the clinicopathological stage I to IV, in each stage the methylated D-loop percentage was markedly higher in the corresponding non-cancerous tissues compared to that in the tumor tissues. Even in stage IV the percentage was 66.7%. This indicates that the D-loop region changes to a demethylation from a methylation status during the initiation and/or progression of colorectal cancer, and the longer the progression is, the more obvious the demethylation will be. Moreover, in the late clinicopathological stages, the paraneoplastic tissue also shows demethylation of the D-loop in mtDNA, which is considered to be an effect of the tumor tissue. There is currently no systemic theory explaining whether the epigenetic change leads to a genetic one in cancer cells. However, research indicates that the epigenetic change is prior to the genetic change during the initiation of the cancer (17), and that it is the epigenetic change that leads to the genetic one (18). Based on the previous data we hypothesized that the demethylation of the D-loop is an early molecular event of colorectal cancer.

The D-loop region is the control region of the mtDNA, and numerous studies have shown that alterations in this region may have a significant effect on the mtDNA expression (19-21). Our results suggest that colorectal cancer tissue with a demethylated D-loop might be associated with the high expression of protein encoded by mtDNA and the high existence of mtDNA copy number.

Western blotting confirmed that the expression of ND2 was significantly increased in the tumor tissues (Fig. 3B). The correlation between the increased ND2 expression and the clinicopathological staging was also analyzed. Fig. 3C shows that this increase is already marked in stage I, which indicates that the increased ND2 expression is an early molecular event during the initiation and progression of colorectal cancer. ND2 is one of the subunits of NADH which is the rate-limiting enzyme of oxidative phosphorylation. Therefore, the increased ND2 might be associated with the increase of NADH, leading to the improvement of oxidative phosphorylation. Our previous study showed that the increased copy number of mtDNA in tumor tissue is an early molecular event of colorectal cancer (22). It is known that cancer cells require more energy than normal cells, and it has been reported that the levels of a number of mitochondrial and nuclear gene transcripts were increased in rat hepatoma (23). Furthermore, numerous studies related to the aging progress have revealed that the high level of oxidative phosphorylation is usually associated with the high level of mtDNA copy number (24,25). Based on previous data, we postulate that demethylation of the D-loop region might regulate the copy number of mtDNA increase, and the increased mtDNA copy number together with the demethylated D-loop region might regulate an increase in the ND2 expression.

We also observed that the differences in ND2 content between the tumor and the corresponding non-cancerous tissues in stages I and II were significantly lower compared to those in stages III and IV. Moreover, this difference was particularly significant between the tumor tissues of different stages, which indicates that the increase in ND2 content might be an early molecular event during the initiation and progression of colorectal cancer. Furthermore, the ratios of corresponding non-cancerous tissues also showed a significant difference. According to the theory known as 'Field Cancerization' introduced by Slaughter *et al*, before the histopathological changes, the corresponding non-cancerous tissues may have molecular alterations which finally result in cancer (26).

To further explore the interrelationship between D-loop region demethylation and ND2 expression, the demethylation experiment was carried out. We treated the primary rat colon cells using 5-Aza, and performed the MSP and western blotting prior to and following the treatment. MSP confirmed that the demethylation treatment was successful. Western blotting showed that the ND2 expression prior to the 5-Aza treatment was lower compared to that following the treatment, and the difference was statistically significant. This result is in line with the epigenetic theory that demethylated DNA is prone to activate gene expression.

In conclusion, our study showed that the expression of ND2 increases in colorectal cancer. The D-loop regions of most tumor tissues were demethylated, while those of most corresponding non-cancerous tissues were methylated. Moreover, these differences between tumor and corresponding non-cancerous tissues were already significant in the early stages of colorectal cancer. These findings suggest that ND2 expression increases during the progression of colorectal cancer. Furthermore, the demethylation experiment confirmed that the increased expression of ND2 was linked with the demethylation of the D-loop of mtDNA. We hypothesize that the demethylated D-loop region is probably involved in the regulation of ND2 expression during the initiation and/or progression of colorectal cancer.

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