Altered expression of mitochondrial cytochrome *c* oxidase I and NADH dehydrogenase 4 transcripts associated with gastric tumorigenesis and tumor dedifferentiation

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Abstract. This study aimed to detect the expression of mitochondrial mRNA in normal and cancerous gastric tissues and discuss their association with clinicopathological characteristics. Semi-quantitative reverse-transcription PCR was used to analyze mitochondrial transcripts in 42 cases of matched cancerous and normal gastric tissues. Expression of these genes was then statistically analyzed to determine the correlation with the clinicopathological characteristics from the patients. Transcripts of cytochrome *c* oxidase I (CO I) and NADH dehydrogenase 4 (ND4) were highly expressed in gastric cancer tissues compared to normal gastric tissues (P=0.005 and P=0.001, respectively). The expression levels of CO I and ND4 were higher in the diffused type of gastric cancer than in the intestinal type (P=0.024 and P=0.0002, respectively). Elevated expression of CO I and ND4 were associated with gastric tumorigenesis and tumor dedifferentiation *ex vivo*.

Introduction

Mitochondria are membrane-enclosed semiautonomous organelles, of diameter between 0.5 and 10 μM, which are found in the majority of eukaryotic cells. They play a vital role in cellular energy production, reactive oxygen species (ROS) generation and apoptosis (1). Recent evidence has also demonstrated that mitochondria are involved in tumor initiation and progression (2-4). In contrast to other organelles present in cells, mitochondria have their own genome, mitochondrial DNA (mtDNA), which is a double-stranded circular DNA of approximately 16.6 kb, encoding 37 genes, including 22 tRNA, 2 rRNA and 13 protein subunits of respiratory complexes. These 13 proteins form 7 subunits of the respiratory complex I (NADH dehydrogenase 1, 2, 3, 4L, 4, 5 and 6), 1 subunit of the respiratory complex III (cytochrome *b*), 3 subunits of the respiratory complex IV (cytochrome *c* oxidase I, II and III), and 2 subunits of the respiratory complex V (ATP6 and ATP8) (5-6). All mtDNA-encoded polypeptides are essential components for the electron transport chain (ETC) and oxidative phosphorylation (7). However, alterations in mtDNA, including mutations, contribute to tumorigenesis and tumor progression by enhancing the metastatic potential of tumor cells (8).

Transcription of mtRNA can be affected by endogenous and exogenous factors, including ROS, hypoxia or irradiation. However, increased mtDNA transcripts are associated with a decreased cell apoptotic rate, and may be associated with tumorigenesis (9). For example, increased ROS production is one feature of mitochondrial dysfunction. Furthermore, defective genetic variations of mtDNA lead to inefficient electron transfer, causing ROS to leak out from the respiratory chain. The cycle of ROS formation and mtDNA damage are synergistic and exponential, leading to a vicious cycle. Therefore, tumor cells harboring different defective or mtDNA-depleted mitochondria have various altered properties, suggesting a complicated mechanism linking mtDNA to tumorigenesis and phenotype (10-12). However, whether or not mitochondrial transcripts are altered in gastric cancer and how these alterations affect tumor cell behavior has yet to be detemined. This study aimed to detect altered transcripts of mitochondrial cytochrome *c* oxidase I (CO I), NADH dehydrogenase 4 (ND4) and 5, cytochrome *b* and ATPase6 in matched gastric cancer and distal normal gastric mucosa tissues using reverse-transcription PCR, and then to associate these mtDNA transcripts with the pathobiological behaviors of gastric cancer.

Materials and methods

Study population. A total of 42 pairs of gastric cancer tissues and their corresponding distal normal gastric mucosae were obtained from patients who underwent curative resection surgery at the Shengjing Hospital of China Medical University (Shenyang, China) between January 2010 and December 2010. These patients included 32 males and 10 females with a median age of 62 years (range, 40-77 years). Tissues were snap-frozen and routinely sectioned to 4 mm for H&E staining. The diagnosis was made according to the WHO histological classification and Lauren's classification of gastric cancer.

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Clinicopathological data were collected from the medical history of the patients. All patients provided written informed consent to participate in the study, which was approved by our institutional review board.

mtRNA extraction from gastric tissues. mtRNAs were extracted from cancerous and normal gastric tissues from all 42 patients for detection of mitochondrial gene expression. Gastric tissues, 50-100 μg, were ground into powder with liquid nitrogen in a chilled mortar and pestle, and dissolved in TRIzol (Life Technologies Corporation, Grand Island, NY, USA). The protein was separated by phenol/chloroform extraction, and RNA was precipitated using isopropanol. The RNA pellet was then washed with ethanol, air-dried and dissolved in diethylpyrocarbonate (DEPC)-treated water. The RNA was then stored at -80˚C until use.

Reverse transcription polymerase chain reaction (RT-PCR) of mitochondrial transcripts. Semi-quantitative RT-PCR was performed on mtRNA from the patients for detection of the mitochondrial transcriptions of CO I, ND4 and 5, cytochrome *b* and ATPase6. The primers for these genes were designed using Oligo 6.0 (Molecular Biology Insights, Inc., Cascade, CO, USA) and Primer Premier 6.0 software (Premier Biosoft International, Palo Alto, CA, USA) (Table I). β-actin mRNA served as a quantitative and a loading control. Reverse transcription was performed using a Takara AMV RT-PCR kit (Takara, Dalian, China) according to the manufacturer's instructions. PCR amplification was conducted in a Biometra personal PCR system at a final volume of 50 μl. The PCR conditions were set with an initial incubation at 94˚C for 4 min, followed by 30 cycles of denaturation at 94˚C for 30 sec, annealing at 55˚C for 30 sec, extension at 72˚C for 2 min, and a final extension at 72˚C for 4 min. The PCR products were then separated on 2% agarose gel and stained with ethidium bromide. The gel images were scanned using a ChemiImager (Alpha Innotech Corporation, San Leandro, CA, USA), and quantitatively analyzed by Image J software to measure the ratio of mtRNA/β-actin.

Statistical analysis. Expression of mtRNA transcripts were quantified and expressed as the mean ± SD. The Student's t-test and one-way ANOVA were applied with SPSS software (version 13.0 for Windows, Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

In this study, we collected 42 samples of gastric cancer and their corresponding distant normal mucosae for a semi-quantitative RT-PCR analysis of transcripts of mitochondrial CO I, ND4 and 5, cytochrome b and ATPase6. We then associated genetic alterations in these genes with clinicopathological data from the patients.

We found that the expression of CO I and ND4 mRNA was significantly higher in gastric cancerous tissues than normal gastric tissues (P=0.005 and P=0.001, respectively; Table II and Fig. 1). However, there was no statistically significant association of NADH dehydrogenase 5, cytochrome *b*, and ATPase6 between the cancerous and normal tissues (all

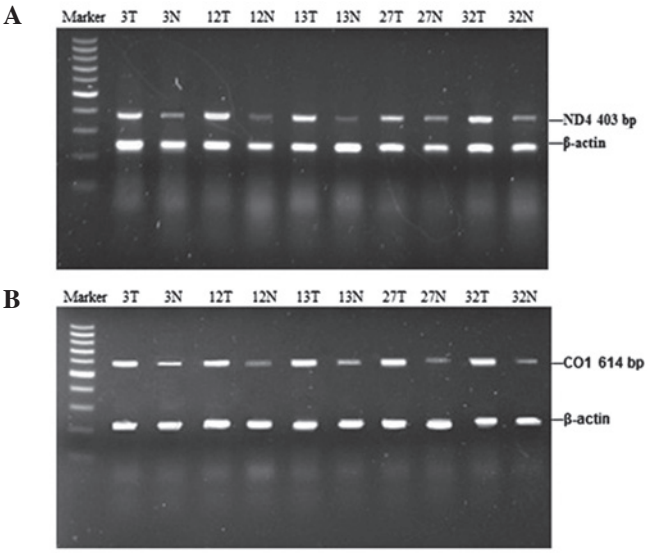

Figure 1. Differential expression of (A) NADH dehydrogenase 4 and (B) cytochrome c oxidase I genes in gastric cancer tissues vs. normal tissues. T, gastric cancerous tissues; N, gastric normal tissues; Marker, molecular marker.

P>0.05). Results of the univariate analysis revealed that the change in the transcripts of mitochondrial CO I and ND4 were negatively associated with tumor differentiation (P=0.034 and P=0.001, respectively). Patients with a poorly differentiated grade of gastric cancer (including poorly differentiated adenocarcinoma, mucous adenocarcinoma and signet-ring cell carcinoma) according to WHO classification had a significantly higher expression of CO I and ND4 than patients with well-differentiated gastric cancers (including papillary adenocarcinoma and well-differentiated adenocarcinoma) (Table II).

Moreover, according to Lauren's classification, the expression levels of CO I and ND4 were higher in diffused gastric cancers compared to intestinal cancers (P=0.024 and P=0.0002, respectively). By contrast, no association was found between the expression of CO I and ND4 and gender, age, Borrmann classification and lymph node metastasis (Fig. 2). In addition, the multivariate analysis revealed that only tumor differentiation was able to markedly affect the expression of CO I and ND4 (P<0.01).

Discussion

In this study, we detected altered transcripts of mitochondrial CO I, ND4 and 5, cytochrome *b* and ATPase6 in normal and cancerous gastric tissues using RT-PCR, and then correlated the expression of these mtDNA transcripts with clinicopathological data from the patients. Our data demonstrated that the expression of CO I and ND4 mRNA was significantly higher in gastric cancer tissues than in normal gastric tissues. Expression of mitochondrial CO I and ND4 was associated with gastric cancer dedifferentiation. However, the remaining three mitochondrial genes were not significantly altered during gastric cancer development. This study suggests that detection of mitochondrial CO I and ND4 expression should be evaluated as biomarkers for the diagnosis of gastric cancer.

The mitochondrial genome is more susceptible to mutagenic changes than nuclear DNA, and alterations in mtDNA

Table I. Primer sequences used to amplify mtRNA.

Genes	Primer	Sequence (5'-3')	Length (bp)	Tm (˚C)	Product (bp)
Cytochrome c oxidase I	F6043	TCTAGGTAACGACCACATCTACAAC	25	62.4	614
	R6656	CGAAGCCTGGTAGGATAA	18	51.9	
ATPase6	F9000	CGCCTAACCGCTAACATTACTG	22	64.2	148
	R9147	AGGCGACAGCGATTTCTA	18	53.8	
NADH dehydrogenase 4	F11581	ATCTGCCTACGACAAACA	18	48.3	403
	R12024	GTGGTGGGTGAGTGAGCCC	19	61.3	
NADH dehydrogenase 5	F13028	CTGACTCCCCTCAGCCATAGA	21	57.2	276
	R13303	TGTGGTTGGTTGATGCCG	18	53.3	
Cytochrome *b*	F15002	GCGCCTCAATATTCTTTATCTGC	23	58.7	305
	R15306	GAAGGGCAAGATGAAGTGAAA	21	53.4	

mtRNA, mitochondrial RNA.

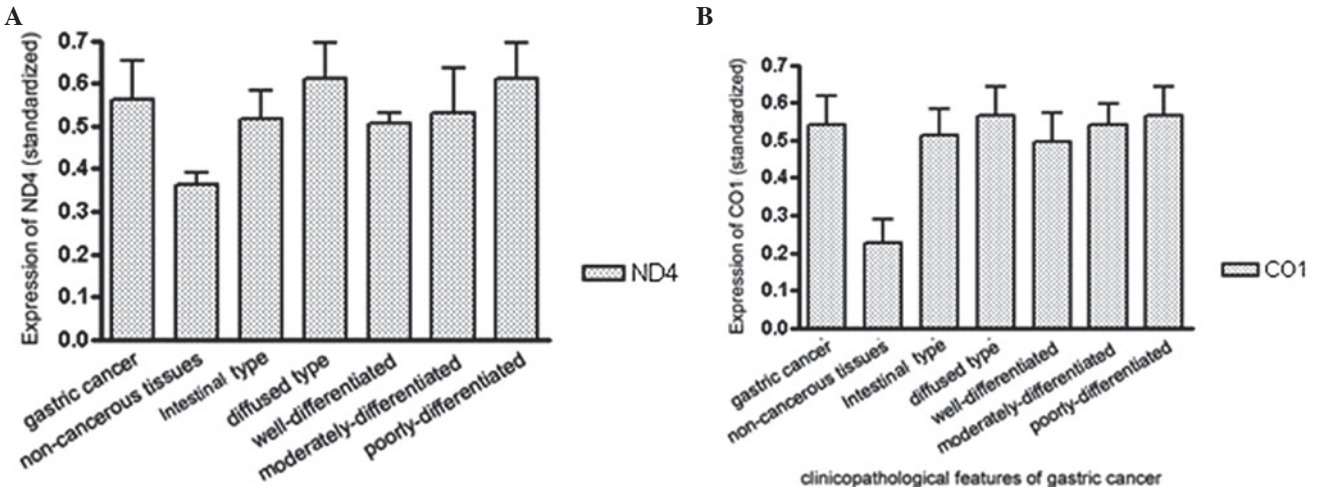

Figure 2. Association of (A) ND4 and (B) CO I expression patterns with different clinicopathological data from gastric cancer patients. ND4, NADH dehydrogenase 4; CO I, cytochrome c oxidase I.

genes contribute to apoptosis, tumor initiation and progression (13-14). Moreover, a previous study reported that there is cross-talk between mitochondrial- and nuclear-derived signals within the cells, and that a coordinated expression of the two genomes is required for proper mitochondrial function (15). Mitochondrial gene expression responds to stimuli, including oxidative stress, hypoxia, calcium load and ceramide, for cell survival or apoptosis. Our current data confirmed that CO I and ND4 transcripts are elevated in gastric cancer tissues, and are associated with tumor dedifferentiation, indicating that the two genes may be involved in gastric tumorigenesis and phenotypes. Another study demonstrated that an elevated expression of other mtDNA genes is associated with reduced cell apoptosis, which may contribute to tumorigenesis (1).

Elevated expression of mitochondrial cytochrome *c* oxidase and NADH dehydrogenase has been reported in different tumors, including hepatoma (16), breast cancer (17) and precancerous diseases (18). Parrella *et al* (17) revealed an increased expression of cytochrome *c* oxidase II in breast cancer compared to matched normal tissues. Yamamoto *et al* (18) demonstrated an induction of NADH dehydrogenase 1 and 16S rRNA expression in familial polyposis coli compared to normal colonic mucosae. Different mitochondrial genes may be elevated to various degrees during tumor progression. Dong *et al* (16) demonstrated that the expression of CO I and II and NADH dehydrogenase 5 in hepatoma were increased 10-, 8- and 5-fold, respectively, whereas mRNA levels of NADH dehydrogenase 1 and ATPase6 were almost identical between tumor and normal hepatocytes. However, our current data only partially supports the findings by Dong *et al* (16). While data from the two studies revealed elevated expression of CO I and no changes in ATPase6, our data did not demonstrate changes in cytochrome *c* oxidase II or NADH dehydrogenase 5. The reason for this discrepancy is unknown, but may be due to the tumor types (hepatocellular carcinoma vs. gastric cancer) and study population, including tumor stage and differentiation.

An increased expression of mtDNA CO I and ND4 was found to be associated with gastric cancer dedifferentiation. Their expression levels were higher in diffused gastric cancers than in intestinal cancers. Lu *et al* (19) performed differential display-hybridization to identify growth and differentiation-related genes in colorectal cancer cells, and found that differentiation

Table II. Association of the expression of cytochrome c oxidase I and NADH dehydrogenase 4 and clinicopathological chracteristics of gastric cancer.

	n	ND4/β-actin	Value of t or F	P-value	CO I/β-actin	Value of t or F	P-value
Gastric cancer	42	0.564±0.089	2.982	0.005	0.541±0.078	3.552	0.001
Normal mucosa	42	0.362±0.028			0.225±0.065		
Gender							
Male	32	0.567±0.086	0.171	0.681	0.539±0.081	0.024	0.878
Female	10	0.554±0.103			0.544±0.069		
Age (years)							
≤60	17	0.556±0.079	0.241	0.627	0.546±0.081	0.14	0.71
>60	25	0.569±0.097			0.537±0.077		
Borrmann classification							
I	6	0.549±0.055	0.260	0.774	0.525±0.089	1.020	0.371
II	20	0.553±0.082			0.557±0.079		
III	16	0.570±0.083			0.579±0.080		
Cancer type							
Papillary adenocarcinoma	5	0.507±0.036	3.911	0.006	0.533±0.069	2.733	0.034
Well-differentiated adenocarcinoma	8	0.505±0.016			0.475±0.075		
Moderately differentiated adenocarcinoma	8	0.532±0.104			0.541±0.055		
Poorly differentiated adenocarcinoma	9	0.603±0.023[a]			0.577±0.064[a]		
Mucous adenocarcinoma	8	0.595±0.129[a]			0.534±0.096		
Signet-ring cell carcinoma	4	0.665±0.049[a]			0.613±0.034[a]		
Lauren's classification							
Intestinal type	21	0.516±0.066	16.93	0.0002	0.514±0.070	5.514	0.024
Diffused type	21	0.612±0.084			0.567±0.077		
Tumor differentiation							
Well	13	0.506±0.024	8.663	0.001	0.497±0.076	3.701	0.034
Moderate	8	0.532±0.104			0.541±0.055		
Poor	21	0.612±0.084[b]			0.567±0.077[c]		
Tumor stage							
T1	5	0.600±0.086	0.790	0.462	0.564±0.068	0.570	0.570
T2	17	0.548±0.071			0.552±0.076		
T3	20	0.573±0.101			0.575±0.054		
Lymph-node metastasis							
No	29	0.557±0.086	0.534	0.469	0.537±0.078	0.231	0.634
Yes	13	0.579±0.092			0.549±0.080		

[a]P<0.01; [b]P<0.01; [c]P=0.01 vs. well-differentiated adenocarcinoma. ND4, NADH dehydrogenase 4; CO I, cytochrome c oxidase I; t, T-test; F, one-way ANOVA.

of human colorectal adenocarcinoma HT-29 cells was correlated with an increased expression of ND4 and 165 rRNA. In their study, Labiche *et al* (20) demonstrated that mitochondrial NADH dehydrogenase 5 transcripts were overexpressed in highly metastatic murine large cell lymphoma RAW117-H10 compared to low metastatic RAW117-P cells. However, our current study did not confirm this observation in gastric cancer with or without lymph node metastasis.

In conclusion, the present study has shown that the expression of mtDNA CO I and ND4 was increased in gastric cancer tissues, which was associated with gastric cancer dedifferentiation. Thus, an altered expression of certain mitochondrial-encoded genes should be evaluated as a biomarker for the diagnosis of gastric cancer.

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