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Alterations in Mitochondrial DNA Copy Numbers and Mitochondrial Oxidative Phosphorylation (OXPHOS) Protein Levels in Gastric Cancer Tissues and Cell Lines

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Alterations in mitochondrial DNA (mtDNA) copy numbers have been reported in patients with stomach cancer and suggested to play a role in gastric carcinogenesis or gastric cancer progression. However, changes in the levels of mitochondrial proteins or mtDNA-encoded oxidative phosphorylation (OXPHOS) proteins in gastric cancer remain unclear. In this study, we investigated mtDNA contents, mitochondrial protein levels, and mtDNA-encoded OXPHOS protein levels in gastric cancer tissues and cell lines. We correlated mtDNA copy numbers with clinicopathologic features of the gastric cancer samples used in this study and used quantitative PCR to analyze the mtDNA copy numbers of the gastric cancer tissues and cell lines. Western blot analysis was used for assessing the amounts of mitochondrial proteins and mtDNA-encoded OXPHOS proteins. Among the 27 gastric cancer samples, 22 showed a reduction in mtDNA copy numbers. The mtDNA content was increased in the other five samples relative to that in normal matched gastric tissues. Mitochondrial protein and OXPHOS protein levels were reduced in some gastric cancer tissues. However, mitochondrial protein and OXPHOS protein levels in gastric cancer cell lines were not always in line with their mtDNA contents. The mtDNA copy numbers were reduced in five gastric cancer cell lines tested in this study. In summary, this study reports a common reduction in mtDNA contents in gastric carcinoma tissues and cell lines, pointing to the possible involvement of mtDNA content alterations in tumorigenesis of the stomach.

Key words : Gastric carcinoma, mitochondria, mitochondrial DNA, mitochondrial DNA copy number, mitochondrial OXPHOS protein

Introduction

One of the hallmarks of eukaryotic cells is the presence of internal membrane-bounded organelles called mitochondria. Although these organelles have a central role in energy generation by using electron transport coupled with oxidative phosphorylation (OXPHOS), mitochondria participate in several other important functions as well. These roles include metabolism, apoptosis, oxygen sensing, signaling, and reactive oxygen species (ROS) production [6, 7, 9, 18, 23, 30]. In recent years, it has become clear that mitochondrial dysfunction contributes to a variety of human diseases including tumor [34, 35].

Mitochondria have their own genomes (mtDNA) which are essential with respect to OXPHOS [14]. Genes encoded in mtDNA are involved in OXPHOS and mitochondrial translation. Human mtDNA encodes 13 protein components of the mitochondrial OXPHOS complexes required for electron transport and ATP synthesis. In addition, mtDNA harbors 2 ribosomal RNA genes and 22 transfer RNA genes required for translation of the mtDNA-encoded OXPHOS subunits in the mitochondrial matrix. In mitochondria, multiple copies of double-stranded, circular mtDNA molecules are packaged into discrete units called nucleiods that contain ~20 different associated proteins [16, 17]. Although these proteins are thought to provide mtDNA with an organization and protection, mtDNA is not tightly protected as nuclear DNA which is well covered by histone proteins. Furthermore, mitochondria possess a fewer DNA repair mechanisms compared to nucleus and are a major site of

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cellular ROS production. Thus, mtDNA is much prone to oxidative damages or to other genotoxic stresses relative to nuclear DNA [29]. In fact, mtDNA mutations accumulate with age, and are reported in many human degenerative diseases including cancer [2, 11, 25].

Given the critical role of mtDNA in maintaining normal mitochondrial function, alterations in mtDNA (*e.g.*, mutations and copy number changes) can affect OXPHOS and mitochondrial ROS production. Defects in OXPHOS and oxidative stress have been proposed to have a causal effect on tumorigenesis [3, 10, 18, 30]. Regardless of their exact mechanistic contributions to tumorigenesis, several reports showed that mutations and deletions of mtDNA have been identified in various types of cancer including gastric cancer [5, 21, 26, 27, 31, 32, 36, 38, 44, 46].

Gastric cancer is one of the most common causes of death in cancer patients throughout the world [24]. Although the precise molecular events leading to the development of this cancer still need to be determined, previous studies have shown evidence that the accumulation of genetic alterations plays an important role in gastric carcinogenesis. For examples, inactivation of the tumor suppressor gene p53 and the chromosomal deletions in 1q, 5q, 17p, and 18q have been reported in most stomach cancers [8, 28, 33]. Mutations of E-cadherin, affecting cell adhesion, or of β-catenin have also been detected in gastric carcinomas, and these changes may contribute to the tumorigenesis of the stomach [1, 42]. In addition to these mutations in nuclear DNA, recent studies have reported various alterations in mtDNA from gastric carcinoma patients [12, 15, 19, 20, 43]. Several studies also have showed a reduction in mtDNA copy number during gastric cancer development [40, 47]. However, a possible relationship between mtDNA copy number alterations and mitochondrial protein and mtDNA-encoded OXPHOS protein levels in gastric cancer still remains unclear.

In order to obtain more insights into effects of alterations in mtDNA amounts on gastric cancer development, here, mtDNA copy numbers were examined using Korean gastric cancer tissues and several gastric cancer cell lines. The correlations between the mtDNA contents and clinicopathologic features of gastric cancer samples were also analyzed. Lastly, to reveal a possible relationship between mtDNA copy numbers with mitochondrial protein and OXPHOS protein levels, amounts of several mitochondrial and OXPHOS proteins from gastric cancer tissues and cell lines were assessed.

Materials and Methods

Gastric cancer tissue samples and gastric cancer cell lines

Total 27 frozen gastric carcinoma and matching normal tissues were obtained from Gyeongsang National University Hospital followed by its Institutional Review Board regulations. Five gastric cancer cell lines were kindly provided by professor Yoo Ji Yoon at Department of Microbiology at Gyeongsang National University. These cell lines include SNU1, SUN484, SNU638, SNU719, and MKN28. These cancer cells were cultured in Roswell Park Memorial Institute 1640 medium (Invitrogen, Carlsbad, USA) supplemented with 10% FBS and 1% Penicillin/Streptomycin at 37℃ in 5% CO_2 humidified incubators.

DNA isolation and quantification

Total cellular DNA was extracted from snap-frozen tissues and cell lines by standard molecular biology techniques. Tissue or cell line samples were suspended in 500 μl lysis buffer. The samples were incubated overnight at 56℃ until the samples were totally dissolved. The DNA was then extracted from each sample with an equal volume of phenol:chloroform: isoamyl alcohol solution (25:24:1). The samples were then centrifuged for 10 min with 12,000 g (at 4℃), and the upper aqueous layer was transferred to a fresh, sterilized microcentrifuge tube. RNase A (10 μl of 10 mg/ml; Thermo Scientific, Pittsburgh, USA) was added, and the solution was incubated at 37℃ for 30 min. Equal volumes of chloroform:isoamyl alcohol solution were added and centrifuged again with 12,000 g (at 4℃) for 10 min. The upper aqueous layer was transferred to a sterilized microcentrifuge tube, and double the volume of chilled isopropanol was added, along with one-tenth volume of 3 M sodium acetate, and chilled at -20℃ for 1 hr for precipitation. Then, the sample was centrifuged at 12,000 g (at 4℃) for 10 min. After decanting the supernatant, 250 μl 70% ethanol was added, and the pellet was dissolved; the mixture was centrifuged at 12,000 rpm for 10 min, and the supernatant was decanted gently. The pellet was air-dried under laminar air flow, and the dried pellet was resuspended in 50 μl nuclease-free water. Then, dissolved DNA was quantified by spectrophotometry using a NanoDrop (Thermo Scientific, Pittsburgh, USA). The quantified DNA was used immediately or frozen at -20℃ for later quantitative PCR analysis.

MtDNA copy number analysis by quantitative PCR

For mtDNA copy number analysis, an aliquot of 12.5 ng DNA was subjected to quantitative PCR using Rotor Gene-Q (Qiagen, Hilden, Germany) to determine the relative abundance of mtDNA versus nuclear 18S rDNA using mitochondrial and nuclear primer sets in two parallel PCR reactions as described previously [41]. The sequence of the PCR primer pairs are as follows: the 18S rRNA fragment was amplified by the primer pair 5′-TAG AGG GAC AAG TGG CGT TC-3′ and 5′-CGC TGA GCC AGT CAG TGT-3-3′; and the COX1 fragment was amplified by the primer pair 5′-CAC CCA AGA ACA GGG TTT GT-3-3′ and 5 ′-TGG CCA TGG GTA TGT TGT TAA-3′. Relative mtDNA copy number was calculated as the ratio of the amount of amplification obtained with mtDNA versus nuclear 18S rDNA primer sets for each sample and plotted normalized to the control group. All experiments were done in triplicates.

Western blot analysis

Gastric cancer tissues, corresponding normal tissue and gastric cancer cell line lysates (15 μg) were resolved on a 10% SDS-PAGE. After electrophoresis, proteins were transferred to a PVDF membrane (Millipore, Billerica, USA), immunoblotted overnight at 4℃ with primary antibodies anti-UQCRC2, anti-MTCO2, anti-ATPB, anti-VDAC (Abcam, England, UK) or anti-Actin (Sigma-Aldrich, St. Louis, USA) at 4℃. Horseradish peroxidase-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Dallas, USA) or goat anti-rabbit IgG (BioRad, Hercules, USA) were used as secondary antibodies. The signal was measured using Bio-Rad Clarity Western ECL substrate (Bio-Rad, Hercules, USA). To assess ratios of target band over actin, resulting blots were quantified using Image J software (NIH, Bethesda, USA). All Western blot analyses were done in triplicates.

Statistical analysis

Statistical analysis was performed using Graphpad Prism 7 software (GraphPad, La Jolla, USA). The Student's two-tailed t-test was used for the determination of statistical relevance between groups. In the figures, *indicates $p<0.05$, **indicates $p<0.01$ and *** indicates $p<0.001$.

Results

Alterations in mtDNA copy number from Korean gastric cancer tissues

In order to examine alterations in mtDNA copy number from the gastric cancer tissues, mtDNA contents was analyzed via quantitative PCR using 27 gastric carcinoma tissues and their matching normal tissues. As shown in Fig. 1A, among the 27 gastric cancer specimens, 22(81.48%) showed a more than 30% reduction in mtDNA amounts relative to their matching normal tissues ($p<0.05$). Interestingly, the remaining 5 gastric cancer cases (18.52%) showed an increase in mtDNA contents. From Fig. 1B, the overall reduction of mtDNA copy number in gastric cancer tissues relative to normal tissues was about 33% ($p<0.001$). Thus, it appears that loss of mtDNA contents might be a common alteration in gastric cancers examined in this study.

Correlation between mtDNA copy number and clinicopathologic features of gastric cancer tissues

Using a statistical analysis, we tested possible correlations between clinicopathologic features and mtDNA contents. Interestingly, as shown in Table 1, we found that an induction in mtDNA copy number correlates with gross type ($p<0.05$). Early-stage gastric carcinoma samples seems to have more mtDNAs relative to remaining advanced gastric

Table 1. Correlation between mtDNA copy numbers and clinicopathologic features in 27 gastric cancer patients

Clinicopathologic Features	mtDNA copy number		*P* value
	Induced	Not induced	
Age			
≤ 55	0	9	0.529
> 55	3	15	
Gender			
Male	2	20	0.474
Female	1	4	
Gross type			
Early	3	7	0.046*
Advanced	0	16	
WHO			
Differentiated	3	8	0.056
Undifferentiated	0	16	
Lauren			
Intestinal	2	14	0.520
Diffuse	0	9	
TNM			
Low	3	10	0.098
High	0	14	

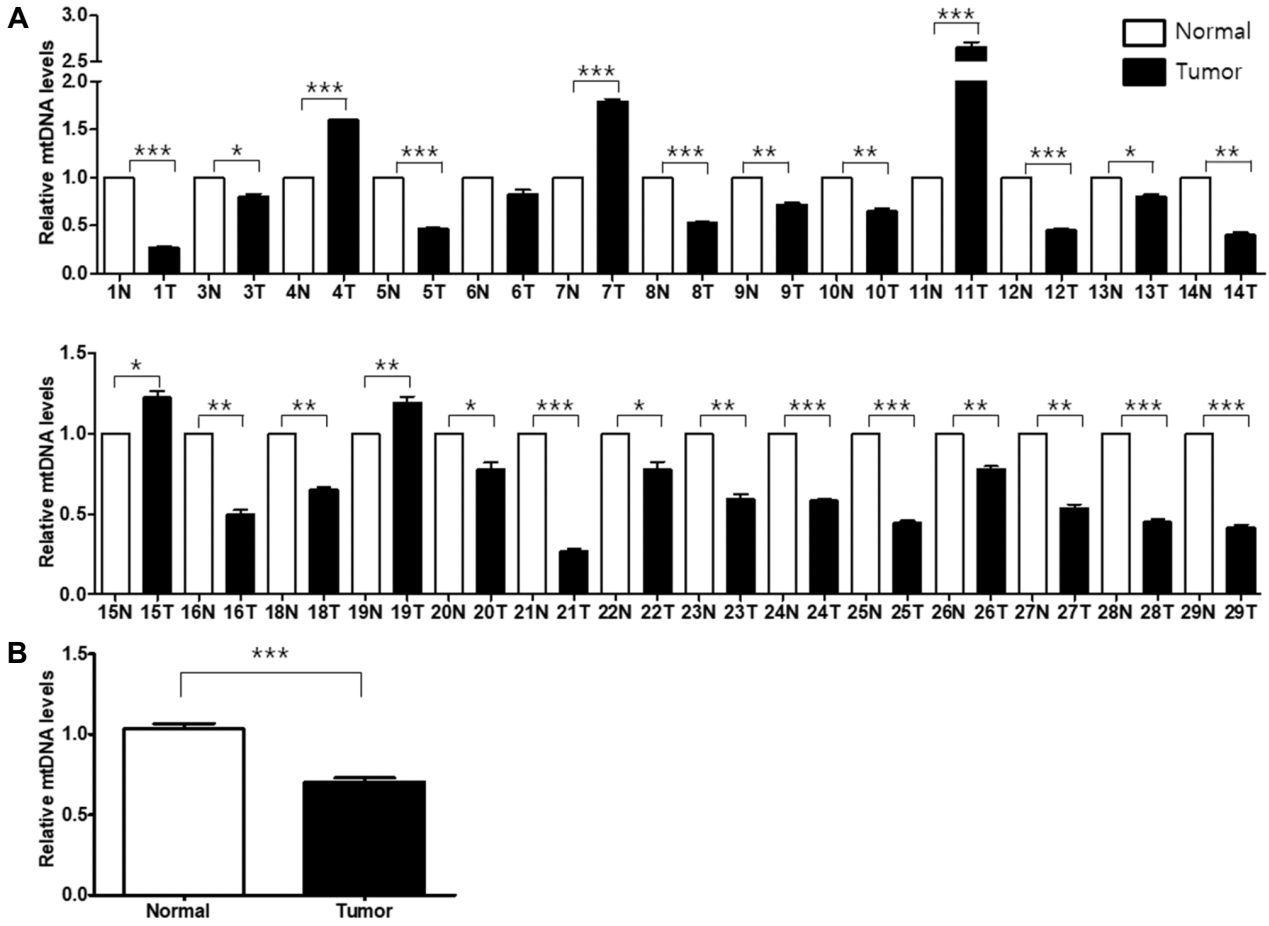

Fig. 1. mtDNA copy numbers in gastric cancer tissues by quantitative PCR. (A) Alterations of mtDNA copy numbers in 27 gastric cancer tissues relative to their matching normal tissues. (B) The overall mtDNA copy number ratio of 27 gastric cancer tissues to their normal tissues. *indicates $p<0.05$, **indicates $p<0.01$, and ***indicates $p<0.001$.

carcinoma. This finding suggests that the up-regulation of mtDNA contents might be involved in preventing tumor progression.

Mitochondrial mass in gastric cancer tissues

In order to obtain an insight into effects of changes in mitochondrial mass on gastric tumorigenesis, we measured mitochondrial mass in gastric cancer tissues. Electron microscopy is the best way to measure mitochondrial amounts in tissue samples. However, the frozen gastric carcinoma tissues used in this study were not suitable for electron microscopy. Thus, to quantify mitochondrial mass, we measured the protein levels of VDAC, a mitochondrial outer membrane protein and a reliable marker for mitochondrial mass, in gastric cancer and matching normal tissues. For Western blot assay, we selected six gastric cancer cases that showed a more than 50% decrease in mtDNA contents relative to their normal tissues. As shown in Fig. 2A, the majority (5 out of 6, 83.33%) of cancer samples with more than 50% reduction in mtDNA contents displayed a reduced expression of VDAC when normalized to Actin. These findings imply that there is a concurrent reduction between mtDNA contents and mitochondrial mass in gastric cancer tissues.

OXPHOS protein levels in gastric cancer tissues

To investigate whether alterations in mtDNA copy number can affect mitochondrial respiratory chain in gastric cancer tissues, we analyzed the expression of mtDNA-encoded OXPHOS protein levels. In this study, we analyzed three gastric cancer tissues that showed a more than 50% reduction in mtDNA copy number. From Fig. 2B, there are apparent reductions in the protein levels of UQCRC2, COX2 and ATPB in gastric cancer tissues compared to corresponding normal tissues. These results suggest that there is a simultaneous reduction between mtDNA copy number and mtDNA-encoded OXPHOS protein levels.

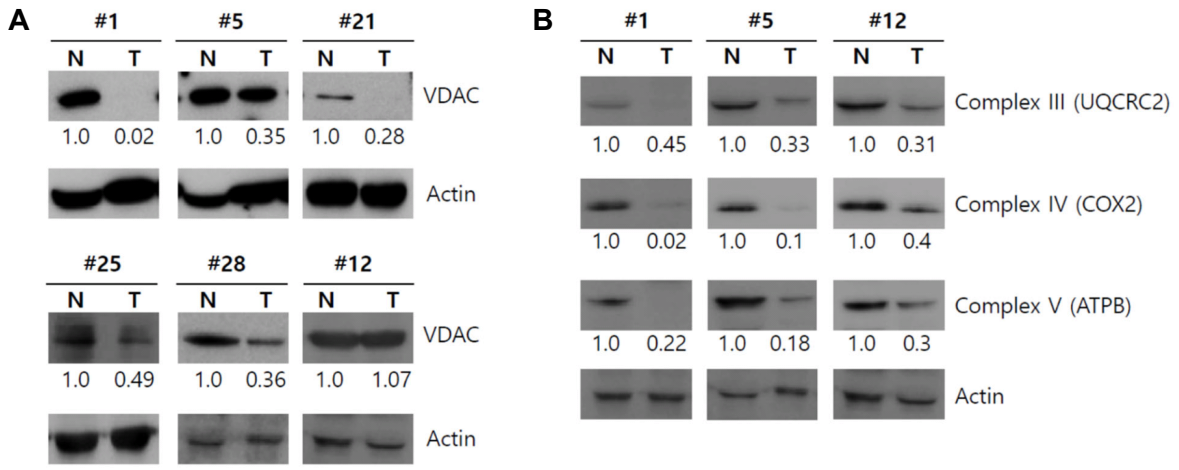

Fig. 2. VDAC and mtDNA-encoded OXPHOS protein levels in gastric cancer tissues by Western blot analysis. (A) VDAC protein levels in six gastric cancer tissues relative to their matching normal tissues. (B) mtDNA-encoded OXPHOS protein (UQCRC2, COX2, and ATPB) levels in three gastric cancer tissues relative to their matching normal tissues. N indicates normal tissue and T indicates gastric cancer tissue. Numbers under the blot images indicate quantitated amounts of corresponding target protein relative to Actin.

Changes in mtDNA copy number, mitochondrial mass, and OXPHOS protein levels in five gastric cancer cell lines

In order to expand our findings in gastric cancer tissues, we also examined mtDNA contents in gastric cancer cell lines. We used five gastric cancer cell lines including SNU-1, SNU-484, SNU638, SNU-719, and MNK-28, which are commonly used cancer cells to study gastric carcinogenesis. As shown in Fig. 3A, gastric cancer cell lines exhibited to have a reduced amount of mtDNA compared to HEK293T cell line which is an immortalized but not a cancer cell line. Next, in order to analyze mitochondrial mass, we assessed the VDAC protein expression levels in these gastric cancer cell lines. HEK-293T was also used as a control cell line. From

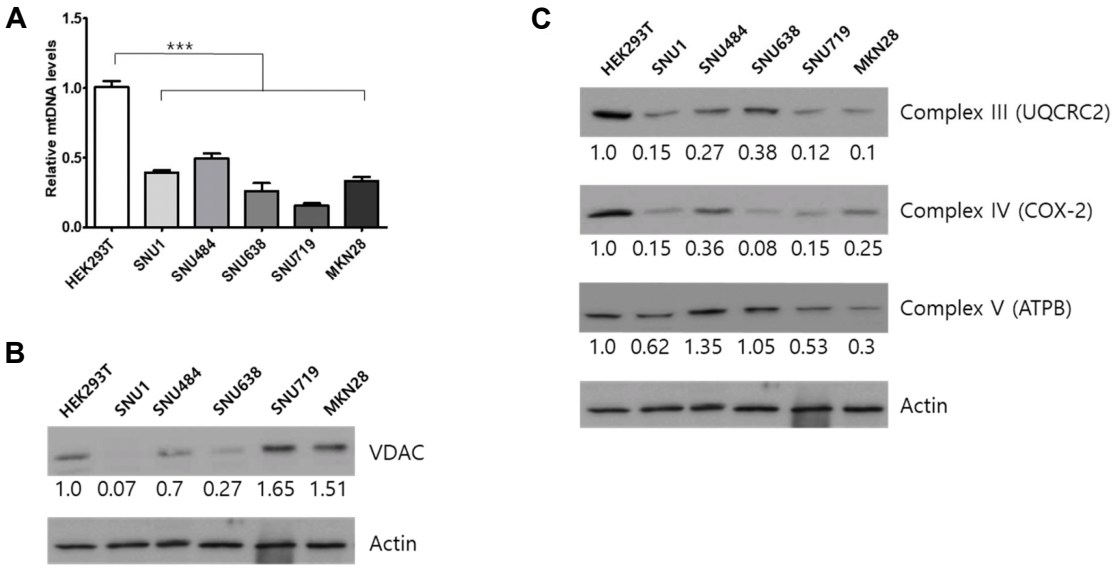

Fig. 3. mtDNA copy numbers, VDAC protein levels, and mtDNA-encoded OXPHOS protein levels in five gastric cancer cell lines (SNU1, SNU484, SNU638, SNU719 and MKN28). (A) mtDNA copy numbers in gastric cancer cell lines were assessed by qPCR. (B) VDAC protein levels in gastric cancer cell lines were assessed by Western blot analysis. (C) mtDNA-encoded OXPHOS protein (UQCRC2, COX2, and ATPB) levels in gastric cancer cell lines were assessed by Western blot analysis. HEK293T cell line was used as a control. Numbers under the blot images indicate quantitated amounts of corresponding target protein relative to Actin. ***indicates $p<0.001$.

Fig. 3B, we found that SNU-1, SNU-484 and SNU-638 showed a significant reduction in VDAC levels compare to HEK-293T. However, SNU-719 and MKN-28 showed a small induction in VDAC levels when normalized to Actin. Lastly, we measured the expression of mtDNA-encoded OXPHOS protein levels to see whether alterations in mtDNA copy number would affect mitochondrial respiratory chain. As shown in Fig. 3C, OXPHOS proteins (UQCRC2, COX2, and ATPB) were often reduced in gastric cancer cell lines relative to HEK-293T. Together, our results indicate that mtDNA contents and mtDNA-encoded OXPHOS protein levels were reduced simultaneously in some gastric cancer cell lines.

Discussion

Dysfunction in mitochondrial OXPHOS in cancer cells was originally reported by Warburg, who proposed a switch in metabolism away from respiration toward glycolysis (so called aerobic glycolysis) is beneficial for tumorigenesis [37]. In addition to metabolic alterations, mitochondria contribute to tumorigenesis and other aspects of cancer development through their direct involvement in apoptosis and the production of ROS [6, 23, 39]. The latter can induce oxidative damage and nuclear genome instability as well as participate in signaling pathways involved in cellular proliferation, differentiation, and adaptation to hypoxia [3, 9, 30].

Given an essential role for mtDNA in OXPHOS as well as genome instability in cancer, it is not surprising that cancer cells harbor alterations in mtDNA. In fact, mtDNA mutations have been documented in a variety of human cancers [2, 4]. For example, the majority of human colon cancer cells contain specific mtDNA point mutations [11, 25] and mtDNA mutations associated with increased ROS production enhance the metastatic potential of tumor cells [13]. In addition, considerable attempts have been done to examine if changes in mtDNA abundance are also associated with tumorigenesis. These efforts have shown that many human cancers do exhibit higher or lower mtDNA copy numbers [22, 45]. Notably, complete loss of mtDNA (via homozygous knock-out of Tfam) inhibits anchorage-dependent growth of cells *in vitro* and Kras-mediated lung tumorigensis in mice [39]. However, in most tumor cells, mtDNA is not absent, suggesting that, if mtDNA is involved in tumorigenesis, mtDNA instability (*i.e.,* increased damage, mutation load and/or altered copy number) and its downstream consequences are likely more biologically relevant than complete loss of mtDNA.

MtDNA instabilities (*i.e.,* alterations in mtDNA molecules) including mtDNA damages, mutations, and content changes will inevitably result in respiratory chain dysfunction because mtDNA is essential for respiration. One possible outcome of the resulting respiratory chain dysfunction could be a ROS production. As mentioned above, ROS have been reported to be involved in tumorigenesis by inducing oxidative stress or altering cellular signaling pathways. Thus, mtDNA harboring ROS-generating mutations could contribute to tumorigenesis and would be maintained or even gained in tumor cells. Another feasible outcome of respiratory chain dysfunction could be a metabolic shift from OXPHOS to glycolysis. The latter is now clearly known to be a much efficient metabolism regarding cell division. This scenario might explain lower levels of mtDNA in some cancers.

In this study, we have shown that mtDNA contents were reduced in many Korean gastric cancer samples. These results were consistent with studies by Wu and colleagues [43] who reported a decrease in mtDNA levels in Chinese gastric cancers. Together, these reports imply that metabolism in gastric cancer cells may be shifted to glycolysis which can confer them with growth advantage. Thus, it will be of interest to examine tumor metabolism in gastric cancer. Regardless of metabolism in gastric cancer, a mtDNA content reduction in gatric cancer presented by this study might be useful for clinical applications (*i.e.,* a novel predictive and diagnostic biomarker). Indeed, although further studies with large number of cases are strongly needed, this study suggested a possible correlation between gross type of gastric carcinomas and relative mtDNA copy number reduction. The finding that advanced gastric carcinoma might have less amounts of mtDNA relative to early-stage carcinoma suggests a possible involvement of mtDNA content alterations in tumor progression. In line with this notion, it will be very interesting to assess mtDNA level changes during adenoma-carcinoma sequence of gastric carcinogenesis.

In summary, this study found a common reduction in mtDNA amounts in Korean gastric carcinomas. The decrease in mtDNA amounts seems to happen together with a reduction in mitochondrial mass. In addition, the decrease in mtDNA amount might be associated with tumor progression. This study also reports mtDNA copy numbers in gastric cancer cell lines. Although, here, limited and small number of gastric cancer cases were investigated, we hope that this at-

tempt will be a step toward understanding a possible involvement of mtDNA content alterations in gastric tumorigenesis.

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The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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초록 : 위암 조직과 세포주에서 mDNA와 OXPHOS 단백질 분석

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위암 환자에서 미토콘드리아 DNA (mtDNA)의 양적 변화가 보고 되고 있으며 이러한 변화가 위암의 발암이나 진행에 관여되는 것으로 추정되고 있다. 그리나 위암에서 미토콘드리아 단백질이나 mtDNA에 의해 암호화된 산화적 인산화(OXPHOS) 단백질의 양적 변화에 관한 연구는 아직까지 미비한 실정이다. 본 연구에서는 위암환자 조직 및 세포주를 이용하여 mtDNA 양 그리고 미토콘드리아 단백질 및 OXPHOS 단백질의 양을 분석하였다. 또한, mtDNA 양적 변화와 위암 환자의 임상병리학적 특징을 연관 분석하였다. MtDNA 양을 분석하기 위하여 qPCR 기법을 그리고 단백질 분석에는 Western blot 기법을 각각 활용하였다. 총 27개의 위암 환자 샘플에서 약 80%에 해당하는 22개의 환자 위암조직에서 정상조직에 비해 mtDNA 양이 감소하였으며, 나머지 환자에서는 mtDNA 양이 증가하였다. 이러한 mtDNA 양이 감소한 위암 조직 샘플에서는 미토콘드리아 단백질 및 OXPHOS 단백질의 양도 같이 감소하였다. 한편, 본 연구에 사용된 총 5개의 위암 세포주 모두에서 mtDNA 양이 감소하였다 그러나 위암 세포주에서는 mtDNA 양적 감소와 미토콘드리아 단백질 및 OXPHOS 단백질의 양적 감소가 항상 일치하지는 않았다. 이러한 연구결과는 위암 조직 및 세포주에서 mtDNA 양의 감소가 흔하며 이는 mtDNA 양적 변화가 위암의 생성에 관여함을 제시한다.