

## RESEARCH ARTICLE

# Is Mitochondrial DNA Copy Number Associated with Clinical Characteristics and Prognosis in Gastric Cancer?

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### Abstract

Alterations in mitochondrial DNA (mtDNA) have been studied in various cancers. However, the clinical value of mtDNA copy number (mtCN) alterations in gastric cancer (GC) is poorly understood. In the present study, we investigated whether alterations in mtCNs might be associated with clinicopathological parameters in GC cases. mtCN was measured in 109 patients with GC by quantitative real-time PCR. Then, correlations with clinicopathological characteristics were analyzed. mtCN was elevated in 64.2% of GC tissues compared with paired, adjacent, non-cancerous tissue. However, the observed alterations in mtCN were not associated with any clinicopathological characteristics, including age, gender, TN stage, Lauren classification, lymph node metastasis, and depth of invasion. Moreover, Kaplan-Meier survival curves revealed that mtCN was not significantly associated with the survival of GC patients. In this study, we demonstrated that mtCN was not a significant marker for predicting clinical characteristics or prognosis in GC.

**Keywords:** Gastric cancer - mitochondrial DNA - copy number - prognosis

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### Introduction

Mitochondrial DNA (mtDNA) is a 16,569 bp, circular, double-stranded DNA molecule, and multiple copies of mtDNA are present in each mitochondrion. The frequency of mutations in mtDNA is 10 to 100-fold higher than that of nuclear DNA because of high concentrations of reactive oxygen species (ROS) in the mitochondrial inner membrane, fewer repair mechanisms, and no mtDNA-coating proteins such as histones in the nucleus (Howell et al., 1996; Paabo, 1996; Zhu et al., 2004). Therefore, mitochondrial genetic studies performed in various cancers demonstrated that most alterations were found in the D-loop, which is a hot spot region (Stoneking, 2000; Bianchi et al., 2001; Lievre et al., 2005; Wang et al., 2005; Jeong et al., 2010; Lee et al., 2011). Because the D-loop, containing the H-strand replication origin, is an essential element for mtDNA replication, mutations in the D-loop may cause a decrease in mtDNA copy number (mtCN) or altered mtDNA gene expression (Shadel, 2008). It has been hypothesized that mutations or decreases in mtCN could lead to a deficiency in oxidative phosphorylation and enhanced generation of ATP by glycolysis. Therefore, mtCN changes may be of clinical significance in cancers (Lee et al., 2004; Yin et al., 2004; Wu et al., 2005; Guo et al., 2013).

Gastric cancer (GC) is highly prevalent in Asia and is the leading cause of death worldwide. Gastric

carcinogenesis is a multi-step process that begins with chronic gastritis, which leads to atrophy, intestinal metaplasia, dysplasia, and finally, invasive cancer (Correa, 1992; Correa and Shiao, 1994). Our previous study also suggested that alteration in mtDNA is an early and important event in gastric carcinogenesis (Jeong et al., 2010). In addition, Wu et al. (2005) suggested that somatic mutations and depletion of mtDNA occurs in GC and that mtDNA depletion is involved in carcinogenesis. It has been reported that mtCN was associated with prognosis in some cancers (Yu et al., 2007; Lin et al., 2008; Cui et al., 2013). However, mtDNA copy number has yet to be studied in a large sample of patients with GC. In the present study, we examined mtCN in GC and then analyzed the clinicopathological characteristics and prognostic value.

### Materials and Methods

#### *Patients and DNA extraction*

We recruited 109 patients who underwent gastrectomy for treating gastric adenocarcinoma from archives of paraffin blocks at Keimyung University Dongsan Hospital from October 1999 to December 2001. Tissue samples were fixed in formalin and embedded in paraffin. All cases were reviewed by an expert panel of two pathologists according to the current criteria of the WHO classification. The clinical data and pathological reports of the patients

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with gastric adenocarcinoma were collected from the medical records. Tumor area and adjacent normal mucosa were selected from slide according to hematoxylin and eosin stained sections by pathologists. 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.

#### Mitochondria copy number

The mtCN was examined using real-time quantitative PCR (qPCR). For the quantitative determination of mtDNA content relative to nuclear DNA (nDNA), primers for specific amplification of the mtDNA COX1 and nDNA-encoded  $\beta$ -actin genes were selected according to previous studies with minor modifications (Lee et al., 2004; Yu et al., 2007; Cui et al., 2013). [12, 17, 19]. Real-time qPCR was then performed using a LightCycler 480 II system (Roche Diagnostics, Germany) with a total reaction volume of 20  $\mu$ l, which contained 10  $\mu$ l SYBR Green Master MIX (Takara, Japan), 8 pmol of each primer, and DNA (50ng). The PCR conditions were 95°C for 1min, followed by 40 cycles of 95°C for 15s and 60°C for 30s. The threshold cycle number (Ct) values of the  $\beta$ -actin gene and the mitochondrial COX1 gene were determined. The mtCN in each tested specimen was then normalized against that of the  $\beta$ -actin gene to calculate the relative mtCN. Each measurement was repeated in triplicate, and five serially diluted control samples were included in

**Table 1. Clinicopathological Characteristics of Mitochondrial Copy Number in Gastric Cancers**

	N	mtCN		p
		Low	High	
Total	109	76 (69.7)	33 (30.3)	
Age				0.57
< 60	54	39 (51.3)	15 (45.5)	
≥ 60	55	37 (48.7)	18 (54.5)	
Gender				0.57
Male	82	56 (73.7)	26 (78.8)	
Female	27	20 (26.3)	7 (29.2)	
pT				0.61
1	46	33 (43.4)	13 (39.4)	
2	26	16 (21.1)	10 (30.3)	
3	2	2 (2.6)	0 (0)	
4	35	25 (32.9)	10 (30.3)	
pN				0.8
1	67	45 (59.2)	22 (66.7)	
2	21	15 (19.7)	6 (18.2)	
3	11	9 (11.8)	2 (6.1)	
4	10	7 (9.2)	3 (9.1)	
Lauren classification*				0.61
Diffuse	26	17 (22.7)	9 (27.3)	
Intestinal	82	58 (77.3)	24 (72.7)	
Lymph node metastasis				0.46
No	76	45 (67.2)	31 (73.8)	
Yes	33	22 (32.8)	11 (26.2)	
Depth of invasion				0.7
Early	46	33 (43.4)	13 (39.4)	
Advanced	63	43 (56.6)	20 (60.6)	

\*One case with mixed type was excluded; mtCN, mitochondrial DNA copy number

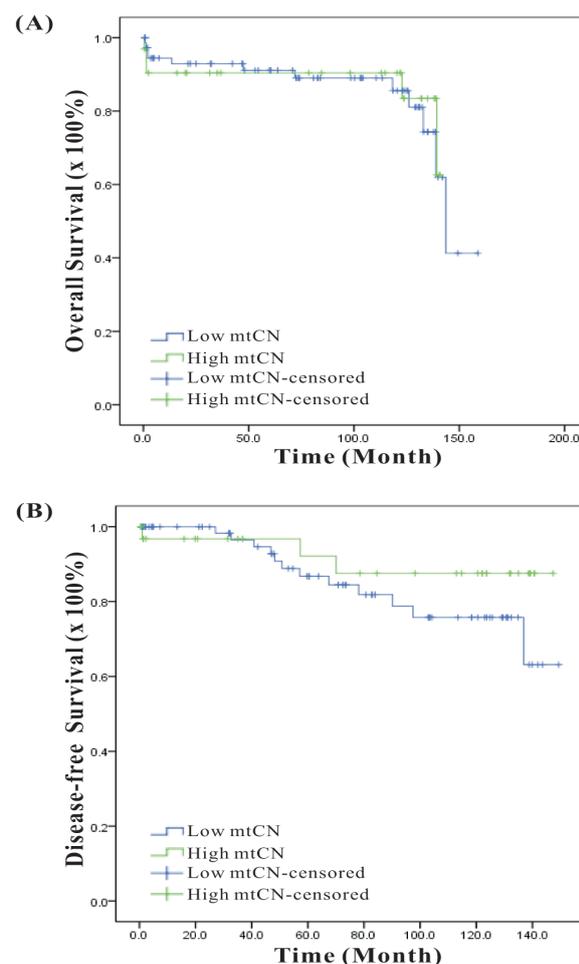
each experiment.

#### Statistical analysis

The SPSS statistical package, version 19.0 for Windows, was used for all statistical analyses. Correlation between mtCN change and clinicopathological characteristics was analyzed by Fisher's exact test or Pearson's Chi square test. Disease-free and overall survivals were measured according to the Kaplan Meier method. Disease free survival was measured from the date of diagnosis to the date of recurrence or the last follow-up. Overall survival was measured from the date of diagnosis to the date of death or the last follow-up visit. Differences between curves were analyzed using the log-rank test. p values <0.05 were considered to indicate statistically significant results.

## Results

The mean age of the 109 patients with gastric adenocarcinoma was 56.2 years (range, 25-82 years). There were 82 (75.2%) male patients and 27 (24.8%) female patients. Early gastric carcinoma that invaded the mucosal or submucosal layer was observed in 46 (42.2%) patients, and advanced gastric carcinoma, which invaded the proper muscle or a deeper layer, was observed in 63 (57.8%) patients. According to the Lauren classification,



**Figure 1. Survival Analysis by Mitochondrial Copy Number in Gastric Cancers. (A) Overall survival; (B) Disease-free survival**

26 (23.9%) patients had diffuse type, 82 (75.2%) patients had intestinal type, and one (0.9%) patient had mixed type.

The mtCN of tumor tissues and corresponding non-cancerous colorectal tissues was analyzed by real-time qPCR. The mean copy number of mtDNA was  $1.68 \pm 1.1$  in tumors and  $1.31 \pm 0.92$  in matched non-cancerous tissue, and the difference was not statistically significant. Seventy cases of GCs (64.2%) showed an increased copy number compared with adjacent non-cancerous colorectal tissues. To further explore the correlation between mtCN and the clinicopathological parameters of GC, we calculated the ratio of the copy number in tumors and paired normal tissues (T/N). Based on the different T/N ratios, patients were categorized into two subgroups according to the median value of the T/N ratios (1.44). The association between mtCN changes and the clinicopathological parameters are summarized in Table 1. However, these results showed no significant correlation.

We then assessed survival in GC to assess the prognostic value of mtCN. Median follow-up of patients for the survival analysis was 80.4 months (1-158 months). The Kaplan-Meier curve showed that mtCN changes were not associated with overall survival ( $p=0.98$ ) and disease-free survival ( $p=0.25$ ) (Figure. 1). When stratifying for the variables, mtCN changes seemed to confer no significant prognostic value.

## Discussion

This article was the first to analyze the clinicopathological characteristics and prognostic value of mtCN in 109 patients with GC. mtDNA copy changes in GC were first described by Wu et al. (2005). According to their study, a significant reduction (below 90%) or increase (above 110%) in mtDNA relative to corresponding non-cancerous tissue was found in 50% and 23% of GC, respectively. mtCN was associated with Borrmann's type, indicating that depletion of mtDNA may be involved in phenotypic changes, tumor progression, and metastasis of GC. However, our study showed that mtCN changes were not associated with clinicopathological characteristics of GC. In contrast, a higher mtCN in GC was found, which was not statistically significant in the present study. According to the review of mtDNA copy in cancers by Yu (2011), an increase in mtDNA was found in some tumors though it is controversial. The observed increase in mtDNA may have resulted from the use of a different method for estimating mtDNA content and extracting DNA. To understand the implication of mtDNA changes in cancers, a standard method for calculating mtDNA content should be used.

Scientists have hypothesized the role of mtDNA in carcinogenesis and suggested that the loss of mtDNA can promote tumor progression (Shadel and Clayton, 1997; Lee et al., 2000; Amuthan et al., 2001; Savagner et al., 2001; Cook and Higuchi, 2012; Dai et al., 2013). According to these studies, alterations in mtCN originate from mutations in the mitochondrial D-loop, p53, mtDNA polymerase  $\gamma$  (POLG), or oxidative stress. Moreover, changes in mtDNA content may affect various cellular processes that promote cancer, such as cell growth, cell

apoptosis, drug resistance, mitochondria-to-nucleus signaling, and more. Based on these results, previous studies suggested that low mtDNA content may be associated with poor prognosis in some cancers (Yu et al., 2007; Lin et al., 2008; Cui et al., 2013). In GC, there were few studies about the association between mtDNA content and prognosis, with only one case-control study being performed (Liao et al., 2011). These studies showed no association between mtDNA copy number levels and risk of developing GC. However, low mtDNA content may have an early effect on the development of GC. To the best of our knowledge, this is the first study to evaluate GC prognosis according to mtDNA copy number. Both overall and disease free survivals in GC were analyzed with long term follow-up (80.4 months). As a result, we observed no association between survival and mtDNA copy number in GC. Through stratified analyses, we also observed no clinical prognostic value of mtCN in GC. However, we cannot rule out a possible association between mtCN and GC prognosis because there was no study that evaluated mtCN in precancerous lesions of GC. Considering the rapid progression of gastric dysplasia to GC (Rugge et al., 1994), future studies should focus on the role of mtDNA content in GC development.

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