Title: Mitochondrial DNA copy numbers in gastric cancer tissues: a possible biomarker for estimating cancer progression

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Abstract

Background: Mitochondria have their own genome (mtDNA), which in humans is a circular multi-copy genome consisting of 16,569 base pairs. Abnormalities in the mtDNA have been reported to correlate with various age-related pathophysiologies.

Methods: Based on a total of 182 DNA samples extracted from gastric cancer tissues, we measured mtDNA copy numbers (mtDNA-CN) using real-time PCR and then examined alongside sex, age, tumor stage, Laurén classification, and the overexpression of Human Epidermal Growth Factor Receptor 2 (HER2).

Results: We found no sex differences in mtDNA-CN and no correlation with age, but significant differences according to tumor stage. The mtDNAcn of intestinal type by Laurén classification was significantly larger than that of diffuse type. There was no significant difference in mtDNA-CN between HER2-positive and -negative tissues. Multiple regression analyses showed that only the tumor stage was a significant variable, while Laurén classification was not.

Conclusion: These results indicate that mitochondrial genomic abnormalities contribute the progression of gastric cancer independently of HER2 overexpression, and may shed light on the emerging role of mtDNA-CN *in situ* as a possible biomarker for estimating cancer progression.

Keywords: biomarker; DNA copy number variation; gastric cancer; mitochondrial DNA; tumor stage

Introduction

In the 1920s, Otto Warburg and his colleagues found that tumor cells metabolize glucose in a different manner than cells in normal tissue [1]. In a characteristic metabolism known today as the “Warburg effect,” cancer cells produce adenosine triphosphate (ATP) using a glycolysis that is less efficient than mitochondrial oxidative phosphorylation [2,3]. The pathophysiology of cancer induces changes in mitochondrial roles, ranging from energy production to the regulation of apoptosis. Today, mitochondria and their own genome (mtDNA) are considered a potential cancer-therapeutic target [4,5].

The mtDNA in humans is a 16,569 base-pair circular multi-copy genome [6], with tens to thousands of copies per cell. The mtDNA copy numbers (mtDNA-CN) in peripheral blood decrease with age [7,8] and are negatively associated with all-cause mortality [9]. According to a previous study that investigated the relationship between blood-derived mtDNA-CN and gene expression in 47 tissues from 419 individuals, mtDNA-CN in the peripheral blood reflected metabolic health across multiple tissues [10]. In addition, cord blood mtDNA-CN correlates with birth outcomes [11]. Thus, mtDNA-CN is one of promising biomarkers in health and disease [12,13].

Gastric cancer is the fifth-most common cancer and the fourth leading cause of cancer-related deaths worldwide [14]. In recent years, in addition to the traditionally used Human Epidermal Growth Factor Receptor 2 (HER2) immunohistochemistry (IHC) test, a total of four biomarker tests, including the Programmed cell Death ligand 1 (PD-L1) IHC test, microsatellite instability (MSI)

/ mismatch repair (MMR) determination test, and Claudin-18.2 (CLDN18) IHC test, have become essential for determining the treatment strategy for advanced gastric cancer [15-17]. Today, the search for biomarkers that correlate with the pathophysiology of gastric cancer becomes more important for diagnosis and treatment.

A previous meta-analysis results showed that genetically predicted mtDNA-CN was not associated with cancer risk [18]. The subgroup analyses indicated no causal association between mtDNA-CN and gastric cancer. However, the accumulation of knowledge using mtDNA-CN in tumors remains insufficient. In this study, we directly analyzed mtDNA-CN *in situ* collected from 182 gastric cancer patients to clarify its clinical significance and utility as a biomarker. We measured mtDNA-CN with real-time PCR using DNA samples derived from gastric cancer tissues and examined the clinical significance of mtDNA-CN in the pathogenesis of gastric cancer by comparing it with accompanying anonymous information (age, sex, tumor stage, Laurén classification, and *HER2* overexpression).

Materials and methods

DNA samples and anonymous information

A total of 182 DNA samples extracted from gastric cancer tissues and the anonymous information were provided from the Department of Gastroenterology and Hepatology at the Sapporo Medical University School of Medicine. In addition,

we obtained DNA samples from non-cancerous lesions in 34 of the 182 patients, which were subsequently analyzed for comparison. These were obtained in a prospective multi-center study, SHERLOCK (Serum Her2/neu elevation in gastric cancer; a multicenter trial in Hokkaido, UMIN ID: 000009773) [19]. The anonymous information included age; sex; tumor stage; Laurén classification, which has been widely used worldwide as a histological classification of gastric cancer since 1965 [20]; and *HER2* overexpression. The patients were enrolled between July 2011 and July 2013, thus tumor staging was based on the seventh edition of the International Union Against Cancer tumor-node-metastasis classification (UICC7). The present study is one of the secondary uses of these biological samples and accompanying information. The study design was approved by the Faculty of Health Sciences Institutional Review Board, Hokkaido University (Approval No. 24-66).

Estimation of mitochondrial DNA copy number

To estimate mtDNA-CN, we used the Human Mitochondrial DNA Monitoring Primer Set and TB Green® Premix Ex Taq™ II (Tli RNase H Plus) (Takara BIO Inc, Shiga, Japan). This contains primer pairs for the amplification of two mtDNA genes (*ND1*, *ND5*) and two nuclear DNA genes (*SLCO2B1*, *SERPINA1*). The base lengths to be amplified were as follows: 73 bp (*ND1*), 99 bp (*ND5*), 139 bp (*SLCO2B1*), and 125 bp (*SERPINA1*). The thermal profile was as follows: 2 min at 98 °C, 40 cycles at 98 °C for 10 s, 15 s at 60 °C, and 30 s at 68 °C.

Statistical analysis

The Spearman's rank correlation test was used to examine the correlation between mtDNA-CN and age. The Wilcoxon signed-rank test was used to assess differences in mtDNA-CN between paired tumor and non-tumor tissue samples. The Mann-Whitney U test was used to examine differences in mtDNA-CN by sex, Laurén classification, and *HER2* overexpression. The Kruskal-Wallis test was used to examine differences in mtDNA-CN by tumor stage. Multiple regression analysis was used to predict independent variables associated with mtDNA-CN. Statistical significance was set at $p < 0.05$ for both sides.

Results

In this study, the mtDNA-CN in tumors for 182 gastric cancer patients were determined and the median was 653.0. As shown in Table 1, the mean age of the patients was 69.8, and males accounted for 122 (67.0%) of the cases. According to UICC7 staging, there were 82 (45.1%) patients with stage IA, 9 (4.9%) with IB, 8 (4.4%) with IIA, 6 (3.3%) with IIB, 5 (2.7%) with IIIA, 5 (2.7%) with IIIB, 7 (3.8%) with IIIC, and 60 (33.0%) with IV. According to Laurén classification, 65 tissues were diffuse type (35.7%) and 117 (64.3%) were intestinal type. *HER2*-positive tissues accounted for 51 (28.0%) samples, and 131 (72.0%) were negative.

There was no significant correlation between mtDNA-CN and age (p = 0.61) (Figure 1A). The median mtDNA-CN for males was 741.1, and for females it was 625.3, with no significant difference (p = 0.86) (Figure 1B). As shown in

Figure 1C, the median mtDNA-CN in tumor tissues was 957.1, while in non-tumor tissues it was 1229.0; however, the difference was not statistically significant ($p = 0.23$).

There was a significant difference in mtDNA-CN according to tumor stage ($p < 0.001$), with a negative trend in each stage, compared to IA (Figure 2A). The median mtDNA-CN of diffuse type by Laurén classification was 437.4 and that of intestinal type was 815.1, showing that the latter was significantly larger ($p < 0.001$) (Figure 2B). The median mtDNA-CN for *HER2*-positive was 606.1 and 747.4 for *HER2*-negative, but this was not significantly different ($p = 0.243$) (Figure 2C).

Table 2 shows the results of the multiple regression analysis. The objective variable was mtDNA-CN in gastric cancer, and the explanatory variables were age, sex, tumor stage, Laurén classification, and *HER2* overexpression. After adjustments for age, sex, Laurén, and *HER2*, the stage still correlated significantly with mtDNA-CN ($p < 0.001$).

Discussion

To the best of our knowledge, this is the first study to demonstrate that mtDNA-CN *in situ* correlates with the tumor stage of gastric cancer. As gastric cancer progressed beyond stage 1B, mtDNA copy numbers decreased markedly. This suggests the clinical significance of measuring mtDNA-CN in lesions detected incidentally by upper gastrointestinal endoscopy for estimating cancer

progression. Our findings support that mitochondrial genomic abnormalities are involved in the progression of gastric cancer. This is consistent with studies showing experimentally that gastric adenocarcinoma progression correlates with the reduction of mtDNA [21].

Interestingly, no significant difference in mtDNA copy number was observed between cancerous and non-cancerous lesions. However, it should be noted that the majority of non-cancerous tissue samples analyzed in this study were derived from patients with stage 1A gastric cancer (Table 1), which may have limited the generalizability of the findings. Additional studies with broader-stage sampling will be necessary to draw more definitive conclusions.

In general, the diffuse type, by Laurén classification, is an isolated-cell carcinoma and induced by active inflammation, which leads to poor prognoses [22]. Also, in gastric adenocarcinoma patients who relapse after resection, the pattern of recurrence varies greatly depending on the Laurén classification [23]. Thus, it appears reasonable that mtDNA-CN correlates with not only UICC7 tumor stage, but also Laurén classification. However, the results of our multiple regression analysis showed that the correlation between mtDNA-CN and tumor stage was significant. Thus, Laurén classification would be a confounding factor.

In this study, there was no significant correlation between mtDNA-CN and *HER2* overexpression in gastric cancer. The protein overexpression/gene amplification of *HER2* is a prognostic factor in breast cancer and a specific therapeutic target for molecular targeted drugs, thus the determination is of clinical importance. However, in gastric cancer, the ToGA study, a global phase

III trial conducted in *HER2*-positive advanced or recurrent gastric cancer patients, showed that trastuzumab, an anti-*HER2* antibody, prolonged overall survival as a first-line treatment for patients with *HER2* overexpression or gene amplification [24]. In addition, a significant improvement in the response rate and overall survival was demonstrated in the anti-*HER2* antibody-drug conjugated trastuzumab deruxtecan in *HER2*-positive advanced gastric cancer after third-line treatment [25]. Thus, it is essential to confirm the expression of *HER2* in tumor tissues prior to treatment. However, the results of this study suggest that abnormal *HER2* expression and mitochondrial genome aberrations in gastric cancer are independent. To investigate the association with other biomarkers, such as *PD-L1*, MSI/MMR, and *CLDN18*, it would also be essential for a further understanding of the role of mtDNA-CN in the progression of cancer.

There are technical limitations to this study. One is sample size and bias. The number of DNA samples limited the statistical power of this study. The lack of information on other risk factors, such as the infection of *H. pylori* [26], may not be sufficient to eliminate bias. The other is the PCR method of measuring mtDNA-CN. Note that the accuracy of relative mtDNA-CN measurements may be reduced due to the genetic instability of cancer cells [27]. In particular, alterations in the nuclear DNA genes *SLCO2B1* and *SERPINA1*—used as reference controls—could potentially affect PCR amplification efficiency. This represents a technical limitation of our study. Further validation studies are needed to confirm the robustness of our findings.

Conclusions

We determined the distribution of mtDNA-CN in gastric cancer tissues from 182 patients, as well as the correlations with age, sex, tumor stage, Laurén classification, and *HER2* overexpression. The results suggested a significant correlation between mtDNA-CN *in situ* and tumor stage, indicating that mitochondrial genomic abnormalities contribute to cancer progression independently of *HER2* pathway. Further investigation is required to validate our findings and may shed light on the emerging role of mtDNA-CN *in situ* as a biomarker for estimating cancer progression.

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Author contributions

Hisanori Fukunaga: Conceptualization, Formal analysis, Funding acquisition, Investigation, Project administration, Writing-original draft, and Writing-review and editing.

Mayuko Fukunaga: Data curation, Investigation, Resource, and Writing-review and editing.

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Conflict of Interest statement

The authors have no conflict of interest.

Data availability

The data used in this study are available upon reasonable request by writing to the corresponding author.

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Figure legends:

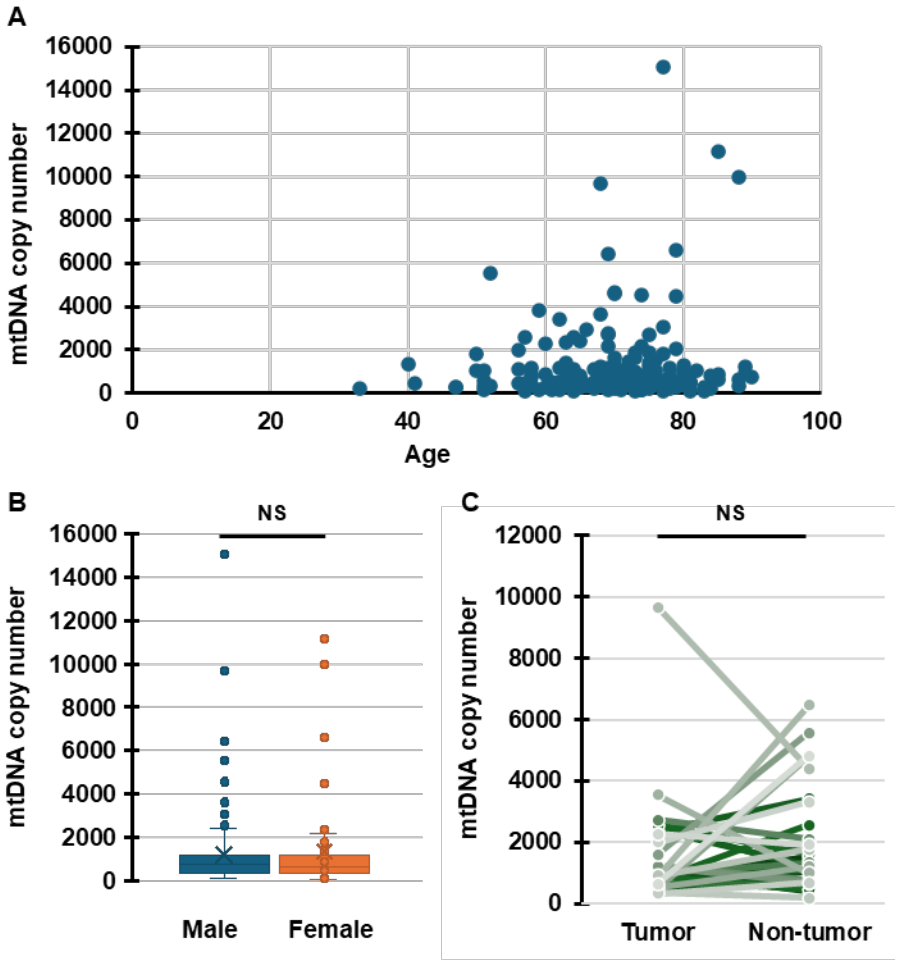

Figure 1. Distribution of mitochondrial DNA copy number (mtDNA-CN). (A) Scatter plot showing the distribution of mtDNA-CN according to patient age. (B) Box-and-whisker plot comparing mtDNA-CN in gastric cancer tissues between male and female patients. (C) Paired comparison of mtDNA copy number between tumor and matched non-tumor tissues in a subset of patients.

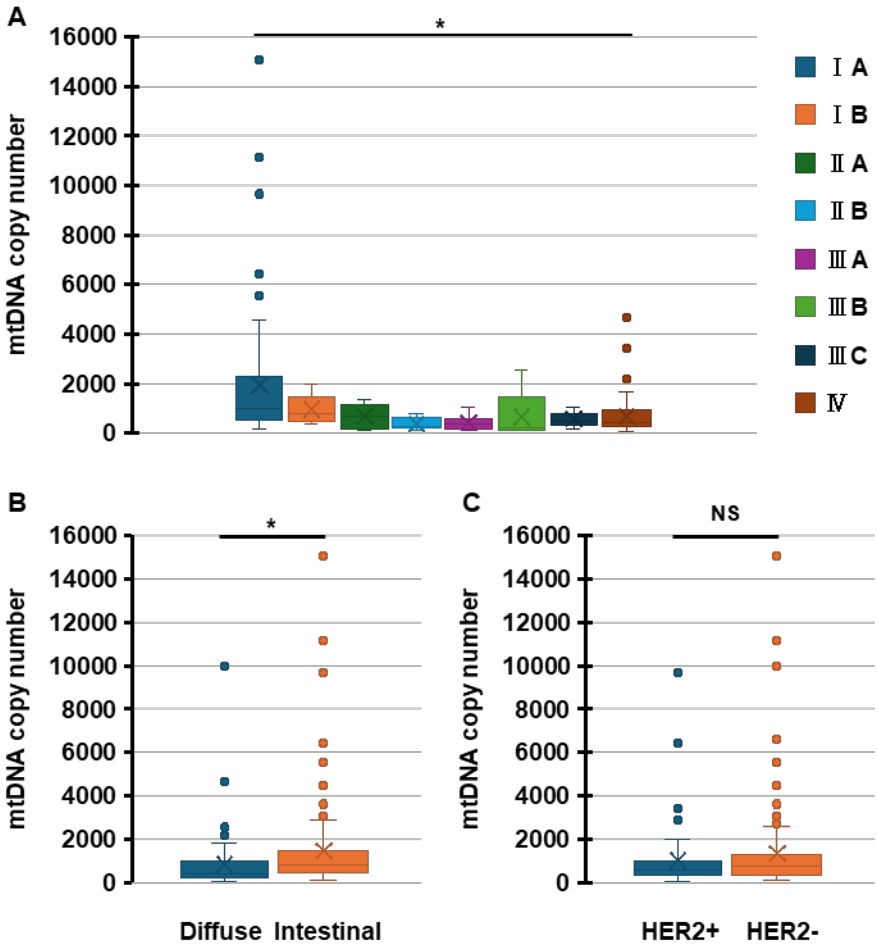

Figure 2. Comparison of mtDNA copy number (mtDNA-CN) according to tumor stage, Laurén classification, and *HER2* status. (A) Box-and-whisker plot showing the distribution of mtDNA-CN by tumor stage. (B) Box-and-whisker plot comparing mtDNA-CN between diffuse-type and intestinal-type gastric cancers, based on Laurén classification. (C) Box-and-whisker plot comparing mtDNA-CN between HER2-positive and HER2-negative tumors. *Statistically significant difference ($p < 0.05$).