Frequent Occurrence of Mitochondrial DNA Mutations in Barrett’s Metaplasia without the Presence of Dysplasia

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Abstract

Background: Barrett’s esophagus (BE) is one of the most common premalignant lesions and can progress to esophageal adenocarcinoma (EA). The numerous molecular events may play a role in the neoplastic transformation of Barrett’s mucosa such as the change of DNA ploidy, p53 mutation and alteration of adhesion molecules. However, the molecular mechanism of the progression of BE to EA remains unclear and most studies of mitochondrial DNA (mtDNA) mutations in BE have performed on BE with the presence of dysplasia.

Methods/Findings: Thus, the current study is to investigate new molecular events (Barrett’s esophageal tissue-specific-mtDNA alterations/instabilities) in mitochondrial genome and causative factors for their alterations using the corresponding adjacent normal mucosal tissue (NT) and tissue (BT) from 34 patients having Barrett’s metaplasia without the presence of dysplasia. Eighteen patients (53%) exhibited mtDNA mutations which were not found in adjacent NT. mtDNA copy number was about 3 times higher in BT than in adjacent NT. The activity of the mitochondrial respiratory chain enzyme complexes in tissues from Barrett’s metaplasia without the presence of dysplasia was impaired. Reactive oxygen species (ROS) level in BT was significantly higher than those in corresponding samples.

Conclusion/Significance: High ROS level in BT may contribute to the development of mtDNA mutations, which may play a crucial role in disease progression and tumorigenesis in BE.

Introduction

Patients with Barrett’s esophagus (BE) have a 30 to 125-fold higher risk of developing esophageal adenocarcinoma (EA) than those without this lesion. The presence of BE confers a 0.5~1% per year risk of the development of EA [1–5]. The prevalence of esophageal cancer is increasing in the world and has a poor prognosis mainly because of presentation at late stage. Progression to esophageal cells to cancer follows the metaplasia-dysplasia-adenocarcinoma sequence. Barrett’s metaplasia of esophagus is defined as replacement of the normal squamous epithelium of distal esophagus by metaplastic glandular epithelium containing intestinal-type goblet cells. The numerous molecular events may play a role in the neoplastic transformation of Barrett’s mucosa such as the change of DNA ploidy, p53 mutation and alteration of adhesion molecules. However, the molecular mechanism of the progression of BE to EA remains unclear.

The mitochondrion is an important micro-organelle that produces the energy for cell development, differentiation and growth by inducing apoptosis [6]. mtDNA repair system is inefficient and there is a higher exposure to reactive oxygen species (ROS) produced in the process of adenosine triphosphate (ATP) synthesis. ROS are commonly released in chronic inflammatory tissue. mtDNA is one of the target of ROS and exacerbate an impairment of mitochondrial function, has been implicated in the etiology of aging, several degenerative pathologies, and tumorigenesis based on the presence of novel hetero-and homoplasmies [9]. Somatic mtDNA mutations play a role in oncogenic transformation and they also can contribute to tumor progression by enhancing the metastatic potential of tumor cells [10]. Thus, the current study is to investigate new molecular events (Barrett’s metaplasia tissue-specific-mtDNA alterations/instabilities) in mitochondrial genome and causative factors for their alterations using the corresponding adjacent normal mucosal tissues and metaplasia tissue from Barrett’s metaplasia with the presence of dysplasia.

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Results

mtDNA Sequence Alterations

In a set of analyses of the mtDNA control region obtained from adjacent NT and BT, characteristic heteroplasmic substitution mutations in the mtDNA control region were observed in two patients (6%), respectively (Table 1, Fig. 1). mtDNA length heteroplasmic mutation at poly C stretch of 303 poly C, 16189 poly C and 514 (CA) repeat were observed in 18 patients (53%) (Table 1, Fig. 2). Overall, BT-specific mtDNA mutations were observed in 53% (18/34) of all patients.

Spectrum of Length Heteroplasmic Mutations

A gene scan (capillary electrophoresis) showed length heteroplasmies in 303 poly C, 16189 poly C and 514 CA repeats which were confirmed by TA cloning. The pattern of BT-specific length heteroplasmic mutation at 303 poly C region was distributed into 8CT6C+9CT6C+10CT6C+11CT6C, 7CT6C+8CT6C+9CT6C+10CT6C+11CT6C+12CT6C, 7CT6C+8CT6C, 8CT6C+9CT6C+10CT6C+11CT6C+12CT6C+13CT6C+14CT6C, 6CT6C+7CT6C+8CT6C+9CT6C+10CT6C, 15C+16C+17C+18C, 14CT6C, 9CT6C+9CT6C+10CT6C (2.9%/each) (Table 1, Fig. 2). The pattern of BT-specific length heteroplasmic mutation at 514 (CA) repeats in HV2 regions disclosed 4(CA) repeats (2.9%) (Table 1). BT-specific length heteroplasmic mutation at 16189 poly C displayed four types of patterns: 9C+10C+11C+12C, 5C+6C+5C+4C, 10C+11C+12C, 9C+10C+11C (2.9%/each) (Table 1). Specimens that showed dissimilarities in length heteroplasmie between patient adjacent NT and BT were 23% (8 cases) for 303 poly C, 29% (1 case) for 514 (CA) repeats and 11% (4 cases) for 16189 poly C. Three patients had BT-specific length heteroplasmic mutations arising in both 303 poly C and 16189 poly C stretch regions. One patient had BT-specific length heteroplasmic mutations from both 303 poly C and 514 (CA) repeats (Table 1).

Table 1. mtDNA substitution and length heteroplasmic mutations in Barrett's metaplasia tissue (BT) and adjacent normal mucosal tissue (NT).

| Mutation status | mtDNA genes or regions | Samples from patients with Barrett’s esophagus | Patient ID | No. | % |
|-----------------|------------------------|-----------------------------------------------|-----------|-----|---|
| Substitution    | Control/HV2            | C174G/T                                       | 2         | 1   |   |
| Control/HV2, HV1| A200G, T16136C, T16172C, A16183C | 27                                               | 1         |     |   |
| Subtotal        |                        |                                               |           | 2   | 5.9|
| Length heteroplasmic mutation | 303 Poly C | 8CT6C+9CT6C+10CT6C | 8CT6C+9CT6C+10CT6C+11CT6C | 1 | 1 |
|                 |                        | 8CT6C+9CT6C+10CT6C                             | 7CT6C+8CT6C+9CT6C+10CT6C+11CT6C+12CT6C | 2 | 1 |
|                 |                        | 7CT6C+8CT6C+9CT6C                             | 7CT6C+8CT6C+9CT6C+10CT6C+11CT6C+12CT6C | 5 | 1 |
|                 |                        | 8CT6C+9CT6C+10CT6C+11CT6C+12CT6C              | 8CT6C+9CT6C+10CT6C+11CT6C+12CT6C+13CT6C+14CT6C | 9 | 1 |
|                 |                        | 6CT6C+7CT6C+8CT6C                             | 6CT6C+7CT6C+8CT6C+9CT6C+10CT6C | 12 | 1 |
|                 |                        | 15C+16C+17C+18C+19C+20C                     | 15C+16C+17C+18C | 13 | 1 |
|                 |                        | 7CT6C+8CT6C+9CT6C                             | 14C+15C+16C+17C+18C | 16 | 1 |
|                 |                        | 8CT6C+9CT6C                                  | 8CT6C+9CT6C+10CT6C | 34 | 1 |
|                 | 16189 Poly C           | 9C+10C+11C+12C+13C                           | 9C+10C+11C+12C | 15 | 1 |
|                 |                        | 4CT4C+5CT4C                                 | 3CT4C+5CT4C | 17 | 1 |
|                 |                        | 9C+10C+11C+12C                              | 10C+11C+12C | 21 | 1 |
|                 |                        | 10C+11C                                      | 9C+10C+11C | 31 | 1 |
|                 | 514 CA                 | CA4+CA5                                     | CA4       | 32 | 1 |
|                 | 303 Poly C             | 7CT6C+8CT6C+9CT6C+10CT6C                     | 5CT6C+6CT6C+7CT6C+8CT6C+9CT6C | 8 | 1 |
|                 | 16189 Poly C           | 9C+10C+11C+12C+13C                          | 10C+11C+12C+13C | 26 | 1 |
|                 | 303 Poly C             | 8CT6C+9CT6C+10CT6C+11CT6C                   | 7CT6C+8CT6C+9CT6C+10CT6C | 26 | 1 |
|                 | 16189 Poly C           | 5CT4C                                      | 4CT4C+5CT4C | 27 | 1 |
|                 | 303 Poly C             | 8CT6C+9CT6C+10CT6C+11CT6C+12CT6C            | 13C+14C+15C+16C+17C | 33 | 1 |
|                 | 16189 Poly C           | 9C+10C+11C+12C+13C                        | 10C+11C+12C+13C | 34 | 1 |
|                 | 303 Poly C             | 6CT6C+7CT6C+8CT6C+9CT6C+10CT6C              | 8CT6C+9CT6C+10CT6C | 34 | 1 |
|                 | 514CA                  | CA4+CA5                                     | CA4       | 32 | 1 |
| Subtotal        |                        |                                               |           | 18 | 52.9|
| Total           |                        |                                               |           | 18 | 52.9|
**mtDNA Copy Number**

The mtDNA copy number (molecules/ul) from BT and adjacent NT showed 4.75±12.14, 1.22±0.78 (Fig. 3). The mtDNA copy number was significantly increased in BT than adjacent NT (P=0.01).

**Hydrogen Peroxide Content**

Hydrogen peroxide was measured from BT and corresponding adjacent NT specimen to investigate the cause of BT-specific mtDNA mutations founded in the current study. The level of hydrogen peroxide significantly elevated in supernatants from BT (90.1±50.7 lM/mg protein) compared with those from adjacent NT (48.9±17.6 lM/mg protein) (Fig. 4). The level of hydrogen peroxide in the BT was significantly higher than those from the corresponding samples (P=0.017).

**Quantitation of mtDNA Large (4977 bp) Deletion**

mtDNA large (4977 bp) deletion in BT and matched adjacent NT were quantified using gene scan and real-time PCR which were developed in the current study. The amounts of mtDNA large deletion were significantly increased in BT compared with those from adjacent NT (P=0.04) (Fig. 5).

**Functional Alteration of Respiratory Chain Enzyme Complex Activity in Barrett’s Tissue**

The activity of mitochondrial respiratory chain enzyme complexes in Barrett’s tissue was impaired in comparison with those of the adjacent normal mucosa: Decreased enzyme activity...
in respiratory chain complexes I, II and III was observed in Barrett’s tissue (Table S3).

Discussion

Carcinogenesis is a long-term, multistep process driven by genetic and epigenetic changes in susceptible cells, which gain a selective growth advantage and undergo clonal expression [11]. BE is a premalignant condition that predisposes to the development of EA. It is detected on endoscopy and confirmed histologically by the presence in the lower esophagus of a metaplastic mucosa, the so-called specialized epithelium, which resembles incomplete intestinal metaplasia in the stomach. The similarities with incomplete intestinal metaplasia is present on histology, mucin histochemistry and immunohistochemistry with various differentiation markers (cytokeratins and MUC antigens). On morphology, the carcinogenic process of Barrett’s mucosa progresses through increasing grades of epithelial dysplasia. Dysplasia, a synonym of intraepithelial neoplasia, is the only marker that can be used at the present time to delineate a population of patients at high risk of cancer.

Although high-grade dysplasia is considered a precursor to invasive adenocarcinoma, detection of this abnormal mucosa remains controversial and currently requires esophagoscopy with biopsy. It has been suggested that epithelial markers, such as increased activity of mucosal ornithine decarboxylase, sulfomucin production, nuclear DNA aneuploidy, and recently molecular analysis, have also been proposed to identify patients at increased risk for malignant degeneration [12].

Among the numerous molecular events that have been shown to play a role in the neoplastic transformation of Barrett’s mucosa, only changes in DNA ploidy, increased proliferation, and alterations of the p53 gene have been suggested to be of potential help in the carcinogenesis [14]. As mentioned in introduction, mtDNA mutation has been regarded as oncogenic factors [15]. However, the study on mitochondrial dysfunction and mtDNA mutation was paucity in BE. The mitochondrion is an important microrganelle that produces the

![Figure 3. Significant increase in mtDNA copy number in Barrett’s metaplasia tissue.](image)

Quantitation of mtDNA copy number was calculated from the ratio of copy number (copies/μl) from mtDNA cytochrome b and nuclear β-actin gene using real-time PCR. The average mtDNA copy number was significantly increased in Barrett’s metaplasia tissues (BT) compared to those from adjacent normal mucosal tissues (NT) \((P=0.01)\). doi:10.1371/journal.pone.0037571.g003

![Figure 4. Production of high level of reactive oxygen species in Barrett’s metaplasia tissue.](image)

Quantitative determination of reactive oxygen species (hydrogen peroxide) from supernats of Barrett’s metaplasia tissue (BT) and adjacent normal mucosal tissue (NT) was measured by commercial kit (Bioxytech H2O2-560). The level of hydrogen peroxide was significantly elevated in supernats from BT compared with those from adjacent NT \((P=0.017)\). doi:10.1371/journal.pone.0037571.g004

![Figure 5. Increase of mtDNA large (4977 bp) deletions in Barrett’s metaplasia tissue.](image)

The amounts of mtDNA large deletions were significantly increased in Barrett’s metaplasia tissue (BT) compared with those from matched adjacent normal tissue (NT). doi:10.1371/journal.pone.0037571.g005
energy for cell development, differentiation and cell growth by inducing apoptosis. Human mtDNA contains 16,569 base pairs and represents 0.1–1.0% of the total genomic DNA. A mammalian cell contains approximately 1,000 mitochondria, and each mitochondrion contains 2 to 10 DNA copies. There is a 10–20 fold greater susceptibility of mtDNA to genetic mutation because mtDNA does not contain introns in comparison to nuclear DNA, the mtDNA repair system is inefficient and there is a higher exposure to ROS produced in the process of ATP synthesis. ROS plays an important role as potential carcinogens in the pathogenesis of GERD, BE and gastritis [16]. Oxidative damage has long been related to mucosal damages of gastrointestinal tracts and their ensuing carcinogenesis. In spite of treatment with anti-secretory medications for reflux esophagitis, considerable portions of patient did not achieve the complete mucosal healings or suffered from sustaining symptoms or development of dread complication like BE, suggesting other damaging factors or impaired mucosal resistance are also involved in their pathogenesis [17,18].

BE occurs due to chronic inflammation which is mainly caused by GERD. An increase in ROS production frequently happens in chronic inflammatory cells and tissue. Thus, the current study attempted to detect BT-specific mtDNA alterations with the assumption that mtDNA aberrations are caused by ROS abundantly produced in chronically inflamed BT. mtDNA mutations in the current study were detected exclusively in BT samples but not in adjacent NT. These mtDNA mutations were observed as base substitutions and length heteroplasmy in the control region containing. The oxidative stress elicited by chronic inflammation increases the number of mtDNA mutations in BT and might correlate with a precancerous status [19]. The level of ROS was significantly higher in the supernatants obtained from BT compared with those from the adjacent NT. This high level of ROS might damage the mitochondria, leading to mtDNA mutations. mtDNA control region contains the mtDNA production regulating fraction and HV region known as a ‘hot spot’ of gene mutation in various degenerative diseases and tumors [20].

Recent reports have showed that severe morphological and functional alterations of cells and tissue are accompanied by a decrease of mtDNA copy number [6]. This is known to be a consequence of mtDNA mutation in the region near the replication origin of mtDNA. In contrast, it has been reported that the number of mtDNA copies increases if ATP synthesis is hindered by aging or chronic inflammation [6]. Concurrent with these reports, the number of mtDNA copies measured in BT was approximately four times higher than those in corresponding adjacent NT in the current study.

Recently one study reports that the frequency of specimens with the 4977 bp deletion increased in relation to the degree of dysplasia, so the mtDNA 4977 bp deletion may be useful as a biomarker to detect the severity [9]. The current study also confirms that the amounts of mtDNA large deletion were significantly increased in BT compared with those from either matched adjacent NT.

Importantly, mutations in the control region might alter the rate of DNA replication by modifying the binding affinity of significant trans-activating factors. These mtDNA alterations in BT might further impair a respiratory chain defect resulting in increasing the mtDNA copy number to compensate for the deficiency in ATP. During this perturbation, mitochondria might produce a large amount of ROS, which causes the vicious cycle observed in other chronic inflammatory diseases [17].

BT-specific mtDNA mutations frequently occurred in both the mtDNA control and minisatellite regions due to excessive production of ROS. High level of ROS in BT may contribute to development of mtDNA mutations, which may play a crucial role in pathophysiology of BE and furthermore progression to EA. Although many risk factors are considered in BE and EA, antioxidant treatment seems to be the therapeutics in the prevention or treatment of BE and moreover it plays the preventive role in disease progression.

Materials and Methods

Patient Specimens and Tissue Staining

Thirty-four patients were enrolled after receiving Institutional Review Board approval and informed consent. Matched Barrett’s metaplasia tissue (BT) and adjacent normal mucosal tissue (NT) was obtained during the endoscopic procedure (Table S1). A diagnosis of BE was based on findings of endoscopy and specialized intestinal metaplasia on Hematoxylin and Eosin (H&E) stained tissue. Following standard tissue preparation, tissue was embedded in paraffin and was sliced with a microslicer. Sliced tissue was embedded into slides and stained with H&E (Fig. 6).

Direct Sequencing of mtDNA Control Region

BT was obtained in a test tube containing 5 ml phosphate buffered saline (PBS, pH 7.4), sliced into small pieces with a sterilized mesh, and dissolved in protease K and tissue lysis buffer solution, from which DNA was extracted. Extracted total DNA was then dissolved in TE buffer (10 mM Tris-HCl, 1 mM Na2EDTA, pH 8.0) and was quantified with the use of a spectrophotometer. Published protocol was used to amplify and sequence mtDNA control region [20]. The mtDNA sequences obtained were analyzed using the Revised Cambridge Reference Sequence (http://www.mitomap.org/), Blast2 program (http://www.ncbi.nlm.nih.gov/blast/b2seq/wblast2.cgi) and MitoAnalyzer (http://www.cstl.nist.gov/biotech/srbase/mitoanalyzer.html) to determine mtDNA aberrations (Table S2).

Determination of mtDNA Length Heteroplasmasies in the Control Region

mtDNA minisatellites are located in the mtDNA non-coding hypervariable regions (HV) 1 and 2. Size-based amplified product separation by capillary electrophoresis based on published protocol was performed in order to detect length heteroplasmic mutations of 16189 poly C (16184CCCCCCCTCCCCC16193, 5CT4C), 303 poly C (303CCCCCCCTCCCCC315, 7CT5C) and 514 (CA) repeat (511CACACACACACA523, (CA)5 repeats) [20,21] (Table S2).

TA Cloning for Confirmation of Length Heteroplasmic Mutations

The procedure including a set of primer pairs and PCR conditions was carried out according to the published protocol [22]. Each purified PCR product of 303 poly C, 16189 poly C and 514 (CA) repeat was inserted into pGEM-T Easy Vector (Promega, Madison, WI, USA). Competent E. coli JM 109 cells were then transformed with the plasmids containing the PCR product inserts. White colonies (8 to 12) including recombinant plasmids were selected and cultured. After plasmids extraction, length heteroplasmic mutations were determined by DNA sequencing (Table S2).

Quantification PCR for Determination of mtDNA Copy Number

To generate a standard curve for quantification of mtDNA, purified PCR product of CYTB and β-actin gene as internal standard were inserted into pGEM-T easy vector and E. coli JM
Quantitative Determination of Hydrogen Peroxide

BT and adjacent NT were homogenized in 500 µl of ice-cold deionized water. An aliquot of homogenate was taken for protein measurement. After centrifugation at 2000 g for 15 min, an aliquot of 200 µl of supernatant was transferred to new tube. Hydrogen peroxide content (µM/mg protein) from supernatant is measured using the commercial kit (Bioxytech_ H2O2-560TM, OXIS International, Foster City, CA, USA), 400 µl of 7.5% hydrogen peroxide solution was mixed with 400 µl of 0.6 mg/mL benzaldehyde and 50 µl of 10% sodium carbonate. After 15 min at room temperature, 300 µl of 10% acetic acid was added to each tube, followed by mixing. The absorbance was measured at 560 nm. The hydrogen peroxide content in the sample is directly proportional to the absorbance. It is calculated using the formula: hydrogen peroxide content (µM/mg protein) = [A560 - blank] / [blank] x 5, where [A560] is the absorbance at 560 nm of the sample and [blank] is the absorbance at 560 nm of the blank.

mtDNA Large(4977 bp) Deletion

For generation of a fluorescent labeled 113 bp fragment of the deleted mtDNA, a mixture of 25 µl containing 12.5 µl of 2x Quantitect SYBR green PCR master mix (Qigagen, Valencia, CA, USA), 400 µM CYTB primers F14909 (5'-TACTTACCGAGCGCTCAACCG-3') and R15396 (5'-TATCGGA ATGGGAGGT GATTC-3') and 6 ng of total DNA was used for PCR with the Rotor-Gene real-time centrifugal DNA amplification system (Corbett Research, Sydney, Australia). For PCR, hot start reactions at 50°C for 2 min and at 95°C for 15 min were followed by 35 cycles of 20 sec at 94°C, 30 s at 56°C, 30 s at 72°C and a melting reaction with a decrease of 1°C per cycle between 72°C and 92°C. The mtDNA copy number was calculated using the following formula: m = (X µg/µl plasmid DNA/4419 (plasmid length) x 600) x 6.022 x 10^23 = Y molecules/ul. The X represents the concentration of plasmid DNA and the Y represents copy number. The mtDNA copy number was reported as the ratio of CYTB and β-actin gene.

Determination of Activity of Mitochondrial Respiratory Chain Enzyme Complexes

Cell homogenates were diluted to 1 g/L total protein with 20 mmol/L potassium phosphate buffer (pH 7.2) before respira-
tory chain complex analysis. Assay conditions were based on previously published spectrophotometric methods [23].

Statistical Analysis

Descriptive data were expressed as mean ± standard deviation. The non-parametric Kruskal-Wallis test was used for the determination of statistical difference in the mtDNA copy number and hydrogen peroxide content between three specimens (BT and adjacent NT). P-value that is lower than 0.05 was considered as statistically significant.

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Supporting Information

Table S1 Age and sex distribution of subjects.

Table S2 Primers for mtDNA control region PCR, direct sequencing, and gene scan.

Table S3 Respiratory chain complex I-IV activities in primary Barrett’s metaplasia tissues and adjacent normal tissues.

Author Contributions

Conceived and designed the experiments: HRK MGS. Performed the experiments: HYK. Analyzed the data: MJH SGB DH. Wrote the paper: MGS HRK. Edited the manuscript: JHS SPS DWR. Collected the samples and provided clinical information: SL.