High incidence of coding gene mutations in mitochondrial DNA in esophageal cancer

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Abstract. The aim of the present study was to detect mutations in the coding genes of mitochondrial DNA (mtDNA) in three esophageal cancer cell lines and in tumor tissues obtained from 30 patients with esophageal cancer, to investigate the relationship between protein- and RNA-coding gene mutations and esophageal cancer. mtDNA was extracted and the coding genes were sequenced and analyzed by comparing the sequencing results with the complete mitochondrial genome of *Homo sapiens*. The results revealed 39 mutations in the three esophageal cancer cell lines; the genes with the highest mutation frequencies included mitochondrially encoded cytochrome B (MT-CYTB), NADH dehydrogenase 5 (MT-ND5) and MT-ND4 gene. A total of 216 mutations were identified in the 30 esophageal cancer tissues, including 182 protein-coding mutations, of which MT-CYTB and MT-ND5 genes exhibited higher mutation frequencies. The results of the present study indicated that mutations in the coding genes of mtDNA in esophageal cancer cells may be related to the occurrence of esophageal cancer.

Introduction

Tumor development is a multifactorial process involving several steps. It is currently considered that the biological characteristics of a tumor not only depend on the nuclear genetic material, but also have a certain relationship with extranuclear genetic material from mitochondrial DNA (mtDNA) (1). As studies on mitochondria increase in number, it has been revealed that the DNA in the mitochondrial basilar membrane is a unique genetic material that only exists outside the nucleus (2). Mitochondria are cellular organelles that are required for oxidative respiration for the production of energy and are the center of aerobic metabolism; therefore, large amounts of oxygen free radicals are produced during oxidative phosphorylation in the mitochondria, which exposes mtDNA to higher concentrations of reactive oxygen species (ROS) (3). Coinciding with this is an imperfect mtDNA damage repair system and a lack of protection of mtDNA against histone and DNA-binding proteins (4-6). Therefore, mtDNA is more susceptible to attacks by carcinogens, leading to damage and mutation with the mutation rate being 10-20 times higher than that of nDNA (7-10). mtDNA is divided into the non-coding displacement (D)-loop region and the coding region. The majority of previous studies concentrated on the D-loop region of the mtDNA. The D-loop region is of great interest in the area of mtDNA mutations in many tumor tissues, and in non-neoplastic diseases and normal tissues; the D-loop region is also of great interest in the area of mtDNA mutations, indicating that a high rate of mutations in the D-loop region is not specific to tumors. Currently, there are few reports on the relationship between coding gene mutations in mtDNA and tumorigenesis. The present study uses 3 esophageal cancer cell lines and 30 tissue samples from patients with esophageal cancer to investigate the presence of mutations in coding sequences in the mtDNA. The mtDNA genes were sequenced to determine the relationship between the mutations and the occurrence of esophageal cancer.

Materials and methods

Esophageal carcinoma cell lines. The EC9706 cell line was a gift from The Key Laboratory of State Molecular Oncology, Chinese Academy of Medical Sciences (Beijing, China). The TE-1 and Eca109 cell lines were gifts from The School of Pharmacy, Zhengzhou University (Zhengzhou, Henan, China).

Cell culture. The Ec9706, TE-1 and Eca109 cells were seeded in separate culture flasks with RPMI-1640 medium containing 10% fetal bovine serum (both Beijing Solarbio Science & Technology Co., Ltd., Beijing, China), which were then placed

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in an incubator at 37˚C and 5% CO_2. The culture medium was replaced every two days and cells were grown to ~90% confluency.

Esophageal cancer tissues. A total of 30 tumor samples were obtained from patients diagnosed with esophageal cancer, who underwent surgery in The First Affiliated Hospital of Zhengzhou University and in The Second Affiliated Hospital of Zhengzhou University (Zhengzhou, China). None of the patients in this study received preoperative treatments, such as chemotherapy or radiotherapy. This study was approved by the Ethics Review Committee of The Second Affiliated Hospital of Zhengzhou University, and all patients provided signed written informed consent. Tumor tissue samples were collected within 30 min following surgical removal and were stored at -80˚C until all samples were collected for combined submission for mtDNA sequencing.

Extraction of mtDNA from cells and tissues. For mitochondrial extraction, ~$5x10^7$ cells from each cell line were required and ~100 mg of each tissue was used. All of the mitochondrial extract was then used to extract mtDNA. Mitochondria were extracted using a Mitochondria Isolation kit (Beijing Solarbio Science & Technology Co., Ltd.) and the mtDNA was isolated using a Mitochondrial DNA Extraction kit (Shanghai Jiemei Gene Pharmaceutical Technology Co., Ltd., Shanghai, China) according to the manufacturer's protocol. The concentration and purity of mtDNA sample were determined by at least three spectrophotometric measurements, with distilled water used as a blank control. The A260/A280 ratio of pure mtDNA samples was 1.8; a ratio >1.9 indicated RNA contamination, and <1.6 indicated contamination with proteins, phenol or other agents.

Coding gene sequencing of mtDNA in esophageal cell lines and tissues. The complete mtDNA sequence was 16,569 bp in length. The mtDNA sequence was pieced together from 15 fragments resulting from the polymerase chain reaction (PCR) method; the primer sequences, the length of the 15 sections and their corresponding locations along the mtDNA are provided in Table I. All primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The 2X Taq PCR MasterMix (Beijing Biomed Gene Technology Co., Ltd, Beijing, China) containing the Taq DNA polymerase and dNTPs was used. The 25 μl PCR reaction system contained 1 μl of mtDNA, 1 μl of forward primer, 1 μl of reverse primer, 12.5 μl of PCR Master Mix (2X) and 9.5 μl of nuclease-free water (Thermo Scientific). The PCR amplification conditions were as follows: Initial denaturation at 95˚C for 3 min, followed by 35 cycles of denaturation at 94˚C for 30 sec, annealing at 55-60˚C for 35 sec, and extension at 72˚C for 50 sec, and a final extension at 72˚C for 8 min. A total of 5 μl PCR product was mixed with 6X Loading buffer (Beyotime Institute of Biotechnology, Shanghai, China) were checked by electrophoresis on 1% agarose gel. A 10,000 bp DNA marker was used to determine the size of the amplified fragments; electrophoresis was performed with 0.5X Tris-borate-EDTA buffer with a constant of 150 V for 20 min. The gel was visualized with ethidium bromide and imaged by a gel image analyzer. PCR products were excised from the gel and purified using the UNIQ-10 Spin Column DNA Gel Extraction kit (Sangon Biotech Co., Ltd.) according to the manufacturer's instructions. The purified PCR products were sent to Sangon Biotech Co., Ltd. for sequencing.

mtDNA mutation analysis. To analyze the collected data, ABACUS algorithm based software (Auto-analysis with GeneMapper 4.0) was provided by Sangon Biotech Co., Ltd. Sequence data were analyzed with Sequencing Analysis Software version 5.2 (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA), Sequence Scanner v1.0 (Applied Biosystems; Thermo Fisher Scientific, Inc.), ChromasPro v2.1.5 (Technelysium Pty., Ltd., South Brisbane, Australia) and EditSeq Lasergene v7.1 (DNASTAR, Inc., Madison, WI, USA). To define the mutation, a revised Cambridge Reference Sequence [rCRS; National Center for Biotechnology Information (NCBI) GenBank accession NC_012920.1; www.ncbi.nlm.nih.gov/nuccore/251831106] was used as the reference sequences.

Results

mtDNA sequencing analysis of three esophageal cancer cell lines. Following sequencing of the mtDNA coding region in the TE-1, EC9706 and Eca109 cell lines, the sequences were compared with the complete mitochondrial genome of *Homo sapiens* (NCBI reference sequence NC_012920.1). The sequencing results identified 39 mutations in the 3 cell lines, 38 of which were base substitutions. The majority of the mutations were A→G or C→T substitutions; 10 of the identified mutations occurred within all three cell lines, including A750G, A1438G, A2706G, A4769G, C7028T, A8860G, G11719A, C12705T, C14766T and A15326G (Table II). There were three mutations identified that led to an amino acid replacement, including A8860G (Thr→Ala) in the mitochondrially encoded ATP synthase 6 (MT-ATP6) gene, and C14766T (Thr→Ile) and A15326G (Thr→Ala) in the cytochrome B (MT-CYTB) gene. A total of 31 protein-coding and 8 RNA-coding mutations were identified (Table III). The mutation frequencies were also assessed by calculating the percentage of the total mutations identified in the 3 cell lines that were also observed in CYTB: Mutation frequency (%) = number of mutations in CYTB / 39. Among the 31 protein-coding mutations, MT-CYTB, MT-ND5 and MT-ND4 exhibited the highest mutation frequencies of 15.4% (6/39), 12.8% (5/39) and 12.8% (5/39), respectively.

mtDNA sequencing analysis of 30 esophageal cancer tissues. Similar to the cell lines, the sequences obtained from the 30 tissue samples were compared with the *H. sapiens* mitochondrial genome NC_012920.1. A total of 216 mutations were identified, including 214 point mutations, one single-nucleotide insertion and one single-nucleotide deletion. A majority of the mutations were A→G and C→T substitutions. Among the identified 216 identified mutations, 84.2% (182/216) occurred in genes involved in mitochondrial respiratory complexes (Table III). MT-ND5, MT-CYTB and MT-ND2 exhibited the highest mutation frequencies at 12.5% (27/216), 11.1% (24/216) and 10.2% (22/216), respectively. In all 30 tissue samples analyzed, the A750G, A4769G, A8860G, G11719A, C14766T and A15326G mutation sites were observed. In addition, the two mutations, A1438G and A2706G, were identified in 29

Table I. List of primers used to obtain full-length human mitochondrial DNA sequence.

Primer name	Primer sequence (5'→3')	Fragment size (bp)	Starting and ending sites
NO1	F: AAACAAAGAACCCTAACACCAGC	1,443	359-1,801
	R: TCATCTTTCCCTTGCGGTACTA		
NO2	F: CCCACTCCACCTTACTACCAG	1,414	1,689-3,102
	R: ATAGAAACCGACCTGGATTACT		
NO3	F: ACCAACGGAACAAGTTACCC	1,479	2,910-4,388
	R: TGATAGGTGGCACGGAGAA		
NO4	F: CCTACCACTCACCCTAGCATTACT	1,455	4,185-5,639
	R: TAAAGTGGCTGATTTGCGTTC		
NO5	F: AAACAATAGCCTCATCATCCC	1,374	5,285-6,658
	R: CCGAAGCCTGGTAGGATAAG		
NO6	F: CAATACCAAACGCCCCTCT	1,290	6,435-7,724
	R: TGAGTGTTAGGAAAAGGGCATA		
NO7	F: GTTTCAAGCCAACCCCATG	1,331	7,479-8,809
	R: TTGGTGTAAATGAGTGAGGCAG		
NO8	F: CCCCACCTCCAAATATCTCA	1,254	8,619-9,872
	R: TTGGCGGATGAAGCAGATA		
NO9	F: CAGGCATCACCCCGCTAA	1,376	9,562-10,937
	R: GGTCGGAGGAAAAGGTTGG		
NO10	F: CACATATGGCCTAGACTACGTACA	1,214	10,718-11,931
	R: ATATTTGATCAGGAGAACGTGGT		
NO11	F: CCACGGGCTTACATCCTCA	1,340	11,713-13,052
	R: CCTTCTATGGCTGAGGGGAG		
NO12	F: ACAGCAGCCATTCAAGCAA	1,450	12,832-14,281
	R: GTCAGGGTTGATTCGGGAG		
NO13	F: TCTTACGAGCCAAAACCTGC	1,310	13,962-15,271
	R: GAGGGTGGGACTGTCTACTGAG		
NO14	F: ACATCGGCATTATCCTCCTG	1,271	15,087-16,357
	R: AAGGGATTTGACTGTAATGTGCT		
NO15	F: ACACCAGTCTTGTAAACCGGA	1,381	15,909-16,569; 1-720
	R: ACTCACTGGAACGGGGATG		

F, forward; NO, number; R, reverse.

Table II. Top 10 common mutations in the three esophageal cancer cell lines.

Locus	Nucleotide position	Nucleotide change	Amino acid change
MT-RNR1	750	A→G	Noncoding
MT-RNR1	1,438	A→G	Noncoding
MT-RNR2	2,706	A→G	Noncoding
MT-ND2	4,769	A→G	Met→Met
MT-COX1	7,028	C→T	Ala→Ala
MT-ATP6	8,860	A→G	Thr→Ala
MT-ND4	11,719	G→A	Gly→Gly
MT-ND5	12,705	C→T	Ile→Ile
MT-CYTB	14,766	C→T	Thr→Ile
MT-CYTB	15,326	A→G	Thr→Ala

ATP6, ATP synthase 6; CYTB, cytochrome B; COX1, cytochrome C oxidase 1; MT, mitochondrially encoded; ND, NADH dehydrogenase; RNR1, 12S RNA; RNR2, 16S RNA.

Table III. Summary of the identified mitochondrial DNA mutations in 3 esophageal cancer cell lines and 30 esophageal cancer tissues.

Source	Mutations in protein-coding genes	Mutations in RNA genes
Cells	31	8
Tissues	182	34

tissue samples, the C7028T mutation occurred in 26 tissue samples and the C12705T mutation was observed in 22 tissue samples (Table IV).

Discussion

mtDNA mutations occur in a variety of human malignancies, and it has been demonstrated that mutations in mtDNA are

Table IV. Top 10 common mutations in esophageal cancer tissues.

Locus	Nucleotide position	Nucleotide change	Amino acid change	Number of mutated tissues
MT-RNR1	750	A→G	Noncoding	30
MT-RNR1	1,438	A→G	Noncoding	29
MT-RNR2	2,706	A→G	Noncoding	29
MT-ND2	4,769	A→G	Met→Met	30
MT-COX1	7,028	C→T	Ala→Ala	26
MT-ATP6	8,860	A→G	Thr→Ala	30
MT-ND4	11,719	G→A	Gly→Gly	30
MT-ND5	12,705	C→T	Ile→Ile	22
MT-CYTB	14,766	C→T	Thr→Ile	30
MT-CYTB	15,326	A→G	Thr→Ala	30

MT, mitochondrially encoded; RNR1, 12S RNA; RNR2, 16S RNA; ATP6, ND, NADH dehydrogenase; COX1, cytochrome C oxidase 1; ATP synthase 6; CYTB, cytochrome B.

closely related to the occurrence and development of malignant tumors (11). In 1998, Polyak *et al* (12) reported for the first time the presence of mtDNA mutations in 7 out of 10 colon cancer cell lines. In that study, 11 of the identified mutations were single-base substitutions and 1 was an insertion; there were 12 somatic mutations and >10 times more mtDNA mutations than nDNA mutations. Another study detected 14 mutations through sequencing of the mitochondrial genome of 15 breast cancer tissues and distant normal tissues, of which 5 were located in the ND genes (2 mutations in *ND2*, 2 mutations in *ND5* and 1 mutation in *ND4*), 2 were located in MT-COX genes, 4 were located in mtRNA, 2 were located in MT-tRNA genes and 1 was located in MT-CYTB (13). One previous study reported that the mutation frequency of mtDNA in lung cancer was approximately 100 times greater than that of normal tissue (14). Among these mutations, two, located within MT-ATP6 and MT-ND3, were significantly associated with the risk of lung cancer.

In the present study, the mtDNA coding regions of esophageal cancer cell lines and tissues were sequenced and compared with the rCRS, NC_012920.1. The sequencing results demonstrated that the majority of mutations in both the cancer cell lines and the cancer tissues were base substitutions between A and G or C and T. Recently, two large-scale studies used DNA sequencing technology to compare >30 tumor types, along with normal tissues collected from >2,000 patients, to map the mutations of the mitochondrial genome (15,16). They found that many somatic cells exhibited dramatic replicative strand bias, predominantly C→T and A→G on the mitochondrial heavy strand. Similar results were observed in another study (17).

A total of 10 mutations were identified as similar between the 3 esophageal cancer cell lines, all of which were base substitution and 3 of the mutations led to amino acid replacement. A previous report identified the same three sites of amino acid changes in a study on the relationship between human life and mtDNA polymorphisms (18). Another study reported that the A8860 G transition existed in hypertrophic cardiomyopathy (HCM), and it was hypothesized that this rare polymorphism may be associated with HCM (19). The A8860G polymorphism was previously reported to reduce the rate of mitochondrial ATP production in yeasts and cultured human cells (20,21). Reanalysis of the CRS was performed by resequencing the original placental mtDNA sample from Andrews *et al* (22). The results of this resequencing confirmed that there are rare polymorphisms in the CRS; 750A, 1438A, 4769A, 8860A and 15326A all represent rare polymorphic alleles (22). Due to its widespread use, it has been recommended that in the CRS for human mtDNA, the rare polymorphic alleles should be retained so that the rCRS is a true reference sequence and not a consensus sequence (22).

In the present study, both esophageal cancer cell lines and tissues exhibited the same phenomenon, MT-ND5 and MT-CYTB had higher mutation frequencies compared with the other identified mutations. A previous study demonstrated that the MT-CYTB mutation may serve a significant role in the oxidation process in mitochondria (23).

The present results indicated that mtDNA mutations may serve a particular role in the occurrence of esophageal cancer. Mutations in mtDNA may be associated with abnormal enzyme functions in the mitochondrial respiratory chain (24). Gene mutations may alter the quality and quantity of the expression products of mitochondria, which may result in changes to the structure of the electron transport chain, leading to defects in mitochondrial function, a decline in oxidative phosphorylation function, a reduction in ATP generation and a significant increase in the number of oxygen free radicals, thereby contributing to the incidence and development of esophageal cancer. A previous study evaluated the roles of the mtDNA mutations identified in cancer cell lines using transmitochondrial cybrids to reveal that individual mtDNA mutations may be responsible for mitochondrial dysfunction (25). Our future research aims to use transmitochondrial cybrids to demonstrate individual mtDNA mutations of esophageal cancer.

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