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ORIGINAL ARTICLE

ANALYSIS OF MITOCHONDRIAL TRANSFER RNA MUTATIONS IN BREAST CANCER

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ABSTRACT

Damage of mitochondrial functions caused by mitochondrial DNA (mtDNA) pathogenic mutations had long been proposed to be involved in breast carcinogenesis. However, the detailed pathological mechanism remained deeply undetermined. In this case-control study, we screened the frequencies of mitochondrial tRNA (mt-tRNA) mutations in 80 breast cancer tissues and matched normal adjacent tissues. PCR and Sanger sequence revealed five possible pathogenic mutations: $tRNA^{Val}$ G1606A, $tRNA^{Ile}$ A4300G, $tRNA^{Ser(UCN)}$ T7505C, $tRNA^{Glu}$ A14693G and $tRNA^{Thr}$ G15927A. We noticed that these mutations resided at extremely conserved positions of tRNAs and would affect tRNAs transcription or modifications. Furthermore, functional analysis suggested that patients with these mt-tRNA mutations exhibited much lower levels of mtDNA copy number and ATP, as compared with controls ($p<0.05$). Therefore, it can be speculated that these mutations may impair mitochondrial protein synthesis and oxidative phosphorylation (OXPHOS) complexes, which caused mitochondrial dysfunctions that were involved in the breast carcinogenesis. Taken together, our data indicated that mutations in mt-tRNA were the important contributors to breast cancer, and mutational analyses of mt-tRNA genes were critical for prevention of breast cancer.

Keywords: breast cancer; mitochondrial tRNAs; mutations; carcinogenesis; pathogenic; mitochondrial dysfunction

INTRODUCTION

Breast carcinoma is one of the most frequent malignancy in females and poses a big threaten to women [1]. Breast carcinogenesis is a very complex progression with unknown etiology [2], knowledge of the genetic causes is still incomplete [3]. Among the genetic factors, mtDNA is involved in energy generation process. Normal cells use mitochondrial OXPHOS for energy production, whereas breast cancer cells depend on aerobic glycolysis to generate energy [4]. The altered metabolic activities can be linked to mitochondrial dysfunction that increases reactive oxygen species (ROS), promotes uncontrolled growth, and causes DNA damage in breast cancer [5].

The human mitochondrial genome is composed of 16569-bp in a double-chain structure characterized by high mutation rate and maternal inheritance [6,7]. Poorly protected mtDNA is sensitive to oxidative stress and other genotoxic damage [8]. mtDNA alternations are considered as the emerging factors which provoke breast cancer formation and progression [9,10]. For instance, mutations in the mitochondrial D-loop may alter the affinity of this region for transcripts involved in promotion of mtDNA replication, transcription, and protein production, leading to the development of breast cancer malignancy [11,12]. Furthermore, recent experimental studies revealed that mutations in OXPHOS genes, which cause impairments of respiration chain function, were involved in the metastasis of breast cancer [13,14].

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Although human mt-tRNA is a relatively small molecule which accounts for only 4-10% of total RNAs, a large amount pathogenic mtDNA mutations are located in this region [15]. In fact, almost every tRNA has a well-conserved secondary structure including four stems and three loops, and plays important roles in mitochondrial translation [16]. Mutations in mt-tRNAs can affect the processes of transcription and translation, which subsequently leads to mitochondrial respiratory chain dysfunction, and are associated with a wide range of clinical diseases [17]. However, to date, no studies have been performed which assess the frequencies of mt-tRNA mutations and breast cancer.

With the purpose of understanding the relationship between mt-tRNA mutations and breast cancer, a total of 80 tissues that are derived from breast cancer patients and 80 matched normal tissues were enrolled for this mutational screening. After genetic amplifications and mtDNA sequence analysis, five possible pathogenic mt-tRNA mutations were identified. To see the contributions of these mutations to mitochondrial dysfunctions, the mtDNA copy number and ATP were analyzed.

MATERIALS AND METHODS

Samples and Clinical Assessments. This study enrolled 80 breast cancer samples, as well as 80 controls. The breast cancer tissues were obtained after surgical resection and stored under -80°C conditions for further experiments. Furthermore, the normal adjacent tissues were assessed by a pathologist. All of these tissues were obtained from Second Affiliated Hospital of Zhejiang University School of Medicine, and this work was approved by the Ethics Committees of Second Affiliated Hospital of Zhejiang University School of Medicine and Zhejiang Chinese Medical University. Every subject provided his/her written informed consent. Notably, participants who received chemotherapy or radiotherapy before the surgical treatment would not be enrolled, as well as patients who had mitochondrial disorders such as neurological disease, cardiovascular diseases, and hearing loss were excluded.

Analysis of Mt-tRNA Mutations. The genomic DNA of fresh frozen tissues was isolated as described previously [18]. The DNA quality and quantity were assessed using a BioSpec Nano spectrophotometer. For the amplification of 22 mt-tRNA genes, a total of 14 primers were used for PCR reaction, as suggested previously [19]. The PCR products were further purified and sequenced. Finally, the mt-tRNA mutations were detected by comparison to the revised Cambridge sequence (GenBank accession number: NC_012920.1) [20].

Analysis of Conservation Index (CI). To determine the CI, 17 species' mtDNA sequences were used. The CI≥75% was believed to have functional significance.

Analysis of MtDNA Haplogroup. The mtDNA haplogroups of seven patients with breast cancer who carried pathogenic mt-tRNA mutations were classified using the PhyloTree database (http://www.phylotree.org) [21].

Analysis of mtDNA Copy Number. Total genomic DNA of seven breast cancer patients with putative pathogenic mt-tRNA mutations and matched controls were isolated, and subsequently the mtDNA copy number was measured by relative quantitative real-time PCR method [22]. Briefly, the DNA was extracted from tissue samples using the NucleoSpin® Tissue kit (Macherey-Nagel, Hoerdt, France), according to the manufacturer's recommendations. The mtDNA specific primers for the *tRNA*$^{Leu(UUR)}$ gene (forward primer: 5'-CACCCAAGAACAGGGTTTGT-3' and reverse primer: 5'-TGGCCATGGGTATGTTGTTAA-3') and nuclear DNA primers for *18s rRNA* gene (forward primer: 5'-TAGAGGGACAAGTGGCGTTC-3' and reverse primer: 5'-CGCTGAGCCAGTCAGTGT-3') were used for quantitative PCR to determine the mtDNA copy number, according to the method described previously [23].

Analysis of ATP Production. The cancer tissues and normal controls from seven patients with mt-tRNA pathogenic/likely pathogenic mutations were used for ATP measurement. The ATP concentrations in tissues were analyzed using ATP assay kit (Molecular Probes, Carlsbad, CA, USA), according to the manufacturer's protocols instructions [24].

Statistical Analyses. All statistical analyses were performed using the unpaired, two-tailed Student's *t* test contained in the GraphPad Prism 5 program (GraphPad Software). $p<0.05$ was considered as statistically significant.

Classifications of the Pathogenic Mt-tRNA Mutations. To assess the potential pathogenicity, Yarham and colleagues designed a weighted scoring system for mt-tRNA mutations [25]: if the score of a tRNA mutation <6 points, it was a "neutral polymorphism"; while a score of 7-10 points was classified as "possible pathogenic", and a score of >11 points was classified as "definitely pathogenic".

RESULTS

Mutational Analysis of mt-tRNA Genes in Breast Cancer. The complete mt-tRNA genes of 80 tumor tissues and normal controls were analyzed by PCR-Sanger sequenc-

ing. The clinical and pathological data of these patients are listed in Table 1. Sanger sequencing suggested the presence of five mutations: $tRNA^{Val}$ G1606A, $tRNA^{Ile}$ A4300G, $tRNA^{Ser(UCN)}$ T7505C, $tRNA^{Glu}$ A14693G and $tRNA^{Thr}$ G15927A (Figure 1). Notably, the G1606A and A4300G mutations were in heteroplasmic forms, whereas the T7505C, A14693G and G15927A mutations were in homoplasmic forms. Interestingly, none of these occurred in control subjects. Moreover, the G1606A, A4300G and G15927A mutations were found in one patient with breast cancer (1.25%), while the T7505C and A14693G mutations were identified in two patients with breast cancer (2.5%), the molecular characterization of these mutations is listed in Table 2.

Assessments of the Pathogenicity. The following criteria were used to evaluate the pathogenic roles of mt-tRNA mutations: (1) had a relative high level of CI (≥75%) [26], (2) found < 1% in control group, (3) had the ability to affect the tRNA functions, (4) affected the mitochondrial functions. As can be seen from Figure 1 and Table 2, one mutation (G1606A) was located at acceptor arm, two mutations (A4300G and G15927A) occurred at anticodon stem, one mutation (T7505C) in D-arm and one mutation (A14693G) in TΨC-loop. Four mutations (G1606A, A4300G, T7505C and G15927A) disrupted the highly conserved Watson-Crick base-pairings. Thus, it may be anticipated that these mutations will disrupt the tRNA stability level and function.

Table 1. Summary of clinico-pathological characteristics of breast cancer patients

Characteristics	**Data (mean ± SD or n (frequency in %))**
Gender	
Male	2 (2.5)
Female	78 (97.5)
Age	
>50 years	45 (56.25)
≤50 years	35 (43.75)
Body mass index (kg/m^2)	25.4±3.3
Histological grade	
I	15 (18.75)
II	28 (35)
III	37 (46.25)
TNM stage	
I	10 (12.5)
II	13 (16.25)
III	19 (23.75)
IV	38 (47.5)
Cancer metastasis	
Positive	38 (47.5)
Negative	42 (52.5)

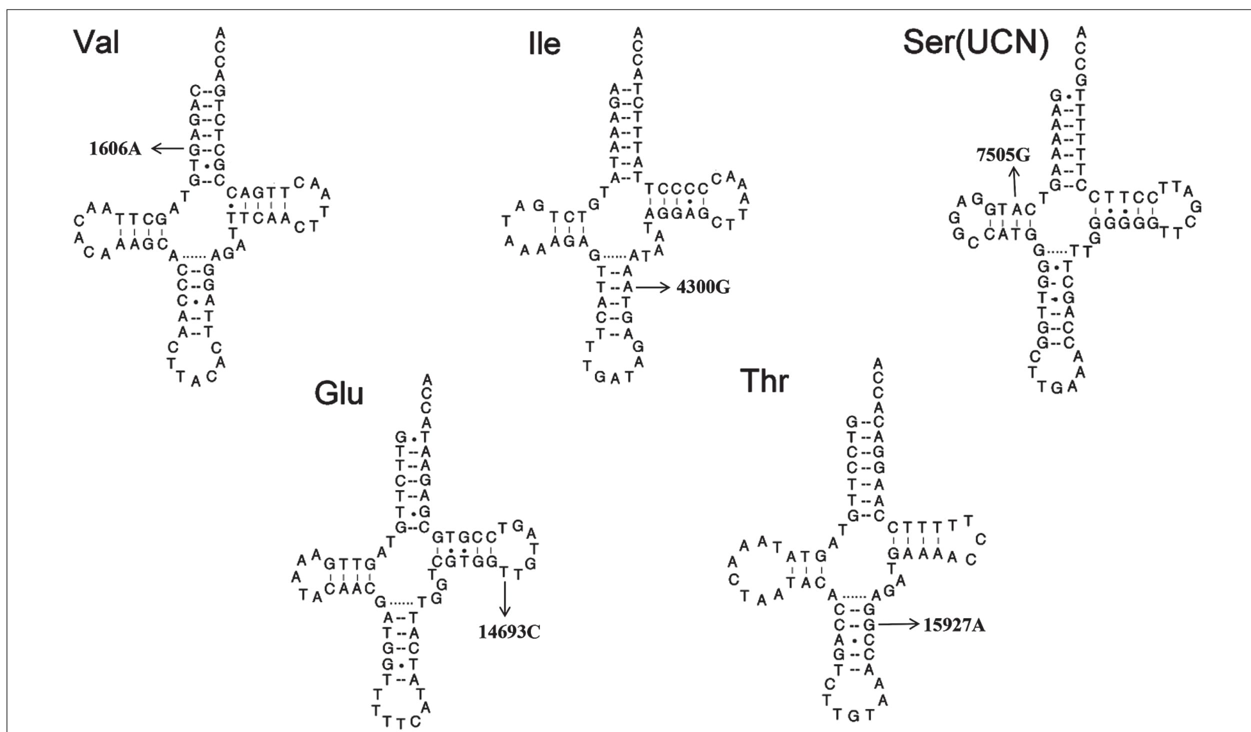

Figure 1. Cloverleaf structures of five mt-tRNAs. Arrows indicate the locations of breast cancer-associated mt-tRNA mutations.

Table 2. mt-tRNA mutations identified in this case-control study

Gene	Sequence alternation	CI (%)[a]	Homoplasmy /Heteroplasmy	Watson-Crick base pairing[b]	Nucleotide at tRNA	Location	Number of 80 breast cancer tissues (%)	Number of 80 matched normal adjacent tissues (%)	mtDNA haplogroup	Disease association
$tRNA^{Val}$	G1606A	100	Heteroplasmy	G-C↓	5	Acceptor arm	1 (1.25)	0	N9a	Progressive ataxia, seizures, mental deterioration, mild myopathy, and hearing loss
$tRNA^{Ile}$	A4300G	100	Heteroplasmy	C-G↓	42	Anticodon stem	1 (1.25)	0	C4c	Cardiomyopathy
$tRNA^{Ser(UCN)}$	T7505C	100	Homoplasmy	A-T↓	10	D-arm	2 (2.5)	0	F1	Deafness
$tRNA^{Glu}$	A14693G	100	Homoplasmy		54	TΨC-loop	2 (2.5)	0	Y2	MELAS, deafness, LHON
$tRNA^{Thr}$	G15927A	100	Homoplasmy	C-G↓	42	Anticodon stem	1 (1.25)	0	B5b	Parkinson's disease, LHON, deafness, CHD,

[a]CI: conservation index;
[b]Classic Watson-Crick base pairing: created (↑) or abolished (↓)
Abbreviations: MELAS: mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episode, LHON: Leber's hereditary optic neuropathy, CHD: coronary heart disease

Table 3. The predicted pathogenicity of breast cancer-associated mt-tRNA mutations

Scoring criteria	G1606A mutation	Score	A4300G mutation	Score	T7505C mutation	Score	A14693G mutation	Score	G15927A mutation	Score	Classification
More than one independent report	Yes	2	Yes	2	Yes	2	Yes	2	Yes	2	≤6 points: neutral polymorphisms; 7~10 points: possibly pathogenic; 11-13 points (not including evidence from single fiber, steady-state level or trans-mitochondrial cybrid studies): probably pathogenic ≥11 points (including evidence from single fiber, steady-state level or trans-mitochondrial cybrid studies): definitely pathogenic
Evolutionary conservation of the base pair	No change	2	No change	2	No change	2	No change	2	No change	2	
Variant heteroplasmy	No	0	No	0	No	0	No	0	No	0	
Segregation of the mutation with disease	Yes	2	No	0	Yes	2	Yes	2	Yes	2	
Histochemical evidence of mitochondrial disease	No evidence	0	No evidence	0	No evidence	0	No evidence	0	No evidence	0	
Biochemical defect in complex I, III or IV	Yes	2	Yes	2	Yes	2	No	0	Yes	2	
Evidence of mutation segregation with biochemical defect from single-fiber studies	No	0	No	0	No	0	No	0	No	0	
Mutant mt-tRNA steady-state level or evidence of pathogenicity in trans-mitochondrial cybrid studies	Strong evidence	5	Strong evidence	5	Strong evidence	5	Weak evidence	3	Strong evidence	5	
Maximum score	Definitely pathogenic	13	Definitely pathogenic	11	Definitely pathogenic	13	Possibly pathogenic	9	Definitely pathogenic	13	

We further analyzed mitochondrial functions in patients carrying these mt-tRNA mutations, as shown in Figure 2. We found that patients with putative pathogenic mt-tRNA mutations had much lower levels of mtDNA copy number and ATP, as compared with the controls (p=0.0138 and 0.0362, respectively)

In addition, the classical pathogenicity scoring system [25] was then used to assess the scores of these mt-tRNA mutations. As a result, the total scores of $tRNA^{Val}$ G1606A, $tRNA^{Ile}$ A4300G, $tRNA^{Ser(UCN)}$ T7505C, $tRNA^{Glu}$ A14693G and $tRNA^{Thr}$ G15927A mutations were 13, 11, 13, 9 and 13 points, which belonged to "definitely pathogenic" and "possibly pathogenic" according to its standard (Table 3).

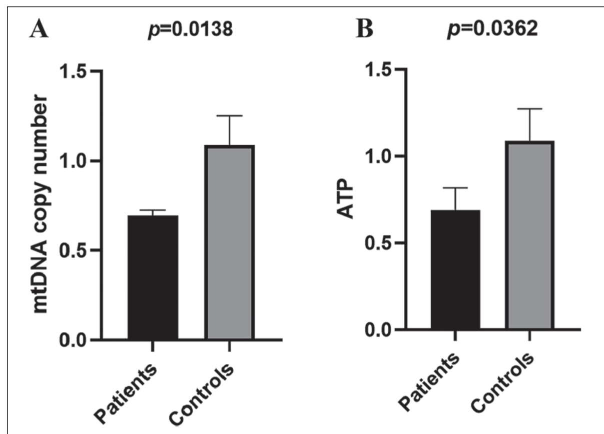

Figure 2. Analysis of mitochondrial functions. A. mtDNA copy number analysis in patients with and without mt-tRNA mutations. B. ATP qualification in patients with and without mt-tRNA mutations.

DISCUSSION

In this study, the frequencies of mt-tRNA mutations in tissue samples of 80 breast cancer patients and matched normal tissues were analyzed by direct sequencing. As a result, we identified five possibly pathogenic mutations: $tRNA^{Val}$ G1606A, $tRNA^{Ile}$ A4300G, $tRNA^{Ser(UCN)}$ T7505C, $tRNA^{Glu}$ A14693G and $tRNA^{Thr}$ G15927A that may be associated with breast cancer. G1606A affected the acceptor arm of $tRNA^{Val}$, which was extremely conserved from different species and was anticipated to disrupt the G5-C68 base-pairing. This mutation was first described in patients with neurological diseases [27]. Single fiber studies suggest that, in *COX*-negative fibers, a markedly increased amount of mutant mtDNA was observed, indicating that the G1606A may lead to mitochondrial dysfunction [28]. Moreover, the heteroplasmic A4300G mutation occurred at very conserved region in $tRNA^{Ile}$. Molecular and biochemical analysis suggested that the A4300G mutation influenced the steady-state level of $tRNA^{Ile}$ and decreased the activities of respiratory chain complexes and has been regarded as a pathogenic mutation for cardiomyopathy [29,30].

In addition, the deafness-associated T7505C mutation was located at position 11 in the conserved base of the D-arm of $tRNA^{Ser(UCN)}$, which abolished the A11-T24 base-pairing [31]. Using the cybrid cells containing the T7505C mutation, mutant cell lines caused a markedly decreased in the steady-state level of $tRNA^{Ser(UCN)}$, as compared with the controls [32]. Furthermore, the T7505C mutation resulted in reductions in Complex I, II, III and IV, and increased ROS production [32]. On the other hand, the A14693G mutation was first reported in a patient with mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) syndrome [33]. A14693G resided at conserved base in the TΨC-loop of $tRNA^{Glu}$ [34]. Previous genetic studies revealed that this mutation enhanced the penetrance of deafness and Leber's Hereditary Optic Neuropathy (LHON)-associated primary mutations in Chinese families [35, 36]. In addition, the G-to-A substitution at 15927 occurred at position 42 in the anticodon loop of $tRNA^{Thr}$, which abolished the very conserved Watson-Crick base-pairing (28C-42G). Functional analysis indicated that G15927A mutation led to an approximately 80% drop in $tRNA^{Thr}$ expression level, as well as the ~39% reduction in aminoacylation ability of $tRNA^{Thr}$ [37]. Moreover, the G15927A mutation decreased the mitochondrial membrane potential (MMP) and ATP production, and enhanced ROS production [38].

We next examined the mtDNA copy number and ATP levels in seven patients with mt-tRNA pathogenic/likely pathogenic mutations and controls. As a result, we noticed that patients with these mutations had lower levels of mtDNA content and ATP when compared with the controls. In fact, the mtDNA copy number represented the number of mitochondria per cell and number of mitochondrial genomes per mitochondrion, being a biomarker of mitochondrial function [39]. Reductions in mtDNA copy number in cells can impair mitochondrial respiration and cause pathology including cancers [40]. Furthermore, reduction in mtDNA copy number will result an increasing in ROS production [41]. The over-production of ROS will lead to serious consequence such as increasing the oxidative stress in cells, damaging DNA; RNA; lipids and contributing to programmed cell death [42]. In addition, the respiratory chain of mitochondria was coupled with the phosphorylation of ADP in the process of electron transfer. Under the action of ATP synthase, ADP and 1-molecule phosphate were combined to form ATP, providing energy for life activities. The activity of respiratory chain complex directly affected OXPHOS function of mitochondria and decreased the ATP production in breast cancer tissues with mt-tRNA mutations. The decreased in mtDNA copy number and ATP suggested the impairment of mitochondrial functions. Therefore, these mt-tRNA mutations caused the failures in tRNA metabolism and led to mitochondrial dysfunctions that were responsible for breast cancer.

In summary, this study suggested that mutations in mt-tRNAs are involved in breast carcinogenesis. Pathogenic mt-tRNA mutations may cause mitochondrial dysfunctions and play active roles in breast cancer. Mutational analysis of mt-tRNA genes were recommended, especially for those patients who had a family history of breast cancer.

Declaration of Interest.
The authors report no conflicts of interest.

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