

Mitochondrial DNA variations in tongue squamous cell carcinoma

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Abstract. Tongue squamous cell carcinoma (TSCC) is the most common type of oral carcinoma. Mitochondrial DNA (mtDNA) is a circular DNA molecule of 16,569 bp, which functionally encompasses a regulatory non-coding region (D-loop) and 37 encoding genes that correspond to 13 subunits of respiratory chain complexes (I, III, IV and V), 22 transfer RNAs and 2 ribosomal (r)RNAs. Recently, mtDNA has been implicated as a mutation hotspot in various tumors. However, to our knowledge mtDNA alteration in TSCC has not been investigated to date. In the present study, the mitochondrial genomes of tongue carcinoma, adjacent non-cancerous tissue and peripheral blood samples from 8 patients with TSCC were sequenced and aligned with the revised Cambridge Reference Sequence. Overall, only one synonymous mutation, which mapped to the NADH:ubiquinone oxidoreductase core subunit 5 gene, was observed in the tongue carcinoma sample from a single patient. A further 21 polymorphisms were identified, including six in the non-coding region (D-loop), five in Complex I, three in Complex III, two in Complex IV, two in Complex V and three in rRNA. In addition, mitochondrial microsatellite instability (mtMSI) was detected in 2/8 tongue carcinoma samples, and localized in the D310 region. These variations, particularly the polymorphisms and mtMSI, imply that the mitochondrial genome may be a hotspot of genome alteration in tongue cancer. Further investigation is expected to reveal the role of mtDNA alteration in TSCC development, as well as its clinical implications.

Introduction

Oral cancer ranks as the 10th most common type of cancer worldwide (1). Tongue squamous cell carcinoma (TSCC) is the most prevalent and aggressive form of oral cancer, accounting for 25-40% of all oral cancer cases (2). Rapid local invasion, high rates of lymph nodal metastasis and high recurrence rates are striking features of TSCC, causing malfunction of mastication, speech and deglutition, and leading to poor survival and quality of life (3). In spite of the advances in chemotherapy, radiotherapy and surgical therapy, the prognosis and overall survival rates of patients with TSCC have not been significantly improved over the last decade, with a current overall five-year survival rate of <50% (4). Epidemiological study has revealed that tongue cancer is associated with a variety of factors, including age, geographic environment, family history, malnutrition, infectious agents, and chronic alcohol, tobacco and betel nut consumption (5). However, the pathogenesis of tongue cancer is yet to be fully understood.

Mitochondria are organelles wherein aerobic respiration is conducted to supply energy for a diverse range of cellular events including fatty acid oxidation (6), calcium signaling (7), apoptosis (8), biosynthesis and biogenesis (9,10). Each mitochondrion possesses its own 16,569 bp circular DNA (mtDNA) (11), characteristically having 37 encoding genes for 13 subunits of respiratory chain complexes (I, III, IV and V), 22 transfer (t)RNAs and 2 ribosomal (r)RNAs (12), and a non-coding region (D-loop) involved in regulation of mtDNA replication and transcription (13-15). Unlike nuclear DNA, mtDNA lacks the protection of histones and efficient repair mechanisms. Thus, mtDNA is more vulnerable to oxidative damage by excessive reactive oxygen species (ROS), a byproduct of oxidative phosphorylation in the mitochondria (16). This may initiate a vicious cycle: Damaged mtDNA can lead to an inefficient oxidative phosphorylation system, which in turn gives rise to more ROS, causing further damage to mtDNA and the genome. Importantly, the resulting genomic instability directly contributes to carcinogenesis.

Early on, it was proposed that injured mitochondria, for instance when there is alteration of oxidative phosphorylation or changes in the number, shape and function of mitochondria, may be an important contributing factor in many cancer types (17). Recently, an increasing number of mtDNA mutations, in the form of point mutations, deletions, insertions and mitochondrial microsatellite instability (mtMSI), have been

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reported in various cancers, including head and neck (18), pancreatic (19), colorectal (20,21) and breast cancers (22-24). However, to date, the mtDNA alterations in TSCC remain poorly defined. To profile the mtDNA alterations in tongue cancer, the present study sequenced and analyzed the mitochondrial genomes of tongue carcinoma, adjacent normal tissue and matched peripheral blood from 8 patients with TSCC.

Materials and methods

Sample collection. A total of 8 patients with tongue cancer who underwent primary surgery between January and December, 2015 at Hunan Cancer Hospital, Changsha, China were enrolled. None of the patients had a history of other known diseases potentially associated with mitochondrial defects including diabetes and hypertension (12). Cancer tissues and adjacent normal tissues at a distance of 2 cm from the tumor margin were divided by experienced surgeons and immediately frozen at -80°C . The tumor node and metastasis staging for the tongue cancer samples was classified by experienced pathologists. Matched peripheral blood samples (2 ml venous blood per patient) were collected. Information regarding age, sex and habits (smoking, alcohol drinking and betel chewing) were also recorded (details are given in Table I). Informed consent was obtained from all subjects and the protocol of the study was approved by the Medical Ethics Committees of the Hunan Cancer Hospital and Family Planning Institute of Hunan Province (Changsha, China).

mtDNA amplification. Genomic DNA was isolated from tumor and adjacent normal tissues with a High Pure Polymerase Chain Reaction (PCR) Template Preparation kit (Roche Diagnostics, GmbH, Mannheim, Germany). Genomic DNA from peripheral blood was extracted with a GeneJET™ Whole Blood Genomic DNA Purification Mini kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The complete mitochondrial genome was obtained using 24 pairs of primers as previously described (25). PCR amplification was performed using high-fidelity long PCR enzyme PrimeSTAR® GXL DNA Polymerase (Takara Bio, Inc., Otsu, Japan) on a Thermal Cycler 9700 machine (Applied Biosystems; Thermo Fisher Scientific, Inc.). The total reaction volume was 50 μl , containing 10 μl of 5X PrimeSTAR GXL buffer (Mg^{2+} plus; Takara Bio, Inc.), 4 μl of 2.5 mM dNTPs mix, 1.25 units polymerase enzyme mix, 1.5 μl of 10 mM of each primer, and 50 ng template DNA, and made up to 50 μl with nuclease-free water. The PCR amplification protocol was as follows: Pre-denaturation at 95°C for 5 min, followed by 32 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 9 min, and a final extension step at 72°C for 4 min. PCR products were purified using a MiniBEST Agarose Gel DNA Extraction kit Ver.4.0 (Takara Bio, Inc.).

Sequence analysis. Purified PCR products were sequenced with a BigDye Terminator v3.1 Cycle Sequencing kit on a 3730 DNA sequencer (both from Applied Biosystems; Thermo Fisher Scientific, Inc.). To detect somatic variations and single nucleotide polymorphisms, the original sequences of the tissues and matched blood samples were aligned against

the revised Cambridge Reference Sequence (rCRS; GenBank access number: NC_012920.1; <https://www.ncbi.nlm.nih.gov/gene/>) using the online software package CodonCode Aligner 6.0.2 (<http://www.codoncode.com>).

mtMSI analysis. A total of 10 microsatellite markers, namely C_7TC_5 (D310), $(\text{CA})_5$ (D514) and C_5TC_4 (D16184) in the D-loop, C_6 in NADH:ubiquinone oxidoreductase core subunit 1 (ND1), A_7 in ND2, C_6 , A_8 and C_3A_3 in ND5, A_7 in cytochrome C oxidase subunit 1 (CO1) and T_7 in CO3, were analyzed for length variations in cancer tissues compared with adjacent normal tissues.

Results

mtDNA mutation in patients with tongue cancer. To investigate the variance of mtDNA in patients with TSCC, the mtDNA genomes of cancer tissues, adjacent normal tissues and matched blood samples were sequenced and aligned to the rCRS. Mutation was defined as a nucleotide variation occurring in the mitochondrial genome of cancer tissues but not in those of adjacent normal tissues and matched blood samples. A variation was considered as a polymorphism when observed within all three samples from a patient. In the present study, which involved 8 patients (Table I), only one synonymous mutation was observed in the ND5 gene in patient no. 2, which was a transition of T-C at nucleotide position (np) 13,830 and caused no substitution of an amino acid (not shown).

mtDNA polymorphisms in patients with tongue cancer. When aligned with the rCRS, 21 nucleotide variations of the mitochondrial genomes of TSCC were identified as polymorphisms (Table II), as these variations were present in not only the cancer tissues, but also the adjacent normal tissues and matched blood samples. All the polymorphisms exhibited a relatively high frequency in the study population, as all were observed in at least 3/8 patients. Furthermore, the polymorphisms were grouped on the basis of their genomic location (Fig. 1). The control region (D-loop) and Complex I gene harbored the most polymorphisms, whereas there was no polymorphism identified in the tRNA gene.

The control region is subdivided into HVS1 (hyper-variable segment 1, np 16024-16383), HVS2 (np 57-372) and HVS3 (np 438-574). The current data exhibited a total of six polymorphisms in the control region, and mainly in HVS2 and HVS3. Of the six polymorphisms, one was located at np 16223 in HVS1, two at np 73 and np 263 in HVS2, two at np 523 and np 524 in HVS3, and one at np 16519 in the 7S DNA region (not shown).

A total of 15 polymorphisms were identified in the mitochondrial coding regions of the eight patients. Of these polymorphisms, five (33.3%) belonged to the Complex I gene, with ND4 having the most polymorphisms and no polymorphisms being detected in the ND1, ND4L and ND6 genes. Three (20.0%) were located in Complex III, and two (13.3%) in Complex IV, one each in CO1 and CO3. Two (13.3%) were located in Complex V, both in the ATP6 gene. The remaining three (20.0%) polymorphisms were seated in mitochondrial rRNA genes (not shown).

Table I. Clinical data of patients with tongue squamous cell carcinoma.

Patient no.	Sex	Age	TNM staging	Stage	Carcinogen use		
					Tobacco	Alcohol	Betel nut
1	M	68	T2N0M0	II	Y	Y	N
2	M	58	T4N2M0	IV	Y	Y	N
3	F	44	T2N0M0	II	N	N	N
4	M	51	T2N1M0	II	N	Y	Y
5	M	59	T4N1M0	IV	Y	N	N
6	M	50	T2N1M0	II	Y	Y	N
7	M	52	T2N0M0	II	Y	N	N
8	M	49	T2N2M0	IV	Y	Y	Y

TNM, tumor, node and metastasis; F, female; M, male.

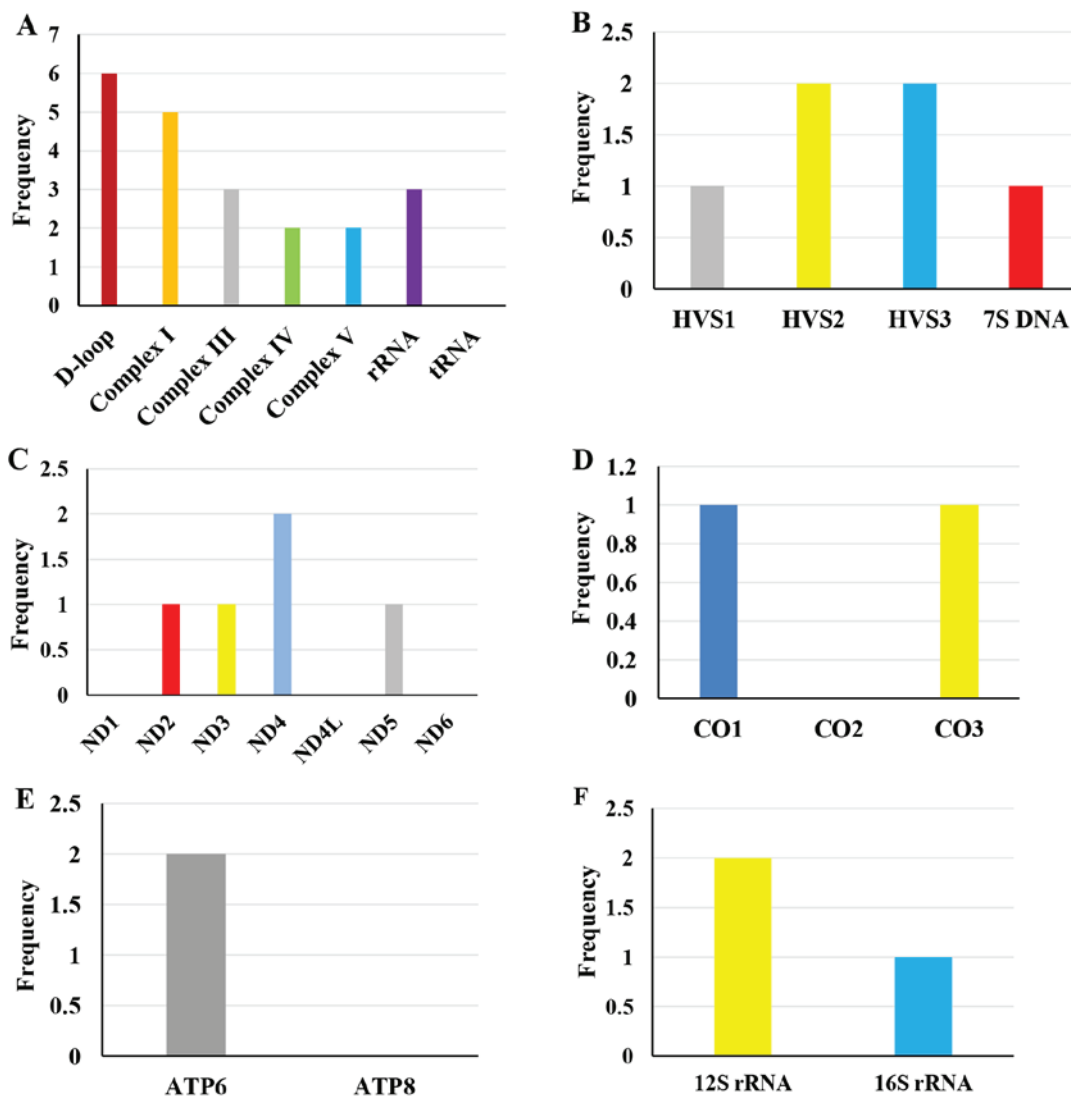


Figure 1. Mitochondrial DNA polymorphisms detected in the complete mitochondrial genome. (A) Overall polymorphisms in the control region, four respiratory chain complexes, rRNA and tRNA; the control region and Complex I harbored the most polymorphisms while there was no polymorphism identified in the tRNA gene. (B) The control region is divided into four functional regions, including the HVS1, HVS2, HVS3 and 7S DNA regions, among which six polymorphisms were mainly identified in the HVS2 and HVS3 regions. (C-F) For the mitochondrial coding regions, 15 polymorphisms were identified in the patients. (C) Complex I: ND4 had the highest frequency of polymorphisms, while no polymorphisms were found in the ND1, ND4L and ND6 genes. (D) Complex IV: One polymorphism each in CO1 and CO3, and none in the CO2 gene. (E) Complex V: Polymorphism was only identified in the ATP6 gene, not in the ATP8 gene. (F) rRNA polymorphisms. HVS, hyper-variable segment; rRNA, ribosomal RNA; tRNA, transfer RNA; ND, NADH:ubiquinone oxidoreductase core subunit; CO, cytochrome C oxidase subunit; ATP, ATP synthase membrane subunit.

Table II. Mitochondrial DNA polymorphisms (in the D-loop, Complexes I, III, IV and V, and rRNA) in tongue cancer patients.

Nucleotide position in mitochondrial genome																					
Sample no.	D-loop				Complex I				Complex III				Complex IV		Complex V		rRNA				
	HVS1		HVS2		HVS3		7S DNA		ND2	ND3	ND4	ND5	CYTB		CO1	CO3	ATP6	12SrRNA	16SrRNA		
	16,223	73	263	523	524	16,519	4,769	10,398	10,873	11,719	12,705	14,766	15,301	15,326	7,028	9,540	8,860	8,701	750	1,438	2,706
1		A-G	A-G	Ad	Cd	T-C	A-G			G-A		C-T	A-G	C-T		A-G		A-G	A-G	A-G	
2	C-T	A-G	A-G			T-C	A-G	A-G	T-C	G-A	C-T	C-T	G-A	A-G	C-T	T-C	A-G	A-G	A-G	A-G	A-G
3		A-G	A-G	Ad	Cd	T-C	A-G			G-A		C-T	A-G	C-T		A-G		A-G			
4	C-T	A-G	A-G	Ad	Cd		A-G	A-G	T-C	G-A	C-T	C-T	G-A	C-T	C-T	T-C	A-G	A-G	A-G	A-G	A-G
5		A-G	A-G			T-C	A-G	A-G		G-A	C-T	C-T	A-G	C-T		A-G		A-G	A-G		
6		A-G	A-G			T-C	A-G			G-A		C-T	A-G	C-T		A-G		A-G	A-G		
7	C-T	A-G	A-G			T-C	A-G	A-G	T-C	G-A	C-T	C-T	G-A	A-G	C-T	T-C	A-G	A-G	A-G	A-G	A-G
8	C-T	A-G	A-G				A-G	A-G	T-C	G-A	C-T	C-T	G-A	A-G	C-T	T-C	A-G	A-G	A-G	A-G	A-G

Polymorphisms occurring in >3/8 patients are shown. A blank field represents no mutation in the respective position. HVS, hyper-variable segment; rRNA, ribosomal RNA; ND, NADH:ubiquinone oxidoreductase core subunit; CYTB, cytochrome B; CO, cytochrome C oxidase subunit; ATP, ATP synthase membrane subunit; d, deletion.

Table III. mtMSI in tongue cancer patients.

Sample no.	D310 locus				D514 locus				D16184 locus			
	Adjacent normal tissue		Blood		Adjacent normal tissue		Blood		Adjacent normal tissue		Blood	
	Tumor	mtMSI status	Tumor	mtMSI status	Tumor	mtMSI status	Tumor	mtMSI status	Tumor	mtMSI status	Tumor	mtMSI status
1	C ₁₃	MSS	C ₁₃	MSS	(CA) ₄	MSS	(CA) ₄	MSS	C ₁₃	MSS	C ₁₃	MSS
2	C ₁₀ TC ₆	MSI	C ₈ TC ₆	MSI	(CA) ₆	MSI	(CA) ₆	MSI	C ₅ TC ₄	MSS	C ₅ TC ₄	MSS
3	C ₈ TC ₆	MSS	C ₈ TC ₆	MSS	(CA) ₄	MSS	(CA) ₄	MSS	C ₅ TC ₄	MSS	C ₅ TC ₄	MSS
4	C ₈ TC ₆	MSS	C ₈ TC ₆	MSS	(CA) ₄	MSS	(CA) ₄	MSS	CTC ₇	MSS	CTC ₇	MSS
5	C ₈ TC ₆	MSS	C ₈ TC ₆	MSS	(CA) ₅	MSS	(CA) ₅	MSS	TC ₄ TC ₄	MSS	TC ₄ TC ₄	MSS
6	C ₇ TC ₆	MSS	C ₇ TC ₆	MSS	(CA) ₅	MSS	(CA) ₅	MSS	C ₅ TC ₄	MSS	C ₅ TC ₄	MSS
7	C ₇ TC ₆	MSS	C ₇ TC ₆	MSS	(CA) ₅	MSS	(CA) ₅	MSS	C ₅ TC ₄	MSS	C ₅ TC ₄	MSS
8	C ₇ TC ₆	MSI	C ₈ TC ₆	MSI	(CA) ₅	MSI	(CA) ₅	MSI	C ₅ TC ₄	MSS	C ₅ TC ₄	MSS

mtMSI/MSS, mitochondrial microsatellite instability/stability.

mtMSI in patients with tongue cancer. mtMSI refers to the change in length of short repetitive sequences of mtDNA between normal and tumor tissues. The present study observed alterations in the length of single nucleotide short repeats within the D310 region in 2/8 patients (Table III), whereas none of the patients exhibited instability at the regions of D514 or D16184 (Table III), in the NADH dehydrogenase subunits ND1, ND2 and ND5, or in the cytochrome oxidase subunits COX1 and COX3 (not shown).

Discussion

Somatic mutations in the mtDNA have been increasingly observed in human cancers over recent years (21,23,24). To contrast the many studies focusing on the control region (24,26), the present study examined the entire mitochondrial genomes in cancer tissues, adjacent normal tissues and matched peripheral blood samples obtained from 8 patients with TSCC. As a result, synonymous mutations, polymorphisms and MSI were identified in TSCC.

In previous studies, the T16519C substitution in the control region has been demonstrated to be correlated with increased risk of breast cancer (22) and with worse prognosis in pancreatic cancer (19), and to exhibit a higher frequency in patients with malignant melanoma and metastasis compared with healthy controls (26). These findings indicate a potential link between the T16519C polymorphism and cancer progression. In addition to variation within the control region, encoding sequence variation in the mitochondrial genome has also been reported as relevant to cancer. The ND3 A10398G non-synonymous polymorphism, which results in the substitution of alanine by threonine within the NADH dehydrogenase subunit of Complex I, has been shown to be associated with an increased risk of breast cancer in European-American women (22). However, in another study, the 10398A variant was reported to increase the risk of breast cancer in African-American women but not in Caucasian women (27). A possible explanation for the conflicting results may be that the A10398G variation was in linkage disequilibrium with other causative polymorphisms in the aforementioned study populations. In the present study, one synonymous mutation and 21 polymorphisms were observed in 8 patients with TSCC. Of note, almost all the polymorphisms were concentrated in the control region and in genes encoding Complexes I, III and V, and rRNA. Theoretically, these variations may have potential impact on mitochondrial function via affecting the replication, transcription or translation events in mitochondria.

In the current study, the MSI of the mitochondrial genome in TSCC was analyzed. It appeared that mtMSI was most frequently observed at the D310 locus (2/8 for the D310 locus vs. undetected for the D514 or D16184 locus). This finding is in line with previous reports (21,28). The D310 locus is considered to be the most variable region in mtDNA, and alterations in this region, including the 303-C insertion, T310C mutation or T deletion, 315-C (CC) insertion and G316C mutation located within a poly-C stretch of HVS II, may lead to MSI. The C-stretch structure is of particular interest, since it is involved in the formation of the persistent RNA-DNA hybrid that leads to initiation of mtDNA heavy-strand replication (29). Therefore, alterations of this

region may have an impact on replication and transcription of the mitochondrial genome. Studies have also suggested that MSI at the non-coding D-loop region could alter the rate of mtDNA replication, thereby disrupting mitochondrion-induced apoptosis (30,31). Regarding the biological significance of mtMSI in cancer, there are studies having associated mtMSI with less differentiated hepatocellular carcinoma, and late-stage progression and poor prognosis of non-small cell lung cancer (32), as well as poor prognosis in colorectal (20) and breast (24) cancer.

In summary, the current preliminary results suggest a relatively high frequency of mtDNA polymorphisms and mtMSI in TSCC. However, the generalization of this phenomenon requires caution, since the present study used a relatively small sample size and lacked healthy controls. Further large cohort studies are needed to validate the findings and identify possible correlations between the mtDNA variations and clinical parameters of patients with TSCC. As increasing evidence supports that mitochondrial genomic variations may serve a role in tumor development, further investigations are expected to uncover the molecular mechanisms by which mtDNA variations affect tumor progression, and their value in cancer risk assessment and management.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

HYZ and BN conceived and designed the study. HYS and HCL were responsible for patient enrollment. HYZ, HCL and HYS performed the data analyses. HYS and HYZ drafted the manuscript. WQX and HYZ critically revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Informed consent was obtained from all subjects and the protocol of the study was approved by the Medical Ethics Committees of the Hunan Cancer Hospital and Family Planning Institute of Hunan Province (Changsha, China).

Patient consent for publication

Informed consent for the publication of patient data was obtained from all patients analyzed in the present study.

Competing interests

The authors declare that they have no competing interests.

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