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HEAD AND NECK

Somatic mutations in the D-loop of mitochondrial DNA in oral squamous cell carcinoma

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Abstract We aimed to characterize somatic mutations in the D-loop of mitochondrial DNA (mtDNA) and their impact on survival in oral squamous cell carcinoma patients in an endemic betel quid chewing area. Histologically confirmed oral cancer and corresponding non-tumor tissues were obtained from 59 patients. The D-loop of mtDNA sequence in a patient's non-cancerous tissues was compared with that of paired oral cancer samples and any sequence differences were identified as somatic mutations. With a median follow-up of 16 months, somatic mutations of the D-loop were observed in 38 (64.4%) patients and most of them occurred in the poly-C tract. There was no significant difference between the mutation group and non-mutation group in age, gender, primary site, histological features, pathological stage, smoking, betel quid chewing, alcohol consumption, and postoperative radiotherapy. However, patients with D-loop mutations have better survival (2 year disease specific survival rate: 73.4 vs. 45.0%, $P = 0.0374$). A high rate of somatic mutations in the D-loop region of mtDNA was found in betel quid-related oral squamous cell carcinoma patients. Somatic mutation of D-loop of mtDNA was associated with better survival.

Keywords Mitochondrial DNA · Oral cancer · Somatic mutation · Squamous cell carcinoma

Introduction

Oral cancer is reported to be the third most common malignancy in developing countries. In Taiwan, oral cancer has been one of the leading causes of death from cancer since 1991 and the annual death toll for oral cancer in males has been increasing rapidly [1]. No significant evolution in the management of oral cancer has been developed in recent years. Even though better combinations of loco-regional treatment modalities have improved the quality of life after diagnosis, the relative 5-year survival rate has not changed much over the past decades [2]. Consequently, recognition of poor prognostic factors has become a focus in the literature.

Mitochondria provide the energy required for the human cells and they play a crucial role in the initiation and execution of apoptosis. Human mitochondrial DNA (mtDNA) is a 16.5-kb circular double-stranded DNA molecule. It contains gene coding for 13 peptides engaged in respiration and oxidative phosphorylation, 2 rRNAs, and a set of 22 tRNAs that are essential for the protein synthesis in mitochondria [3]. In addition, mtDNA contains a non-coding region that includes a unique displacement loop (D-loop) that controls replication and transcription of mtDNA. It has

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been recognized that the mitochondrial genome is particularly vulnerable to oxidative damage and mutation because of the high rate of reactive oxygen species generation and inefficient DNA repair system in the organelle [4].

Somatic mutations of mtDNA have been detected in many human cancers including hepatocellular carcinoma, gastric carcinoma, colorectal cancer, breast cancer, etc. [3–6]. Most of the mtDNA mutations are located in the D-loop region of mtDNA. To date, only two studies pointed out that 56.7–66.7% of the oral squamous cell carcinoma cases had somatic mutations in the D-loop of mtDNA [7, 8]. However, the relationship between somatic mutations of the D-loop of mtDNA and prognosis of oral cancer patients has never been reported. The aim of this study was to characterize somatic mutations in the D-loop of mtDNA and investigate the survival impact on oral cancer patients in an endemic betel quid chewing area.

Methods

This study was approved by the Institutional Review Board of Taichung Veterans General Hospital. From August 2008 to March 2010, patients scheduled to undergo excision of oral cancer were eligible for this study. All patients were informed about the study and consents were obtained before enrollment. Those who switched to non-surgical modalities, had tumors other than squamous cell carcinoma, had inadequate chart records, or were reluctant to participate in this study were excluded. Basic demographic data including age, gender, tumor location, tumor staging, and histological features were obtained. Clinical treatments for all patients were according to the consensus guideline of the head and neck cancer team of our hospital. In addition, all surgical procedures were performed by single head and neck surgeon (SA Liu).

Human oral squamous cell carcinoma tissues and DNA extraction

Histologically confirmed oral squamous cell carcinoma and corresponding non-tumor tissues were obtained from 59 patients with their informed consent at Taichung Veterans General Hospital. All these samples were microdissected so as to minimize normal contamination that could skew the mitochondrial alteration distribution. The samples were obtained from the center of tumors. All the tissues were placed in liquid nitrogen immediately after microdissection. Total DNA was extracted by the QIAamp DNA Mini kit (QIAGEN) according to the instructions of the manufacturer. The final DNA was dissolved in doubly distilled water and frozen at −30°C until use.

Direct sequencing of the D-loop region of mtDNA

The D-loop region of mtDNA was analyzed for mutations by direct sequencing of the polymerase chain reaction (PCR) products. The primer pairs L16190 [nucleotide position (np) 16190–16209, 5′-CCCCATGCTTACAAGCA AGT-3′) and H602 (np 602-583, 5′-GCTTTGAGGAGGT AAGCTAC-3′) were used for amplification of a 982-bp DNA fragment from the D-loop region of mtDNA. All the PCR products were purified and sequenced with an ABI Big Dye Terminator (version 3.1) cycle sequencing ready reaction kit and an ABI PRISM 3100 sequencer (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The DNA fragments from non-cancerous oral tissues and oral cancer specimens of the same patient were analyzed. The D-loop of mtDNA sequence in a patient's non-cancerous oral tissues was compared with that in paired oral cancer samples and any DNA sequence differences were identified as somatic mutations.

Statistical analysis

We used descriptive statistics for general data presentation. Comparisons of nominal or ordinal variables between patients with somatic mutations of the D-loop of mtDNA and those without were analyzed by the chi-square test, whereas continuous variables were analyzed with Student's *t* test. Survival analysis was performed by the Kaplan–Meier method. The differences between subgroups were examined by the log-rank test. All statistics were calculated by SPSS for Windows version 10.1 (SPSS, Chicago, IL) and a $p < 0.05$ was regarded as statistically significant.

Results

A total of 64 patients scheduled to undergo surgical excision of oral cancer were enrolled for this study. Five patients were excluded because 1 refused any treatment, two underwent non-surgical treatment, and two patients had pathological report of cancer other than squamous cell carcinoma. Adequate data were obtained from 59 patients. The average age was 49.3 ± 8.8 years and males accounted for 94.9% ($n = 56$) of all patients. Fifty-one (86.4%) patients had habits of smoking while 43 (72.9%) and 47 (79.7%) patients had habitual betel quid chewing and alcohol consumption. Two patients were followed for 33 months and the average follow-up period in this study was 16 months (±8.8 months). Twenty-five patients were followed for more than 2 years and 15 patients of them were still alive. Using direct sequencing, we found 64.4% (38 out of 59) of the oral squamous cell carcinoma carried somatic mutations in the D-loop of mtDNA. Other descriptive statistics are

Table 1 Descriptive and bivariate analysis of oral cancer patients with or without somatic mutations of D-loop of mitochondrial DNA

Variables	Total no. of patients (% in column)	No. of patients (%)		*P* value
		Mutation group ($n = 38$)	Non-mutation group ($n = 21$)	
Age (year)				
<45 years	21 (35.6%)	16 (76.2%)	5 (23.8%)	0.262
≥45 years	38 (64.4%)	22 (57.9%)	16 (42.1%)	
Gender				
Female	3 (5.1%)	2 (66.7%)	1 (33.3%)	0.999[†]
Male	56 (94.9%)	36 (64.3%)	20 (35.7%)	
Primary tumor sites				
Lip	1 (1.7%)	1 (100%)	0 (0%)	0.557
Gingival	2 (3.4%)	1 (50.0%)	1 (50.0%)	
Floor of mouth	1 (1.7%)	0 (0%)	1 (100%)	
Anterior tongue	32 (54.2%)	22 (68.8%)	10 (31.3%)	
Buccal	18 (30.5%)	11 (61.1%)	7 (38.9%)	
Hard palate	4 (6.8%)	3 (75.0%)	1 (25.0%)	
Retromolar trigone	1 (1.7%)	0 (0%)	1 (100%)	
Histological features				
Well differentiated	2 (3.4%)	2 (100%)	0 (0%)	0.552
Moderately differentiated	37 (62.7%)	23 (62.2%)	14 (37.8%)	
Poorly or undifferentiated	20 (33.9%)	13 (65.0%)	7 (35.0%)	
Pathological stage				
Stage I-II	20 (33.9%)	15 (75.0%)	5 (25.0%)	0.352
Stage III-IV	39 (66.1%)	23 (59.0%)	16 (41.0%)	
Postoperative radiotherapy				
Yes	47 (79.7%)	29 (61.7%)	18 (38.3%)	0.509
No	12 (20.3%)	9 (75.0%)	3 (25.0%)	
Smoking				
Yes	51 (86.4%)	32 (62.7%)	19 (37.3%)	0.783
No	8 (13.6%)	6 (75.0%)	2 (25.0%)	
Betel quid chewing				
Yes	43 (72.9%)	26 (60.5%)	17 (39.5%)	0.465
No	16 (27.1%)	12 (75.0%)	4 (25.0%)	
Alcohol consumption				
Yes	47 (79.7%)	28 (59.6%)	19 (40.4%)	0.232
No	12 (20.3%)	10 (83.3%)	2 (16.7%)	

[†] Fisher's exact test

detailed in Table 1. Most of the mtDNA mutations in oral cancers were heteroplasmic (26/38, 68.4%). Twenty-seven oral cancers (71.1%) displayed alterations in the mononucleotide repeat located in the polycytidine stretch over np 303 of mtDNA. Details of the somatic mutations in the D-loop region of mtDNA are listed in Table 2.

After dividing all the patients into two groups on the basis of somatic mutations of the D-loop of mtDNA, the mutation group consisted of 38 patients and the non-mutation group consisted of 21 patients. Comparisons of variables between the two groups are presented in Table 1. No significant difference was noted in the distribution of age between the two groups (mutation group vs. non-mutation group: 48.5 ± 9.1 vs. 50.7 ± 8.2 years, $P = 0.366$). In addition, there was no significant difference between two groups in gender, primary tumor location, smoking, betel quid chewing, alcohol consumption, histological features, pathological stage, and postoperative radiotherapy.

Twenty patients died during follow up period. Among them, 9 patients were in the mutation group while 11 patients were in the non-mutation group. All of them were died of cancer recurrence or distant metastasis except two

Table 2 Somatic mutations in the D-loop region of mtDNA of oral cancers

Patient code	Nucleotide position	Somatic mutation	Homoplasmy
01	303	C8 → C8-9	Heteroplasmy
05	317	C5 → C6	Yes
11	303	C7 → C7-9	Heteroplasmy
15	303	C8 → C9	Yes
	16257, 16316	T → C	
16	205	T/C → T	Yes
	312	C8 → C9	
17	312	C8-9 → C8	Yes
	16340	A → G	
	16524	T → C	
21	303	C8 → C8-9	Heteroplasmy
25	303	C15 → C15-18	Heteroplasmy
26	312	C8 → C7-9	Heteroplasmy
28	303	C9 → C8	Yes
34	369, 414, 467	G → A	
	414	C → G/C	Heteroplasmy
35	312	C8 → C9	Yes
36	303	C7 → C7-8	Heteroplasmy
39	303	C7 → C7-8	Heteroplasmy
40	2288	G → G/T	Heteroplasmy
42	303	C8 → C7	Yes
44	303	C8 → C8-9	Heteroplasmy
45	303	C8 → C8-9	Heteroplasmy
47	303	C8 → C8-9	Heteroplasmy
53	146, 150	C → T	
	199, 16261	T → C/T	Heteroplasmy
56	303	C8 → C8-9	Heteroplasmy
58	369, 392, 567	G/A → A/G	Heteroplasmy
59	66	Deletion G	Heteroplasmy
	369	G/A → A/G	
60	303	C7 → C7-8	Heteroplasmy
61	303	C8 → C8-9	Heteroplasmy
	66	G6 → G5-6	
62	303	C8 → C8-9	Heteroplasmy
63	303	C8 → C8-9	Heteroplasmy
64	303	C8-9 → C8	Yes
	13135	G → A	
	13152	A → G	
65	303	C8 → C8-9	Heteroplasmy
	10230	G → G/A	
73	303	C8 → C8-9	Heteroplasmy
75	303	C8-9 → C8	Yes
	9053	G → A	
	10310	G → A	
76	303	C8 → C8-9	Heteroplasmy
79	303	C8-9 → C8	Yes
	9824	T → C	
80	303	C8 → C8-9	Heteroplasmy
	3882	A → A/G	
	6392	T → T/C	
	10398	A → A/G	
83	303	C8-9 → C8	Yes
84	3882	A/G → G	Yes
	3970	C/T → C	
88	303	C8 → C8-9	Heteroplasmy
93	303	C8 → C8-9	Heteroplasmy

mtDNA mitochondrial DNA

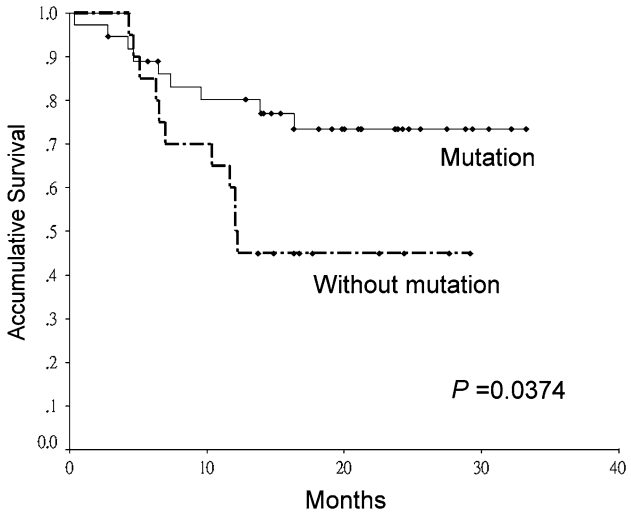

Fig. 1 Univariate analysis of the effect of somatic mutations in the D-loop of mitochondrial DNA on survival of oral squamous cell carcinoma patients

patients. One patient in mutation group was died of surgical complication whereas another patient in non-mutation group was died of pneumonia. Based on the Kaplan–Meier survival analysis, patients with somatic mutations of the D-loop of mtDNA had better prognosis when compared with those without (2 year disease specific survival rate: 73.4 vs. 45.0%, $P = 0.0374$) (Fig. 1).

Discussion

The D-loop region of mtDNA is a crucial site for replication and expression of the mitochondrial genome because it holds the leading-strand origin of replication and the main

promoters for transcription [9]. Due to its distinctive triple-stranded DNA structure, the D-loop region is hypervariable and vulnerable to somatic mutations. Mitochondrial mutations may change the role of a functional oxidative phosphorylation chain as both a metabolic gatekeeper to avoid inappropriate alteration to a glycolytic metabolic phenotype and a caretaker to prevent inappropriate production of genotoxic reactive oxygen species [10].

Our study found more than half of the oral squamous cell carcinoma had somatic mutations of the D-loop of mtDNA. The mutation rate was different from the rates in previous studies. Zhou et al. [10] found 24 of 83 (28.9%) head and neck tumor samples had alteration in the D-loop region.[10] Other studies showed about one fifth of head and neck cancer samples had somatic mutation in the D-loop region [9, 11]. The difference might be explained by the diverse studied population. The abovementioned studies included a variety of head and neck cancer patients whereas our study only enrolled oral squamous cell carcinoma patients. Tan et al. [7] in their study of betel quid-related oral cancer patients found that somatic D-loop mutations developed in 66.7% (12/18) samples.[7] Another study about oral squamous cell carcinoma also found high rate of somatic mutations in the D-loop region (17 out of 30, 56.7%) [8]. The high mutation rate in aforementioned studies was comparable with the rate in our study.

To the best of our knowledge, this is the first study about correlation between prognosis and mitochondrial D-loop mutations in oral squamous cell carcinoma patients. Although there was no significant difference between the mutation group and non-mutation group in age, gender, primary site, histological features, pathological stage, smoking, betel quid chewing, alcohol consumption, and postoperative radiotherapy, patients with somatic mutations of mtDNA D-loop had better survival status when compared with those without. Lievre et al. in their study of clinico-pathological correlation of mitochondrial D-loop mutations in head and neck carcinoma found that the presence of D-loop mutation was not associated with prognosis. The 5-year overall survival of patients with somatic D-loop mutation was 81% compared to 70% for patients without mutation ($p = 0.71$) [11]. Nevertheless, their study included a variety of head and neck cancer patients while the current study enrolled only oral cancer patients. According to Ha's study [12], it was suggested that the mutation of the D-loop region was an early event in head and neck carcinogenesis. They found the D-loop mutations rate increased from 22% in premalignant lesions to 50% in lesions of severe dysplasia and even 61% in carcinomas in situ. We speculate that oral squamous cell carcinoma patients without somatic mutations of the D-loop of mtDNA might have other genetic defects that worsen their prognosis.

A previous study found a higher rate of mutations in the D-loop of mtDNA in cancer patients with advanced stage [3]. The findings suggested the concept that the alterations increased in cancer during its progression stage because of highly unstable nature of mtDNA [3]. However, our result did not suggest such a conclusion. In a study regarding premalignant lesions of the head and neck, Ha et al. found that the mtDNA alterations were an early event in carcinogensis [12]. It might explain why the mutations rate of the D-loop of mtDNA is not associated with tumor stage. In addition, differences in distribution of tumor sites between studies may lead to diverse results.

Our study demonstrated that the most frequent alterations in the D-loop of mtDNA were found in the polycytidine stretch. The results were comparable with those of previous studies [3, 7, 8]. The D-loop was found to be highly susceptible to oxidative damage as compared with the other regions of mtDNA [13]. The extensive oxidative damage to the polycytidine sequences may cause slipping and/or misincorporation during replication or repair of mtDNA by mitochondrial DNA polymerase.

A previous study pointed out that the mutations of mtDNA correlated positively with p53 mutations [10]. Other studies also found that the mutations of the D-loop were significantly associated with tobacco consumption and betel quid chewing [7, 11]. However, as this study didn't collect relevant data, no comparison could be made.

Challen et al. [14] in their study about mtDNA mutations in head and neck cancer found somatic mutation of D-loop of mtDNA were infrequent and may not significantly influence the prognosis.[14] However, aforementioned study was conducted in non-endemic betel quid chewing area. Besides, they include a variety of head and neck cancer such as, oral cavity, hypopharynx, oropharynx, and larynx. Cancers from oral cavity consisted less than 50% of all samples. Conversely, our study included 59 specimens and corresponding normal tissues from oral cavity cancer patients.

Accumulation of mutations in mtDNA was found to increase proliferation rate and inhibit apoptosis [15]. It was also found that mutations in mtDNA played a role in the development of cancer [16]. Although mutation in mtDNA might be involved in the carcinogenesis, it did not necessarily have impact on the survival. For example, human papillomavirus (HPV) is closely associated with oropharyngeal cancer yet patients with HPV-related oropharyngeal cancer have better survival than those without HPV infection [17]. Zhang et al. [18] indicated that genetic polymorphisms in the D-loop are independent poor prognostic markers for patients with esophageal squamous cell carcinoma, which was complete different from our results.[18] The reasons might be that they investigated patients with esophageal cancer and they analyzed peripheral blood instead of tumor

specimens. In addition, abovementioned study examined absolute mutation rather than somatic mutation. Furthermore, aforementioned study didn't provide the frequency of single nucleotide polymorphisms in their study population. Though our study found patients with somatic mutation of D-loop of mtDNA were associated with better survival, further study is needed to clarify the exact mechanism.

There were some limitations in our study. First, external validity of the findings is limited as it was conducted at a single hospital. Second, the sample size is not large enough and the follow-up period was relatively short. Finally, this study included oral cavity cancers from various subsites and the different characteristics among various locations inevitably existed.

In conclusion, a high rate of somatic mutations in the D-loop region of mtDNA was found in our oral cancer patients from an endemic betel quid chewing area. The presence of somatic mutations in the D-loop region was associated with better prognosis in these patients. Further study with larger population and longer follow-up period is undergoing in order to confirm the relationship between the D-loop mutations and survival of oral cancer patients.

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Conflict of interest None declared.

References

1. Wong YK, Tsai WC, Lin JC et al (2006) Socio-demographic factors in the prognosis of oral cancer patients. Oral Oncol 42:893–906
2. Liu SA, Tsai WC, Wong YK et al (2006) Nutritional factors and survival of patients with oral cancer. Head Neck 28:998–1007
3. Lee HC, Yin PH, Lin JC et al (2005) Mitochondrial genome instability and mtDNA depletion in human cancers. Ann N Y Acad Sci 1042:109–122
4. Lee HC, Li SH, Lin JC, Wu CC, Yeh DC, Wei YH (2004) Somatic mutations in the D-loop and decrease in the copy number of mitochondrial DNA in human hepatocellular carcinoma. Mutat Res 547:71–78
5. Chatterjee A, Mambo E, Sidransky D (2006) Mitochondrial DNA mutations in human cancer. Oncogene 25:4663–4674
6. Yin PH, Wu CC, Lin JC, Chi CW, Wei YH, Lee HC (2010) Somatic mutations of mitochondrial genome in hepatocellular carcinoma. Mitochondrion 10:174–182
7. Tan DJ, Chang J, Chen WL et al (2004) Somatic mitochondrial DNA mutations in oral cancer of betel quid chewers. Ann N Y Acad Sci 1011:310–316
8. Prior SL, Griffiths AP, Baxter JM et al (2006) Mitochondrial DNA mutations in oral squamous cell carcinoma. Carcinogenesis 27:945–950
9. Fliss MS, Usadel H, Caballero OL et al (2000) Facile detection of mitochondrial DNA mutations in tumors and bodily fluids. Science 287:2017–2019
10. Zhou S, Kachhap S, Sun W et al (2007) Frequency and phenotypic implications of mitochondrial DNA mutations in human squamous cell carcinoma of the head and neck. Proc Natl Acad Sci USA 104:7540–7545
11. Lievre A, Blons H, Houllier AM et al (2006) Clinicopathological significance of mitochondrial D-loop mutations in head and neck carcrinoma. Br J Cancer 94:692–697
12. Ha PK, Tong BC, Westra WH et al (2002) Mitochondrial C-tract alteration in premalignant lesions of the head and neck: a marker for progression and clonal proliferation. Clin Cancer Res 8:2260–2265
13. Mamba E, Gao X, Cohen Y, Guo Z, Talalay P, Sidransky D (2003) Electrophile and oxidant damage of mitochondtial DNA leading to rapid evolution of homoplasmic mutations. Proc Natl Acad Sci USA 100:1838–1843
14. Challen C, Brown H, Cai C et al (2011) Mitochondrial DNA mutations in head and neck cancer are infrequent and lack prognostic utility. Br J Cancer 104:1319–1324
15. Pietka G, Kukwa W, Bartnik E et al (2008) Mitochondrial DNA mutations in the pathogenesis in the head and neck squamous cell carcinoma. Otolaryngol Pol 62:158–164 (Article in Polish)
16. Chatterjee A, Dasgupta S, Sidransky D (2011) Mitochondrial subversion in cancer. Cancer Prev Res 4:638–654
17. Chung CH, Gillison ML (2009) Human papillomavirus in head and neck cancer: its role in pathogenesis and clinical implications. Clin Caner Res 15:6758–6762
18. Zhang R, Wang R, Zhang F et al (2010) Single nucleotide polymorphisms in the mitochondrial displacement loop and outcome of esophageal squamous cell carcinoma. J Exp Clin Cancer Res 29:155