The role of mitochondrial DNA alterations in esophageal squamous cell carcinomas

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Objective: The study objective was to evaluate the roles of mitochondrial DNA alterations in esophageal squamous cell carcinoma, with emphasis on the changes in the copy number and D310 variants of mitochondrial DNA.

Methods: Paired samples microdissected from esophageal muscles, noncancerous esophageal mucosa, cancerous esophageal squamous cell carcinoma nests, and metastatic lymph nodes of 72 patients with esophageal squamous cell carcinoma were subjected to DNA extraction. The copy number and D310 variants of mitochondrial DNA were determined by quantitative real-time polymerase chain reaction and direct sequencing, respectively.

Results: Fifty-six patients (77.8%) with somatic D310 mutations had lower survival probability ($P = .027$). From noncancerous esophageal mucosa to cancerous esophageal squamous cell carcinoma nests and metastatic lymph nodes, the D310 variants were decreased from 2.2 to 1.7 and 1.5, respectively, with a trend to homoplasmy ($P = .0009$). Concurrently, the mitochondrial DNA copy number was increased from 0.159 to 0.192 and 0.206, respectively, ($P = .024$), especially in cigarette smokers ($P = .014$) and heavy wine drinkers ($P = .005$). Notably, a decrease in D310 variants ($1.5, P < .001$) and an increase in the incidence of the homoplasmic D310 pattern ($P = .005$) were observed in the matched esophageal muscle tissues. Among the 56 esophageal squamous cell carcinoma cancer nests with somatic D310 mutations, 51 (91.1%) had D310 variants in association with their corresponding noncancerous esophageal mucosa, including 36 (64.3%) fully related and 15 (26.8%) partially related pairs.

Conclusion: We demonstrated that somatic D310 mutations and increase in the copy number of mitochondrial DNA are of clinical importance in esophageal squamous cell carcinoma. We also propose a model of DNA instability and clonal expansion during the carcinogenesis and progression of esophageal squamous cell carcinoma from the viewpoint of mitochondrial DNA transmission. (J Thorac Cardiovasc Surg 2010;139:189-97)

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Esophageal squamous cell carcinoma (ESCC) is an aggressive malignant neoplasm in patients in Asian countries. Despite advances in therapeutic modalities during the past

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decades in Taiwan, the survival outcome remains dismal.¹⁻³ Several clinical and molecular prognostic factors have been investigated in patients with ESCC.¹⁻³ However, most studies are concerned with the roles of nuclear DNA (nDNA) mutations, and less attention has been paid to mitochondrial DNA (mtDNA) alterations. Thus, appraisal of the role of mtDNA alterations in the pathophysiology of ESCC is of great clinical importance.

Each human cell contains several hundred to 1000 mitochondria, and each mitochondrion has 2 to 10 copies of mtDNA. In contrast with the heterozygotic characteristics of nDNA inherited from parents, mtDNA is transmitted through the maternal lineage. In addition, the majority of mtDNA copies in the cells of post-mitotic tissues of an individual are identical at birth with a pattern termed “homoplasmy.”^{4,5} Human mtDNA accumulates damages and mutations at a rate approximately 17 times greater than does the nDNA.^{5,6} When the mutated mtDNA molecules coexist with the wild-type mtDNA, the homoplasmy is disrupted and shifted to the so-called heteroplasmy.⁴

Human mtDNA is a 16,569 base-pair, double-stranded, closed-circular DNA molecule that encodes 13 polypeptides, 2 rRNAs, and a set of 22 tRNAs required for protein synthesis in mitochondria. The displacement loop located at nucleotide position (np) 16024–576 with 1.1 kb in size is a noncoding

Abbreviations and Acronyms

ANOVA	= analysis of variance
ESCC	= esophageal squamous cell carcinoma
mtDNA	= mitochondrial DNA
nDNA	= nuclear DNA
np	= nucleotide position
PCR	= polymerase chain reaction

region essential for the replication and transcription of mtDNA.⁷ The displacement loop has been discovered as a hot spot for mutation in several human cancers, especially in the D310 region.⁸

There is a polycytidine tract consisting of repeated monocytydine between np 303 and np 316 with a thymidine interposed at np 310 (ie, -C₃₀₃CCCCC-T₃₁₀-CCCCC₃₁₆-). Generally, the cytidine number before thymidine is 7 (C-7, wild type), but 6, 8, 9, 10, and 11 (C-6, C-8, C-9, C10, C-11 variants) have also been reported in different individuals (<http://www.mitomap.org>).⁹ Such variations among human populations are referred to as D310 polymorphisms or D310 variants. Somatic D310 mutation (ie, altered D310 variants between matched cancerous and noncancerous counterparts) was recently found in several human cancers, including head and neck, lung, gastric, breast, and prostate cancers.^{10,11} However, little is known about such mutations in ESCC.^{12,13} Because of the regulatory role of the displacement loop in mtDNA replication, it is important to determine whether such D310 mutations are associated with quantitative changes in mtDNA copy number. Although changes in mtDNA copy number have been reported in several human cancers, as an increase in head and neck cancers¹⁴ or a decrease in advanced gastric cancer, breast cancer, and hepatocellular carcinomas,^{11,15-17} their clinical significance remains unclear.

In this study, we investigated the mtDNA alterations, mainly focused on D310 mutations and change in mtDNA copy number, and their association with the clinical outcomes of ESCC. Possible factors, including demographic data, history of cigarette smoking or alcohol drinking, and tumor characteristics, which might be related to mtDNA alterations, were also evaluated. Finally, we propose a model to explain the role of mtDNA alterations in the pathophysiology of ESCC.

MATERIALS AND METHODS**Patient Selection**

Patients with resectable ESCC and without obvious distant organ metastasis on preoperative assessments who received complete surgical resection (including transthoracic subtotal esophagectomy, gastric tube reconstruction with esophagogastric anastomosis in the neck, and radical lymph node dissection) as their primary treatment modality at Taipei Veterans General Hospital were enrolled between 2000 and 2003. None of the patients received preoperative neoadjuvant chemotherapy or radiotherapy. The patients' pathologic data (TNM status according to the American Joint

Committee on Cancer¹⁸) and clinical data (eg, smoking status, alcohol consumption, and overall survival period) were recorded in detail for systemic analysis. All patients were followed up until December 2006, and the preliminary results have been reported.³ The patients' clinical data and pathologic slides were subject to review, and a total of 72 ESCC cases were recruited for analysis in this study. Institutional review board approval from Taipei Veterans General Hospital was obtained to conduct this retrospective study (VGHIRB No: 95-11-15A).

Tissue Collection and DNA Extraction

With the assistance of an experienced pathologist, representative tumor foci without necrosis and lymphocyte infiltration on pathologic slides were located, and thin slices approximately 5 μ m in thickness from corresponding paraffin-embedded tissue blocks were prepared for laser microdissection. Under the guidance of the hematoxylin-eosin Y stain, paired pathologically normal non-cancerous esophageal mucosa and underlying esophageal muscle tissues and the cancerous ESCC nests from each patient were microdissected using a laser beam under a light microscope (Figure E1). Among the 72 patients with ESCC, 44 had lymph node metastasis, and pathologic blocks containing the metastatic lymph nodes of 37 patients were retrieved adequately for laser microdissection. The tissue samples were stored in 0.5 mL Eppendorf vials and mixed with 60 μ L QuickExtract DNA extraction solution (Epicenter, Madison, Wis) to extract total cellular DNA at 65°C for 3 hours.¹⁹ The quality of the extracted DNA was verified by the ratio between the absorbance at 260 and 280 nm, and the existence of a DNA band polymerase chain reaction (PCR) amplified from 18S rRNA gene after electrophoresis and ethidium bromide staining. The DNA sample was kept at -20°C until use.

Sequencing of the D310 Region

The D310 region was PCR amplified from the extracted DNA and then subjected to direct sequencing. Each 50- μ L PCR reaction contained 25 μ L of AmpliTaq Gold PCR Master Mix (Applied Biosystems, Foster City, Calif), 22 μ L of PCR-grade H₂O, 1 μ L of each primer (H76-1: 5'-CACGC GATAGCATTGCGA-3' and L335: 5'-TAAGTGCTGTGGCCAGAAGC-3'), and 1 μ L of sample DNA.²⁰ The PCR conditions were set at 95°C for 5 minutes followed by 40 cycles of amplification at 95°C for 15 seconds, 58°C for 15 seconds, and 72°C for 30 seconds, and the final extension at 72°C for 7 minutes. PCR products were subject to electrophoresis on a 3% agarose gel to separate the DNA bands and visualized by ultraviolet light illumination after ethidium bromide staining. The DNA band of interest was then cut out of the gel and subject to direct sequencing. The results were compared in pairs with reference to the human mitochondrial genome database (<http://www.mitomap.org>).⁹

Determination of Mitochondrial DNA Copy Number

The mtDNA copy number was determined as mtDNA/nDNA ratio by quantitative real-time PCR. For each 10- μ L reaction, 1 μ L of unknown DNA was amplified in a capillary containing 0.25 μ L of each primer (20 μ M), 1.2 μ L 3 mmol/L MgCl₂, 1 μ L LightCycler SYBR Green mixed reagent (Roche Applied Science, Mannheim, Germany), and 6.3 μ L PCR-grade H₂O. Also amplified were DNA samples from 143B cells (1.25 ng/ μ L) and PCR-grade H₂O as the positive and negative controls, respectively. The PCR was performed with hot start at 95°C for 10 minutes followed by 40 cycles of 95°C for 20 seconds, 62°C for 20 seconds, and 72°C for 20 seconds. The sequences of primers used to amplify mtDNA (ND1 region) were mtF3212, 5'-CACCCAAGAA CAGGGTTGT-3' and mtR3319, 5'-TGGCCATGGGATTGTTGTAA-3'. The sequences of primers used to amplify nDNA (18S rRNA gene) were 18S1546F 5'-TAGAGG GA CAAGTGGCGTTC-3' and 18S1650R 5'-CGCTGAGCCAGTCAGTGT-3'.²¹ Standard curves representing the quantities of both mtDNA and nDNA related to replication threshold cycles were established by using DNA from 143B osteosarcoma cells ($R^2 = 0.9973$ for mtDNA and $R^2 = .9973$ for nDNA) as previously reported.²¹ The mtDNA copy number of

the 143B cell was used as the internal standard and defined as 1. The relative mtDNA copy numbers of clinical samples were then determined accordingly. Each analysis was done in duplicate, and the value of the mean was used for data presentation.

Statistics

All statistical analyses in this study were performed using the Statistical Package for the Social Sciences, 12.0 (SPSS Inc, Chicago, Ill) and Statistics Analysis System 9.1 (SAS Institute Inc, Cary, NC). The continuous variables between groups were compared using the Student *t* test/Mann-Whitney test, analysis of variance (ANOVA)/Kruskal-Wallis test, or paired *t* test when appropriate. Categorical variables between groups were compared using the chi-square test, Fisher's exact test, or Cochran-Armitage test for trend when appropriate. Survival probability was calculated and presented by the Kaplan-Meier plot. The log-rank test was used to compare different survival probabilities among different levels within each categorical variable. Possible prognostic factors associated with survival probability at a significance level of .20 or less were considered in a multivariable Cox's proportional hazard regression analysis.

RESULTS

Clinical Data

Between January 2000 and December 2003, 72 patients with ESCC (62 men, 86.1%) with a mean age of 60.1 years were eligible for analysis. The demographic data are summarized in Table 1. Fifty-six patients (77.8%) smoked cigarettes with a mean smoking index (packages smoked per day times years of smoking; pack-year) of 35.8 ± 22.2 , and 57 patients (79.2%) drank alcohol with a mean alcohol consumption index (bottles consumed per day times years of drinking; bottle-year) of 23.6 ± 18.5 . On the basis of the American Joint Committee on Cancer staging system, 6 patients (8.3%) were in stage I, 21 patients (29.2%) were in stage II, 18 patients (25.0%) were in stage III, and 27 patients (37.5%) were in stage IV of nonregional lymph node metastasis. The overall mean and median survival periods were 30.7 months (95% confidence interval, 25.0–36.3) and 24.2 months (95% confidence interval, 16.8–31.6), respectively. Eleven patients (15.3%) underwent incomplete R1 resection because of the presence of microscopically atypical cells (3 dysplasia, 4 carcinoma in situ, 4 invasive carcinoma) in the proximal resection margin of the cervical esophagus. Although all the distal resection margins over the gastric cardia were free of carcinoma invasion, 9 patients (12.5%) were found to have ESCC cancer nests in the gastric cardia.

Distribution of Major D310 Variants in Examined Tissues of Patients With Esophageal Squamous Cell Carcinoma

Distributions of the major D310 variants of the examined tissues are summarized in Table 2. By taking the noncancerous esophageal mucosa of patient A01 as an example, 3 kinds of D310 variants were found (C-8, C-9, and C-10; heteroplasmic), and C-8 was the major variant (Table E1). Among the major D310 variants in the examined tissues, C-8 was the most common followed by C-7. C-8 as the major variant was noted in

56.9% ($n = 41$) of 72 muscle tissues, 38.9% ($n = 28$) of 72 noncancerous mucosa, 45.8% ($n = 33$) of 72 ESCC nests, and 48.6% ($n = 18$) of 37 metastatic lymph nodes.

D310 Sequence Variations in Noncancerous Esophageal Mucosa and Paired Cancerous Esophageal Squamous Cell Carcinoma Nests

Changes in D310 variants between the noncancerous esophageal mucosa and the paired cancerous ESCC nests in each patient were compared, and 5 types of alterations were classified, including 16 patients (22.2%) with type I (homoplasmic to the same homoplasmic variant), 8 patients (11.1%) with type II (homoplasmic to heteroplasmic), 23 patients (31.9%) with type III (heteroplasmic to heteroplasmic), 23 patients (31.9%) with type IV (heteroplasmic to homoplasmic), and 2 patients (1.5%) with type V (one homoplasmic variant to another homoplasmic variant) alterations (Tables 3 and E1; Figure E2). We defined type II, III, IV, and V alterations as D310 mutations ($n = 56$, 77.8%) resulting from any shift in the proportions of D310 variants between noncancerous esophageal mucosa and paired cancerous ESCC nests, and type I alteration ($n = 16$, 22.2%) was regarded as no mutation. By using patient A01 as an example (Table E1), C-8 (major), C-9, and C-10 variants coexisting in the noncancerous esophageal mucosa were changed to C-9 (major), C-8, and C-10 variants coexisting in the paired cancerous ESCC nest. We thus defined this patient as having type III D310 alteration with D310 mutation.

Relationship Between D310 Variants in Cancerous Esophageal Squamous Cell Carcinoma Nests and Matched Noncancerous Esophageal Mucosa

The relationship between D310 variants in the cancerous ESCC nests and the corresponding noncancerous esophageal mucosa of each patient was analyzed and grouped as follows: (1) fully related ($n = 52$, 72.2%): All the D310 variants of the cancerous ESCC nests already existed in the paired noncancerous esophageal mucosa (eg, patient A01), including 16 (22.2%) without D310 mutations and 36 (50%) with D310 mutations; (2) partially related ($n = 15$, 20.8%): Some but not all of the D310 variants of the cancerous ESCC nests existed in the paired noncancerous esophageal mucosa (eg, patient B08), and all of them harbored D310 mutations; (3) not related ($n = 5$, 6.9%): None of the D310 variants of the cancerous ESCC nests existed in the paired noncancerous esophageal mucosa (eg, patient E12), and all of them harbored D310 mutations (Tables 3 and E1).

Prognostic Roles of Somatic D310 Mutation and Other Factors

There were 56 patients (77.8%) with ESCC harboring D310 mutations with poorer prognosis ($P = .027$, log-rank test) (Figure 1, A; Table 1). Other clinical factors, including

TABLE 1. Demographic data and possible prognostic factors affecting survival probability in patients with esophageal squamous cell carcinoma

Demographic data/prognostic factors (%)	Overall survival Mean (95% CI) (mo)	Univariable <i>P</i> (log-rank)	Multivariable		
			Hazard ratio	95% CI	<i>P</i> (Cox regression)
D310 mutation		.027			.002
Yes (n = 56) (77.8)	26.9 (20.7–33.0)		1.000		
No (n = 16) (22.2)	40.9 (30.3–51.5)		0.258	0.109–0.614	
Gender		.615			
Male (n = 62) (86.1)	30.0 (23.8–36.3)				
Female (n = 10) (13.9)	34.9 (23.0–46.8)				
Presenting symptoms					
Difficulty in swallowing		.017			.329
Yes (n = 58) (80.6)	25.9 (20.5–31.3)				
No (n = 14) (19.4)	46.4 (33.4–59.5)				
Body weight loss		.829			
Yes (n = 20) (27.8)	29.4 (19.3–39.4)				
No (n = 52) (72.2)	30.3 (23.8–36.7)				
Special habit					
Cigarette smoking		.258			
Yes (n = 56) (77.8)	28.7 (22.4–35.0)				
No (n = 16) (22.2)	36.0 (24.8–47.1)				
Alcohol drinking		.5773			
Yes (n = 57) (79.2)	29.7 (23.4–36.1)				
No (n = 15) (20.8)	31.4 (21.4–41.5)				
Pathologic findings					
Esophageal resection margin invasion*		.073			.081
Yes (n = 11) (15.3)	18.7 (9.67–27.7)				
No (n = 61) (84.7)	32.5 (26.3–38.7)				
Gastric cardia invasion		.001			.383
Yes (n = 9) (12.5)	10.1 (3.5–16.7)				
No (n = 63) (87.5)	33.2 (27.3–39.2)				
T-status (depth of tumor invasion)		.005			.004
T1 (n = 7) (9.7)	45.0 (26.9–63.1)		T1 vs T4	0.232	0.060–0.896
T2 (n = 13) (18.1)	38.4 (27.4–49.4)		T2 vs T4	0.235	0.092–0.599
T3 (n = 29) (40.3)	30.7 (23.4–38.0)		T3 vs T4	0.314	0.149–0.658
T4 (n = 23) (31.9)	14.5 (9.3–19.6)		T4 vs T4	1.000	
N-status		<.001			.007
N(–) (n = 28) (38.9)	44.0 (35.2–52.7)			0.410	0.197–0.855
N(+) (n = 44) (61.1)	21.5 (15.9–27.2)			1.000	
M-status		.117			.689
M0 (n = 45) (62.5)	35.2 (28.0–42.5)				
M1a (n = 12) (16.7)	22.4 (12.5–32.3)				
M1b (n = 15) (20.8)	22.9 (11.8–34.0)				
Cancer stage (AJCC)		.043			.363
I (n = 6) (8.3)	48.6 (28.4–68.9)				
II (n = 21) (29.2)	38.0 (28.8–47.2)				
III (n = 18) (25.0)	22.2 (14.1–30.3)				
IV (n = 27) (37.5)	22.4 (14.9–29.8)				
Postoperative adjuvant therapy					
Radiotherapy		.9395			
Yes (n = 22) (30.6)	29.4 (20.5–38.2)				
No (n = 50) (69.4)	30.5 (23.6–37.3)				
Chemotherapy		.2378			
Yes (n = 26) (36.1)	25.3 (18.5–32.1)				
No (n = 46) (63.9)	33.3 (25.6–40.9)				

AJCC, American Joint Committee on Cancer; CI, confidence interval. *Cervical esophageal resection margins invasion indicates R1 resection, that is, atypical cells (3 dysplasia, 4 carcinoma in situ, 4 invasive carcinoma) were found in the resection margin during microscopic examination.

TABLE 2. Distribution of D310 patterns and mtDNA copy numbers among the esophageal muscular tissues, noncancerous esophageal mucosa, cancerous esophageal squamous cell carcinoma nests, and metastatic lymph nodes in 72 patients with esophageal squamous cell carcinoma

	Reference tissues	Target tissues			P value	P value¶	P value#
	Esophageal muscular tissues	Noncancerous esophageal mucosa	Cancerous ESCC nests	Metastatic lymph nodes			
	n = 72 (100)	n = 72 (100)	n = 72 (100)	n = 37 (100)			
D310 parameters							
Pattern of D310 distribution							
Homoplasmic (%)	43 (59.7)	26 (36.1)	41 (56.9)	25 (67.6)	.005*	.012*	<.001‡
Heteroplasmic (%)	29 (40.3)	46 (63.9)	31 (43.1)	12 (32.4)			
No. of D310 variants							
Mean ± SD	1.5 ± 0.8	2.2 ± 1.0	1.7 ± 1.0	1.5 ± 0.8	<.001†	.002†	.001§
Median	1.0	2.0	1.0	1.0			
Major D310 variant							
C-7 (%)	25 (34.7)	29 (40.3)	27 (37.5)	13 (35.1)	.491*	.732*	.589‡
Non C-7 (%)	47 (65.3)	43 (59.7)	45 (62.5)	24 (64.9)			
C-8 (%)	41 (56.9)	28 (38.9)	33 (45.8)	18 (48.6)			
C-9 (%)	6 (8.3)	11 (15.3)	9 (12.5)	5 (13.5)			
Others (%)	0 (0)	4 (5.6)	3 (4.2)	1 (2.7)			
mtDNA copy no.							
Mean ± SD	ND	0.159 ± 0.056	0.192 ± 0.111	0.206 ± 0.113	ND	.004†	.024§

ESCC, Esophageal squamous cell carcinoma; SD, standard deviation; ND, not determined. *Chi-square test. †Paired *t* test. ‡Cochrane–Armitage test for trend. §ANOVA. ||Compared between matched esophageal muscular tissues and noncancerous esophageal mucosa. ¶Compared between matched noncancerous esophageal mucosa and cancerous ESCC nests. #Compared among noncancerous esophageal mucosa, cancerous ESCC nests, and metastatic lymph nodes.

advanced T status ($P = .005$), positive lymph node metastasis ($P < .001$), late cancer stage ($P = .043$), gastric cardia invasion ($P = .001$), invasion of the cervical esophageal resection margins ($P = .073$), and symptoms of swallowing difficulty ($P = .017$), were associated with shorter survival. After conducting a Cox’s regression with stepwise analysis, we found that D310 mutation ($P = .002$), advanced T status ($P = .004$), and positive lymph node metastasis ($P = .007$) were independent risk factors related to worse outcomes (Table 1). We found that patients with a higher degree of D310 heteroplasmy (ie, >2 D310 variants) in the noncancerous mucosa had a lower survival probability than did patients with a lower degree of D310 heteroplasmy (ie, ≤ 2 D310 variants) ($P = .005$; Figure 1, B).

Alterations of the D310 Patterns and Mitochondrial DNA Copy Numbers in Tissues of Patients With Esophageal Squamous Cell Carcinoma

On average, each noncancerous esophageal mucosa harbored 2.2 kinds of D310 variants, each cancerous ESCC nest harbored 1.7 kind of D310 variants, and each metastatic lymph node harbored 1.5 kinds of D310 variants. There was a stepwise decrease of D310 variants when ESCC progressed ($P = .001$, ANOVA) and an increase in the incidence of homoplasmic D310 pattern from noncancerous esophageal mucosa (26/72, 36.1%) to cancerous ESCC nests (41/72, 56.9%) and to metastatic lymph nodes (25/37, 67.6%) ($P = .0009$, Cochrane–Armitage test for trend) (Table 2). The mean mtDNA copy number showed a stepwise increase from the noncancerous esophageal mucosa

(0.159, $n = 72$) to the cancerous ESCC nests (0.192, $n = 72$) ($P = .004$, paired *t* test) and then to the metastatic lymph nodes (0.206, $n = 37$) ($P = .024$, ANOVA) (Table 2) during ESCC progression. In the pathologically normal tissues, the kinds of D310 variants in the esophageal muscle (1.5) were significantly less than those in paired noncancerous esophageal mucosa (2.2) ($P < .001$, paired *t* test), with a higher incidence of heteroplasmic pattern in the noncancerous esophageal mucosa ($P = .005$, χ^2 test) (Table 2).

Factors Related to Somatic D310 Mutation and Mitochondrial DNA Copy Number Alteration

The possible contributory factors for somatic D310 mutations are old age ($P = .015$), heteroplasmy with multiple D310 variants ($P < .001$), and major D310 variants other than C-7 in noncancerous esophageal mucosa ($P < .001$) (Table 4). To compare the changes of mtDNA copy number, we defined the mtDNA copy ratio as the mtDNA copy number of the cancerous ESCC nest over the paired noncancerous esophageal mucosa in each patient. Male patients ($P = .012$), cigarette smokers ($P = .014$), alcohol drinkers ($P = .005$), and presence of the heteroplasmic D310 pattern ($P = .015$) and major variants other than C-7 ($P = .014$) in the noncancerous esophageal mucosa are associated with an elevated mtDNA copy ratio (Table 4).

Quantitative Changes of Mitochondrial DNA Copy Number When Harboring D310 Mutation

Among the 56 patients with somatic D310 mutations, 23 (41.1%) had type IV alteration (heteroplasmic to

TABLE 3. Differences of mtDNA copy ratios according to types of D310 alteration and D310 clonal relationships

Classification	Case No. (%)‡	mtDNA copy ratio	P value
Without somatic D310 mutation (n = 16)	16 (22.2)	1.09 ± 0.41	.319†
Type of D310 alteration			
I (homoplasmic to homoplasmic)	16 (22.2)	1.09 ± 0.41	
Clonal relationship			
Fully related	16 (22.2)	1.09 ± 0.41	
Partially related	ND	ND	
Not related	ND	ND	
With somatic D310 mutation (n = 56)	56 (77.8)	1.28 ± 0.72	.008†
Type of D310 alteration			
II (homoplasmic to heteroplasmic)	8 (11.1)	0.91 ± 0.31	.048*
III (heteroplasmic to heteroplasmic)	23 (31.9)	1.21 ± 0.69	
IV (heteroplasmic to homoplasmic)	23 (31.9)	1.50 ± 0.80	
V (A homoplasmic to B homoplasmic)	2 (1.5)	1.12 ± 0.43	
Clonal relationship			
Fully related	36 (50)	1.35 ± 0.68	.047*
Partially related	15 (20.8)	1.02 ± 0.67	
Not related	5 (6.9)	1.52 ± 1.05	

ND, Not determined; mtDNA copy ratio, mtDNA copy number of cancerous ESCC nest/paired noncancerous esophageal mucosa. *ANOVA/Kruskal–Wallis. †Paired *t* test (changes of the mtDNA copy number between the cancerous ESCC nest and the paired noncancerous mucosa). ‡Overall percentage.

homoplasmic, ie, shifted to a single D310 variant) and 36 (69.2%) had D310 variants in the ESCC cancerous nests fully related to their noncancerous esophageal mucosa (Table 3). Those with type IV alteration had a higher mtDNA copy ratio than did those with types II, III, and V alterations ($P = .048$). Moreover, patients with D310 variants in the cancerous ESCC nests fully related to their paired noncancerous esophageal mucosa also had a higher mtDNA copy ratio compared with those who harbored only partially related D310 variants ($P = .047$) (Table 3).

DISCUSSION

Somatic D310 mutations and their clinical significance in human cancers have been recognized in the past decade,^{10,11,15–17} especially in head and neck cancers.²² In recent years, somatic D310 mutations have been detected in ESCC.^{12,13} We demonstrated in this study that somatic D310 mutation is an independent prognostic factor in patients with ESCC (Table 1). Heteroplasmic D310 variants and major variants other than C-7 in the noncancerous esophageal mucosa are associated with somatic D310 mutations (Table 4). Previous studies of human lung cancers showed that somatic D310 mutations occur more frequently if the major D310 variants in matched blood lymphocytes

are non-C-7 variants and heteroplasmic.^{10,23} Generally, the incidence of D310 mutations in cancers in the Caucasian population is approximately 20%, with the highest in gastric cancer (62.5%) and lowest in prostate and ovarian cancers.¹⁰ For patients with ESCC, the incidence of D310 mutation was approximately 30%.^{12,13} However, the incidence of D310 mutation was higher (77.8%) in our series. D310 polymorphisms (C-7 vs non-C-7) among different populations may explain this disparity. C-7 (wild type) is the most common variant in Caucasian populations, whereas most patients with ESCC in Taiwan (43/72, 59.7%) harbored major D310 variants other than C-7 in the noncancerous esophageal mucosa. As mentioned above, non-C-7 variants are a risk factor for somatic D310 mutation, and patients with ESCC would be prone to somatic D310 mutation when the corresponding noncancerous esophageal mucosa harbors D310 variants other than C-7, which might explain the high mutation rate in Taiwanese patients with ESCC. Another possible explanation is that the samples we studied were obtained from laser microdissection and free from contamination with different cell types, and thus of higher homogeneity. We believe that the power to differentiate D310 mutations is higher when tissues are obtained from microdissection.

It is noteworthy that a heteroplasmic to homoplasmic shift of D310 variants and an increase in mtDNA copy number were observed in the progression from noncancerous esophageal mucosa to cancerous ESCC nests and then to metastatic lymph nodes (Table 2). This can be explained by Nowell's²⁴ hypothesis of cancer evolution, which states that most cancers arise from heterogeneous precancerous lesions (with heteroplasmic D310 pattern), in which specific clones acquire a proliferation advantage (increase of copy number), followed by subsequent expansion of these clones (with homoplasmic D310 pattern). To confirm the notion, we further evaluated the associations of D310 patterns, mtDNA copy number, and D310 clonal relationship between the noncancerous esophageal mucosa and the paired cancerous ESCC nests among 56 patients with ESCC harboring D310 mutations (Table 3). We found that 23 (41.1%) of the 56 patients exhibited a type IV heteroplasmic to homoplasmic D310 alteration (ie, to a single clone) and had a higher mtDNA copy ratio (ie, increased clonal quantity) (Table 3, $P = .048$). Fifty-one (91.1%) of the 56 cancerous nests had D310 clonal relationship with their noncancerous esophageal mucosa, including 36 (64.3%) fully related and 15 (26.8%) partially related. Moreover, 36 (64.3%) of the 56 patients who had mutant D310 variants in the cancerous ESCC nests fully related to their noncancerous esophageal mucosa had a higher mtDNA copy ratio than did those patients with a partially related relationship (Table 4, $P = .047$). On the basis of these findings, we propose a model of clonal expansion during ESCC progression from the viewpoint of mtDNA transmission

FIGURE 1. A, Comparison of survival between patients with ESCC with and without somatic D310 mutations of mtDNA. Patients without D310 mutations (n = 16) had better survival probability and lower hazard ratio than those with D310 mutations (n = 56). B, Comparison of the survival between patients with ESCC according to the degree of heteroplasmic D310 variation in noncancerous esophageal mucosa. Patients with lower degree heteroplasmy of mtDNA (n = 43) had better survival probability and lower hazard ratio than those with higher degree heteroplasmy of mtDNA (n = 29). C, Proposed model for the role of mtDNA alterations in ESCC is illustrated. During the aging process with accumulative damages, heteroplasmic mtDNA variants may coexist in the pathologically normal esophageal mucosa, and the quantity of total mtDNA may increase to compensate for the defects in mtDNA. When carcinogenesis is initiated or cancer is progressed, specific mtDNA variants harbored by the original malignant clone(s) are amplified during the process of clonal expansion. *HR*, Hazard ratio; *ROS*, reactive oxygen species; *ESCC*, esophageal squamous cell carcinoma; *mtDNA*, mitochondrial DNA.

(Figure 1, C, middle and right). Moreover, cigarette smoking ($P = .014$) and alcohol consumption ($P = .005$) were highly associated with the increase in the mtDNA copy number (Table 4).

Generally, the mtDNA molecules in an individual are assumed identical at birth, and the characteristics of D310 variants in normal esophageal muscles and paired noncancerous esophageal mucosa are supposed to be identical and concordant.⁴ However, the percentage of homoplasmic D310 variants in esophageal muscle tissue (43/72, 59.7%)

was higher than that of the paired noncancerous esophageal mucosa (26/72, 36.1%) ($P = .005$), and esophageal mucosa had more D310 variants than did the corresponding esophageal muscle (2.2 vs 1.5, $P = .002$, Table 2). This discordance may be explained by a model of accumulated damages in mtDNA during the aging process,^{4,5} because mucosa is the barrier of the underlying muscles (Figure 1, C, left). This indicates that the pathologically normal esophageal mucosa may be molecularly abnormal because of accumulated damage at the DNA level.

TABLE 4. Factors related to the D310 mutation and mtDNA copy ratios in patients with esophageal squamous cell carcinoma

Clinical parameters	D310 mutation			mtDNA DNA copy ratio [‡]	
	Yes (n = 56)	No (n = 16)	P value	Mean $\pm$ SD	P value
Age (y) (mean $\pm$ SD)	62.1 $\pm$ 12.1	53.3 $\pm$ 13.4	.015*	ND	
Gender					
Male (n = 62)	48	14	.855†	1.28 $\pm$ 0.70	.012*
Female (n = 10)	8	2		0.97 $\pm$ 0.24	
Cigarette smoking					
Yes (n = 56)	43	13	.705†	1.33 $\pm$ 0.71	.014*
No (n = 16)	13	3		0.90 $\pm$ 0.24	
Alcohol drinking					
Yes (n = 51)	42	15	.164†	1.31 $\pm$ 0.71	.005*
No (n = 21)	14	1		0.96 $\pm$ 0.29	
D310 variants in noncancerous esophageal mucosa					
No. of variants (mean $\pm$ SD)	2.5 $\pm$ 1.0	1.0	<.001*	ND	
Distribution pattern					
Heteroplasmy (n = 46)	46	0	<.001†	1.35 $\pm$ 0.76	.015*
Homoplasmy (n = 26)	10	16		1.02 $\pm$ 0.38	
Major D310 variant					
C-7 (n = 29)	13	16	<.001†	1.03 $\pm$ 0.39	.014*
Non-C-7 (n = 43)	43	0		1.37 $\pm$ 0.77	
Cancer stage					
T-status					
T1 (n = 7)	6	1	.388†	1.37 $\pm$ 0.93	.382‡
T2 (n = 13)	10	3		1.32 $\pm$ 0.80	
T3 (n = 29)	24	5		1.06 $\pm$ 0.47	
T4 (n = 23)	16	7		1.36 $\pm$ 0.70	
N-status					
N (–) (n = 28)	22	6	.897†	1.20 $\pm$ 0.65	.745*
N (+) (n = 44)	34	10		1.25 $\pm$ 0.68	
M-status					
M0 (n = 45)	36	9	.564†	1.34 $\pm$ 0.71	.158‡
M1a (n = 12)	9	3		1.18 $\pm$ 0.71	
M1b (n = 15)	11	4		0.97 $\pm$ 0.32	
Cancer stage (AJCC)					
I (n = 6)	5	1	.346†	1.11 $\pm$ 0.71	.271‡
II (n = 21)	18	3		1.34 $\pm$ 0.73	
III (n = 18)	13	5		1.42 $\pm$ 0.73	
IV (n = 27)	20	7		1.06 $\pm$ 0.53	

AJCC, American Joint Committee on Cancer; SD, standard deviation; ND, not determined. * χ^2 test/Mann–Whitney test. †Chi-square test. ‡ANOVA/Kruskal–Wallis.

The aforementioned findings led us to conclude that (1) heteroplasmic D310 variants of mtDNA in pathologically normal esophageal mucosa may be a result of accumulated damage, (2) D310 mutations of mtDNA in cancerous nests are highly associated with the prognosis of patients with ESCC, (3) the change from heteroplasmic to homoplasmic D310 variants and an increase in mtDNA copy number

suggest a clonal expansion during ESCC progression, and (4) cigarette smoking and alcohol consumption are highly associated with an increase in mtDNA copy number.

Observation of multiple D310 variants in the noncancerous esophageal mucosa led us to examine whether the degree of heteroplasmy of the mtDNA in pathologically normal mucosa affected the clinical outcome of ESCC. On the basis of the mean and median number of D310 variants in the noncancerous esophageal mucosa being 2.2 and 2.0, respectively, we defined those who had less than 2 D310 variants (n = 43) as the low heteroplasmy group and those who had more than 2 D310 variants (n = 29) as the high heteroplasmy group. The results showed that high heteroplasmy was associated with lower survival probability ($P = .005$; Figure 1, B). This suggests that unstable mtDNA with multiple variants (heteroplasmic mtDNA) in the noncancerous esophageal mucosa is a poor prognostic factor for patients with ESCC, even if the pathologic examination of their mucosa looked “normal.”

The mtDNA copy number has been shown to change in many human cancers. An increase in the mtDNA copy number was reported in head and neck cancers¹⁴ and ESCC as shown in this study. A decrease in mtDNA copy number was noted in advanced hepatocellular carcinoma, gastric cancer, breast cancers, and advanced lung cancers with drug-resistance after neoadjuvant chemotherapy.^{11,15–17,21} On the basis of the mechanism of clonal expansion, the increase in mtDNA copy number might be a compensation for the damaged mtDNA to maintain mitochondrial function.¹⁴ When cancer progresses to advanced stages because of excess damage, decompensation may be followed with a decrease in mtDNA copy number, which results in impaired mitochondrial function and the Warburg effect.²⁵ The roles of mtDNA copy number alterations in human cancers warrant further study.

CONCLUSIONS

We have demonstrated that mtDNA alterations are associated with the progression and prognosis of ESCC. The somatic D310 mutations, which indicate mtDNA instability, may play a role in the pathophysiology of ESCC. Whether such mtDNA alterations affect the bioenergetic function of mitochondria in affected tissues of patients with ESCC requires further investigation.

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FIGURE E1. Laser microdissection (LMD) adopted for the harvest of samples from noncancerous esophageal mucosa, cancerous ESCC tissues, metastatic lymph nodes, and esophageal muscular tissues of patients with ESCC. First panel: Noncancerous esophageal mucosa before (*left*) and after (*right*) LMD. Second panel: Cancerous ESCC tissues before (*left*) and after (*right*) LMD. Third panel: Metastatic lymph nodes before (*left*) and after (*right*) LMD. Fourth panel: Esophageal muscle tissues before (*left*) and after (*right*) LMD. *ESCC*, Esophageal squamous cell carcinoma.

FIGURE E2. Types of somatic D310 alterations of mtDNA between noncancerous esophageal mucosa (*left*) and cancerous ESCC tissues (*right*). Arabic number above the *red arrow* denotes the cytidine number before the indicated thymidine peak, which is shown in red color during D310 sequencing. Type I, homoplasmic to homoplasmic (first row): The example shows homoplasmic C-7 variant to homoplasmic C-7 variant without D310 mutation. Type II, homoplasmic to heteroplasmic (second row): The example shows homoplasmic C-7 variant to heteroplasmic C-8, C-7, C-9 variants with D310 mutation. Type III, heteroplasmic to heteroplasmic (third row): The example shows heteroplasmic C-8, C-9, C10 variants to heteroplasmic C-9, C-8, C10 variants with proportional change of the D310 variants with D310 mutation. Type IV, heteroplasmic to homoplasmic (fourth row): The example shows heteroplasmic C-8, C-7 variants to homoplasmic C-8 variant with D310 mutation. Type V, homoplasmic to homoplasmic with variant shift (fifth row): The example shows homoplasmic C-7 to homoplasmic C-8 with D310 mutation. *ESCC*, Esophageal squamous cell carcinoma.

TABLE E1. Summary of the D310 variants between the paired noncancerous esophageal mucosa and the cancerous esophageal squamous cell carcinoma nests in 72 patients with esophageal squamous cell carcinoma

Somatic D310 mutation	Types of D310 alteration	Patient	Noncancerous esophageal mucosa			Cancerous ESCC nests			Relationship
			D310* variants	Major	Pattern	D310* variants	Major	Pattern	
No (n = 16)	I (homoplasmic to homoplasmic)	A06	7	7	Homoplasmy	7	7	Homoplasmy	Fully related
		A10	7	7	Homoplasmy	7	7	Homoplasmy	Fully related
		A12	7	7	Homoplasmy	7	7	Homoplasmy	Fully related
		B03	7	7	Homoplasmy	7	7	Homoplasmy	Fully related
		B09	7	7	Homoplasmy	7	7	Homoplasmy	Fully related
		C08	7	7	Homoplasmy	7	7	Homoplasmy	Fully related
		C10	7	7	Homoplasmy	7	7	Homoplasmy	Fully related
		D02	7	7	Homoplasmy	7	7	Homoplasmy	Fully related
		D07	7	7	Homoplasmy	7	7	Homoplasmy	Fully related
		D09	7	7	Homoplasmy	7	7	Homoplasmy	Fully related
		E06	7	7	Homoplasmy	7	7	Homoplasmy	Fully related
		E07	7	7	Homoplasmy	7	7	Homoplasmy	Fully related
		E10	7	7	Homoplasmy	7	7	Homoplasmy	Fully related
		F06	7	7	Homoplasmy	7	7	Homoplasmy	Fully related
		F07	7	7	Homoplasmy	7	7	Homoplasmy	Fully related
		F10	7	7	Homoplasmy	7	7	Homoplasmy	Fully related
Yes (n = 8)	II (homoplasmic to heteroplasmic)	A03	7	7	Homoplasmy	7, 8	7	Heteroplasmy	Partially related
		A07	7	7	Homoplasmy	7, 9	7	Heteroplasmy	Partially related
		B10	8	8	Homoplasmy	8, 9	8	Heteroplasmy	Partially related
		C06	7	7	Homoplasmy	7, 10	7	Heteroplasmy	Partially related
		E01	7	7	Homoplasmy	7, 9, 10, 11	7	Heteroplasmy	Partially related
		E02	7	7	Homoplasmy	7, 8, 6	7	Heteroplasmy	Partially related
		F11	7	7	Homoplasmy	8, 7, 9	8	Heteroplasmy	Partially related
		G03	8	8	Homoplasmy	7, 8	7	Heteroplasmy	Partially related
Yes (n = 23)	III (heteroplasmic to heteroplasmic)	A01	8, 9, 10	8	Heteroplasmy	9, 8, 10	9	Heteroplasmy	Fully related
		A02	10, 9, 11, 8	10	Heteroplasmy	9, 10, 8, 11	9	Heteroplasmy	Fully related
		A04	9, 8, 10, 11	9	Heteroplasmy	9, 10, 8, 11	9	Heteroplasmy	Fully related
		B01	8, 9	8	Heteroplasmy	8, 9	8	Heteroplasmy	Fully related
		B04	8, 9	8	Heteroplasmy	8, 7, 6	8	Heteroplasmy	Partially related
		B06	8, 9, 10, 11	8	Heteroplasmy	8, 9, 10	8	Heteroplasmy	Fully related
		B08	9, 8, 10	9	Heteroplasmy	7, 8	7	Heteroplasmy	Partially related
		B12	7, 9, 8	7	Heteroplasmy	9, 7, 8	9	Heteroplasmy	Fully related
		C02	7, 8	7	Heteroplasmy	10, 9, 8	10	Heteroplasmy	Partially related
		C04	8, 7, 9, 10	8	Heteroplasmy	9, 8, 7, 10	9	Heteroplasmy	Fully related
		C09	9, 8, 7	9	Heteroplasmy	10, 9	10	Heteroplasmy	Partially related
		C11	8, 9	8	Heteroplasmy	8, 9	8	Heteroplasmy	Fully related
		D05	8, 9	8	Heteroplasmy	8, 7, 6	8	Heteroplasmy	Partially related
		D06	9, 8	9	Heteroplasmy	9, 8	9	Heteroplasmy	Fully related
		D08	11, 10, 9	11	Heteroplasmy	9, 8	9	Heteroplasmy	Partially related
		E04	9, 7, 10, 8	9	Heteroplasmy	8, 9, 10	8	Heteroplasmy	Fully related
		E05	8, 9, 7	8	Heteroplasmy	8, 9, 7	8	Heteroplasmy	Fully related
		E09	9, 8	9	Heteroplasmy	9, 8	9	Heteroplasmy	Fully related
		F02	8, 7, 9	8	Heteroplasmy	8, 9	8	Heteroplasmy	Fully related
		F03	8, 9, 10	8	Heteroplasmy	8, 9	8	Heteroplasmy	Fully related
		F05	9, 10, 8	9	Heteroplasmy	8, 9	8	Heteroplasmy	Fully related
		F08	8, 9, 10, 7	8	Heteroplasmy	9, 10, 8, 7	9	Heteroplasmy	Fully related
		G02	7, 8	7	Heteroplasmy	7, 9	7	Heteroplasmy	Partially related

TABLE E1. Continued

Somatic D310 mutation	Types of D310 alteration	Patient	Noncancerous esophageal mucosa			Cancerous ESCC nests			Relationship
			D310* variants	Major	Pattern	D310* variants	Major	Pattern	
Yes (n = 23)	IV (heteroplasmic to homoplasmic)	A05	8, 7, 9	8	Heteroplasmy	8	8	Homoplasmy	Fully related
		A08	8, 9, 10	8	Heteroplasmy	8	8	Homoplasmy	Fully related
		A09	8, 9, 10	8	Heteroplasmy	8	8	Homoplasmy	Fully related
		A11	10, 9	10	Heteroplasmy	8	8	Homoplasmy	Not related
		B02	9, 8, 10	9	Heteroplasmy	8	8	Homoplasmy	Fully related
		B05	8, 7, 9, 10	8	Heteroplasmy	8	8	Homoplasmy	Fully related
		B07	8, 9, 7	8	Heteroplasmy	8	8	Homoplasmy	Fully related
		B11	8, 10, 9	8	Heteroplasmy	8	8	Homoplasmy	Fully related
		C01	8, 9, 10	8	Heteroplasmy	8	8	Homoplasmy	Fully related
		C03	7, 8	7	Heteroplasmy	8	8	Homoplasmy	Fully related
		C05	8, 9	8	Heteroplasmy	8	8	Homoplasmy	Fully related
		C07	9, 8, 10	9	Heteroplasmy	8	8	Homoplasmy	Fully related
		C12	8, 9, 10	8	Heteroplasmy	8	8	Homoplasmy	Fully related
		D01	8, 9, 7	8	Heteroplasmy	7	7	Homoplasmy	Fully related
		D03	9, 8	9	Heteroplasmy	8	8	Homoplasmy	Fully related
		D04	8, 10, 9	8	Heteroplasmy	8	8	Homoplasmy	Fully related
		D10	8, 7	8	Heteroplasmy	8	8	Homoplasmy	Fully related
		D12	8, 9	8	Heteroplasmy	8	8	Homoplasmy	Fully related
		E08	7, 8	7	Heteroplasmy	7	7	Homoplasmy	Fully related
		E11	10, 9	10	Heteroplasmy	7	7	Homoplasmy	Not related
		F04	9, 8, 10, 11	9	Heteroplasmy	4	4	Homoplasmy	Not related
		F09	8, 7	8	Heteroplasmy	8	8	Homoplasmy	Fully related
		G04	8, 9, 7	8	Heteroplasmy	8	8	Homoplasmy	Fully related
Yes (n = 2)	V (A homoplasmic to B homoplasmic)	E12	7	7	Homoplasmy	8	8	Homoplasmy	Not related
		G01	7	7	Homoplasmy	8	8	Homoplasmy	Not related

*The cytidine numbers of D310 variants are listed in order according to the detected sequencing peaks.